Production sélective et directionnelle des prostaglandines dans l'endomètre.

Thèse

Nicolas Lacroix-Pépin

Doctorat en Médecine moléculaire
Philosophiae Doctor (Ph.D.)

Québec, Canada

© Nicolas Lacroix-Pepin, 2014

Résumé

Les prostaglandines (PG) sont impliquées dans plusieurs processus et un bon équilibre dans leur production relative est important pour la cyclicité et la fertilité à la fois chez le bovin et l'humain. Les principales PG produites dans l'endomètre sont la PGE2 et la PGF2α. Leur action est régulée au niveau de la biosynthèse, du catabolisme et de la transduction de leur signal. Le but du présent projet était d'améliorer notre compréhension des mécanismes de production du PGF2α, étant moins connus que ceux du PGE2. Tout d'abord, chez le bovin, nous avons établi la voie impliquée dans l'induction du PGF2α par l'ocytocine (OT) et constaté qu'elle nécessite la transactivation d'EGFR. Deuxièmement, nous avons démontré que la protéine 4 associée à la résistance multiple aux médicaments (MRP4) est un transporteur fonctionnel sous le contrôle de l'OT et conduit à la libération polarisée des prostaglandines. Ensuite, comme plus d'une prostaglandine F2α synthase sont dentifiées dans l'endomètre humain, nous avons vérifié quelles enzymes encore non identifiées pourraient avoir le même rôle chez le bovin. Nous avons identifié et confirmé que les protéines recombinantes d'AKR1A1 et d'AKR1B1 sont les synthases les plus puissantes dans les deux espèces. Troisièmement, nous avons décrit l'association entre AKR1B1 et l'augmentation de la production PGF2α par les cellules endométriales humaines après stimulation par l'interleukine-1β (IL-1β). Comme plusieurs synthases sont exprimées simultanément dans l'endomètre, une preuve définitive du rôle d'AKR1B1 exigeait le KO du gène. Nous avons utilisé une approche utilisant l'ARN-guidée DNase Cas9 et les courtes répétitions palindromiques interespacées par grappes régulières (CRISPR) pour abolir l'expression du gène dans les cellules stromales. Le clone généré a produit une perte d'expression d'AKR1B1 et de production du PGF2α mais maintenu sa capacité à augmenter la production de PGE2 en réponse à l'IL-1β. En testant cela, nous avons aussi constaté que la PGF2α est capable de réguler la production de PGE2 en agissant sur le récepteur FP. Nos résultats confirment qu'AKR1B1 est fortement impliqué dans la synthèse de PGF2α et que cette activité représente une nouvelle et importante cible pour réguler les réponses inflammatoires et ischémiques associées à plusieurs pathologies humaines.

Abstract

Prostaglandins (PG) are key regulators of reproductive processes and a good balance in the relative production of different members is important for cyclicity and fertility in females. The main PG produced in the endometrium are PGE2 and PGF2α. Their action is controlled at the level of biosynthesis, catabolism and signal transduction. The aim of the present project was to improve our understanding of the production of PGF2α as it is underdocumented. In cattle, we established that induction of PGF2α by oxytocin (OT) required the transactivation of EGFR. We also showed that multidrug resistance protein 4 (MRP4) is a functional PG carrier under the control of OT contributing to the polarized release of prostaglandins in the bovine endometrium. Since two different enzymes exhibit PGFS activity in the human endometrium, we investigagted wheter other proteins could play the same role in the bovine endometrium. We have identified and confirmed that AKR1A1 and AKR1B1 recombinant proteins were the most powerful synthases in both species. Finally, AKR1B1 is a very effective PGF2α synthase in the bovine and human endometrium. We have proposed that AKR1B1 play a leading role in PGF2α production stimulation following interleukin-1β (IL- 1β) stimulation in human endometrial cells. However, because multiple synthases are expressed simultaneously in the endometrium, a definitive proof of the role of AKR1B1 would require knockout of its gene. Accordingly, we used an editing genome approach using RNA-guided cas9 DNase and clustered regularly interspaced short palindromic repeats (CRISPR) to abolish the expression of AKR1B1 gene in stromal cells. The resulting clone exhibited a complete loss of AKR1B1 expression and PGF2α production, but maintained the ability to increase PGE2 production in response to IL-1β. While testing this, we also found that PGF2α was able to regulate the production of PGE2 through a feedback loop involving the FP receptor. Our results confirm that AKR1B1 is the leading enzyme responsible for the synthesis of PGF2α thus representing a new and important target for the treatment of inflammatory and ischemic responses associated with human diseases.

Table des matières

Liste des tableaux

Liste des figures

Liste des abréviations et sigles

AA	acide arachidonique / arachidonic acid
ABCC	cassette C de liaison à l'ATP / ATP-binding cassette C
AC	adénylate cyclase / adenylate cyclase
AINS/NSAID	anti-inflammatoires non-stéroïdiens / non steroidal anti-inflammatory drug
AKR	aldo-céto réductase / aldo-keto reductase
AKR1A1	aldo-céto réductase A1 / aldo-keto reductase A1
AKR1B1	aldo-céto réductase B1 / aldo-keto reductase B1
AKR1C1	aldo-céto réductase C1 / aldo-keto reductase C1
AKR1C3	aldo-céto réductase C3 / aldo-keto reductase C3
AKR1C4	aldo-céto réductase C4 / aldo-keto reductase C4
AMPc/cAMP	adenosine monophosphate cyclique / cyclic adenosine monophosphate
ARI	inhibiteur d'aldose réductase / aldose reductase inhibitor
ATP	adénosine triphosphate / adenosine triphosphate
bEEL	cellules endométriales épithéliales bovines/ *bovine endometrial epithelial cells* (Notre laboratoire)
BEND	cellules endométriales bovines / *bovine endometrial cells* (Lignée commerciale)
CBR1	réductase de carbonyle 1 / carbonyl reductase 1
COX	cyclooxygénase / cyclooxygenase
cPGES	synthase de la prostaglandine E cytosolique / cytosolic prostaglandin E synthase
cPLA2	phospholipase A2 cytosolique / cytosolic phospholipase A2
CREBP	protéine liant l'élément de réponse à l'AMPc / cAMP response element-binding protein
CRISPR	courtes répétitions palindromiques interespacées par grappes régulières / clustered regularly interspaced short palindromic repeats
CSC	cellules stromales de caroncule bovin / bovine carancular stromal cells

DAG	diacylglycérol / diacylglycerol
DD3 (DDBX)	dihydrodiol déhydrogénase 3 / dihydrodiol dehydrogenase 3
DP	récepteur de la prostaglandine D2 / prostaglandin D2 receptor
EGFR	récepteur du facteur de croissance épidermique / epidermal growth factor receptor
ELISA	essai d'immuno-absorption enzymatique / enzyme-linked immunosorbent assay
EP	récepteur de la prostaglandine E2 / prostaglandin E2 receptor
ERK1/2	kinase ½ régulée par signal extracellulaire / extracellular signal regulated kinase ½
ERα	récepteur-α aux oestrogènes / estrogen receptor-α
E_2	oestradiol / estradiol
FGF	facteur de croissance des fibroblastes / fibroblast growth factor
FP	récepteur de la prostaglandine F2α / prostaglandin F2α receptor
GEF	facteur d'échange de nucléotide guanine / guanine exchange factor
GM-CSF	facteur stimulant les colonies granulocytes-macrophages / granulocyte-macrophage colony-stimulating factor
GMPc/cGMP	guanosine monophosphate cyclique / cyclic guanosine monophosphate
GPCR	récepteurs couplé aux protéines G / guanine nucleotide-protein coupled receptor
GSK3	glycogène synthase kinase 3 / glycogene synthase kinase 3
GST-μ	glutathion s-transférase / glutathione s-transferase
HIEEC	cellules épitéliales endométriales humaines immortalisées / *human immortalized endometrial epithelial cells*
HIESC	cellules stromales endométriales humaines immortalisées / *human immortalized endometrial stromal cells*
IBMX	isobutylméthylxanthine / isobutylmethylxanthine
ICSC	cellules stromales intercaronculaires / intercaruncular stromal cells
IL-1	interleukine-1 / interleukin-1
IL-6	interleukine-6 / interleukin-6
IFN	interféron / interferon

IFN- τ	interféron-τ / interferon-τ
IP3	inositol 1,4, 5 triphosphate / inositol 1,4, 5 triphosphate
IRAK	kinases associées au récepteur de l'interleukine-1 / interleukin receptor associated kinase
IL-1R	récepteur de l'interleukine 1 / interleukin 1 receptor
ISG	genes stimulés par l'interféron / interferon stimulated genes
JAK	janus kinase
LPS	lipopolysaccharide
MAPK	protéines kinases activées par des agents mitogènes / mitogen-actived protein kinase
MAPEG	protéines associées à la membrane dans le métabolisme des eicosanoïdes et du glutathion / membrane-associated proteins in eicosanoid and glutathione metabolism
MIF	facteur d'inhibition des macrophages / macrophage inhibitory factor
mPGES	synthase microsomale de la prostaglandine E / microsomal prostaglandin E synthase
MRP	protéine associée à la résistance à plusieurs médicaments / multidrug resistance protein
MRP4	protéine 4 associée à la résistance à plusieurs médicaments / multidrug resistance protein 4
NADH	nicotinamide adénine dinucléotide / nicotinamide adenine dinucleotide
NADP	nicotinamide adénine dinucléotide phosphate / nicotinamide adenine dinucleotide phosphate
NOS	protoxyde d'azote / nitrous oxide
OAS	2',5'-oligoadénylate synthétase / 2',5'-oligoadenylate synthetase
OATP	polypeptide de transport d'anion organique / organic anion transporting polypeptide
OD	densité optique / optical density
OT	ocytocine / oxytocin
OTR	récepteur à l'ocytocine / oxytocin receptor
P4	progestérone / progesterone
PG	prostaglandine / prostaglandin
PGD2	prostaglandine D2 / prostaglandin D2
PGE2	prostaglandine E2 / prostaglandin E2
PGES	prostaglandine E2 synthase / prostaglandin E2 synthase

PGF2α	prostaglandine F2α / prostaglandin F2α
PGFS	prostaglandine F2α synthase / prostaglandin F2α synthase
PGH2	prostaglandine H2 / prostaglandin H2
PGHS	prostaglandine H2 synthase / prostaglandin H2 synthase
PGI2	prostaglandine I2, prostacycline / prostaglandin I2, prostacyclin
PGEM	métabolite de la prostaglandine E2 / prostaglandin E2 metabolite
PGFM	métabolite de la prostaglandine F2α / prostaglandin F2α metabolite
PGT	transporteur de prostaglandine / prostaglandin transporter
PI3K	phosphoinositide 3-kinase / phosphoinositide-3-kinase
PKA	protéine kinase AMPc-dépendante / protein kinase cAMP dependant
PKC	protéine kinase C / protein kinase C
PLC	phospholipase C
PLA_2	phospholipase A_2
PMA	acétate et myristate de phorbol / phorbol myristate acetate
PQ	9,10-phénanthrènequinone / 9,10-phenanthrenequinone
PR	récepteur à la progestérone / progesterone receptor
P_4	progestérone / progesterone
RAS	protein du sacrome du rat / rat sarcoma protein
ROS	dérivé réactif de l'oxygène / reactive oxygen species
RTK	récepteur à activité tyrosine kinase / receptor tyrosine kinase
STAT	activateur de la transcription et transducteur de signal / signal transducer and activator of transcription
SV40	virus simien 40 / simian virus 40
TALEN	nucléases effectrices semblable à des activateurs de transcription / transcription activator-like nucleases
TNFα	facteur-α de nécrose de tumeur / tumor necrosis factor-α
TP	récepteur de la thromboxane / thromboxane receptor

TXA2	thromboxane A2
TXB2	thromboxane B2
ZFNases	nucléases à doigt de zinc / zinc finger nuclease

Cette thèse est dédiée à Marie-Josée, Loic et mes parents. Pour vous remercier pour tout le support que vous m'avez donné lors de ces travaux et de la rédaction de cet ouvrage. Sans vous, tout ce travail n'aurait pas le sens qu'il a aujourd'hui.

Remerciements

Les travaux présentés dans cette thèse n'ont pas uniquement été réalisé par moi et je souhaite en conséquence souligner ma reconnaissance envers ceux qui m'ont guidé, aidé et appuyé. Je remercie d'abord le Dr Michel A. Fortier pour avoir dirigé ma formation au cours des cinq dernières années. Je souligne également l'apport inestimable de M. Pierre Chapdelaine et Mme. Eva Bresson à la réalisation de mes travaux. Leur aide technique, leurs connaissances et support ont grandement contribué au succès de mes travaux. Je souhaite également remercier les équipes de Robert Sullivan et d'André Tchernof pour les collaborations et les prêts de matériel. Je remercie également les étudiants passés du laboratoire qui m'ont fait profiter de leur expérience scientifique, plus particulièrement Narayanan Krishnanswamy qui a amorcé ma formation à mon arrivée.

Avant-propos

Cette thèse est présentée avec insertion d'articles et comporte 8 chapitres.

L'introduction présente un résumé sur les prostaglandines, les différents facteurs les modulant, leur rôle dans le cycle reproducteur femelle chez l'humain et le bovin ainsi que l'hypothèse et les objectifs de cette thèse. Cette introduction vise à mettre en contexte les résultats qui sont présentés dans 6 articles correspondant à autant de chapîtres.

Les auteurs de l'article 1 qui a été publié dans la revue *Endocrinology* sont Krishnaswamy N (études préliminaires, expériences, rédaction), Lacroix-Pepin N (expériences, rédaction); Chapdelaine P (contribution au design expérimental); Taniguchi H; Kauffenstein G; Chakravarti A; Danyod G; Fortier MA (superviseur de la thèse, contribution au design expérimental et révision du manuscrit).

Les auteurs de l'article 2 qui a été publié dans la revue *Endocrinology* sont Lacroix-Pepin N (desing et stratégie expérimentale,toutes les expériences sauf qPCR, rédaction); Danyod G (études préliminaires, résultats qPCR); Krishnaswamy N (desing et stratégie expérimentale); Chapdelaine Pierre (contribution au design expérimental); Fortier MA (superviseur de la thèse, contribution au design expérimental et révision du manuscrit).

Les auteurs de l'article 3 qui a été publié dans la revue *Prostaglandins and Other Lipid Mediators* sont Lacroix-Pepin N (desing et stratégie expérimentale,expériences, rédaction); Chapdelaine P (contribution au design expérimental, aide technique); Fortier MA (superviseur de la thèse, contribution au design expérimental et révision du manuscrit).

Les auteurs de l'article 4 qui a été publié dans la revue *Frontiers Pharmacology* sont Bresson E (étude de AKR1B1 dans les lignées immortalisées, discussions et rédaction); Lacroix-Pepin N (fait les expériences bovines, conceptions des figures schématisées, discussions et rédaction); Boucher-Kovalik S (contribution aux expériences de AKR1B1 chez l'humain); Chapdelaine P (expériences sur les promoteurs, discussions et révision du manuscrit); Fortier MA superviseur de la thèse, contribution au design expérimental et rédaction du manuscrit).

Les auteurs de l'article 5 qui a été publié dans la revue *Molecular Human Reproduction* sont Lacroix-Pepin N (desing et stratégie expérimentale, expériences, rédaction); Chapdelaine P (contribution au design expérimental, modification des sgRNA, aide technique); Rodríguez Y (expériences complémentaires); Tremblay PJ (aide pour la conception des CRISPRs) et Fortier MA (superviseur de la thèse, contribution au design expérimental et révision du manuscrit).

L'article 6 est un résumé de résultats non-soumis présentés sous forme d'article du format *Prostaglandins and Other Lipid Mediators*. Les auteurs sont Lacroix-Pepin N (desing et stratégie expérimentale, expériences, rédaction); Rodríguez Y (aide pour les résultats humains); Fortier MA (superviseur de la thèse, contribution au design expérimental et révision du manuscrit).

Enfin, le dernier chapitre énonce les principales conclusions et perspectives de cette thèse.

L'annexe est constituée d'un article de collaboration publié dans la revue PLOS ONE. Les auteurs sont Michaud A (expériences, rédaction), Lacroix-Pepin N (fait les dosages de prostaglandines, contribution au design expérimental), Pelletier M, Veilleux A, Noël S, Bouchard C, Marceau P, Fortier MA (superviseur de la thèse, contribution au design expérimental et révision du manuscrit), André Tchernof (contribution au design expérimental et rédaction du manuscrit)

Note: Étant donné que les études présentées ont été effectuées chez l'humain et le bovin, dans la discussion, la nomenclature des aldo-céto réductases a été changée pour maintenir la clareté. Exemple: AKR1B1 et AKR1A1 deviennent hAKR1B1 et hAKR1A1 chez l'humain et bAKR1B1 et bAKR1A1 chez le bovin.

.

Chapitre 1: Introduction

1.1 Les prostaglandines

1.1.1 Présentation des prostaglandines

La biosynthèse des prostaglandines est illustée à la Figure 1. Les prostaglandines sont des molécules de signalisation dérivées de l'acide arachidonique, un acide gras à 20 carbones, et font donc partie de la famille des eicosanoïdes et ont une masse d'environ 350 g/mol. Caractérisées pour la première fois dans le sperme en 1935, elles sont reconnues comme des médiateurs de la douleur, de la fièvre, de l'inflammation et de l'hypertension (Hata and Breyer 2004; Jabbour and Sales 2004; Miller 2006; Ricciotti and FitzGerald 2011). Elles jouent aussi un rôle important dans l'angiogenèse (Wang and DuBois 2004), l'adhésion (Mayoral, Fernandez-Martinez et al. 2005) et la prolifération de certains types cellulaires (Yu, Wu et al. 2009). La PGE2 et la PGF2α sont les deux principales prostaglandines produites par l'endomètre qui sont fortement impliquées au niveau de la reproduction bovine (Poyser 1995) et humaine (Fortier, Krishnaswamy et al. 2008). Le tractus génital femelle et en particulier les cellules stromales et épithéliales de l'endomètre sont le site d'une intense production de ces deux prostaglandines, et il n'est donc pas étonnant qu'elles aient une importance marquée dans des fonctions telles que l'ovulation, la lutéolyse, l'implantation, et la parturition (Poyser 1995; Lim, Paria et al. 1997; McCracken, Custer et al. 1999; Arosh, Banu et al. 2004).

La première étape de leur formation est la libération de l'acide arachidonique (AA) à partir des phospholipides membranaires par une phospholipase cytosolique de type A2 (Arosh, Banu et al. 2004). Par la suite, la conversion de l'AA en prostaglandine H2 (PGH2) s'effectue par une cyclooxygénase (COX, PTGS ou PGHS). Par la suite, des synthases terminales spécifiques transformeront le PGH2 en prostaglandines actives (PGE2, PGF2α, PGI2, PGD2, TXA2) (Fortier, Krishnaswamy et al. 2008). Une fois relâchées, ces prostaglandines agiront via leurs récepteurs membranaires respectifs pour activer des voies de signalisations différentes telles que la protéine kinase C (PKC) ou la protéine kinase AMPc-dépendante (PKA) et ainsi induire des réponses physiologiques variées (Jabbour and Sales 2004). Des effets variés pour une même prostaglandine s'expliquent par le fait que certaines possèdent plusieurs récepteurs décrits à la section 1.1.6. C'est le cas du PGE2 qui peut agir via 4 récepteurs distincts (EP1 à 4 dont certains ont des rôles opposés, contrairement au PGF2α qui agira toujours via le récepteur FP (Hata and Breyer 2004)).

Étant chargées négativement, les PGs sont hydrophile et les membranes cellulaires sont subséquemment réfractaires à leur passage. Elles doivent par conséquent passer par un transporteur ou une forme d'endo/exocytose pour pénétrer ou sortir des cellules (Schuster 1998; Schuster 2002). L'endocytose des prostaglandines n'ayant jamais été démontrée et comme elles ne peuvent pas passer à travers les

cannaux ioniques, il doit donc y avoir un transporteur pour qu'elles puissent passer les membranes avec efficacité. Une fois libérées, les PGs exercent une action locale de courte durée terminée par leur dégradation spontanée (PGI1, TXA2) ou leur entrée dans la circulation où elles sont dégradées dès le premier passage au niveau des poumons (Piper, Vane et al. 1970; Shrestha, Beg et al. 2012).

Comme aucune prostaglandine ne subit d'accumulation dans les tissus et vu leur courte vie dans la circulation, elles servent principalement de messagers locaux (action paracrine et autocrine) dans des contextes variés (Ricciotti and FitzGerald 2011). Elles sont cependant essentielles pour plusieurs processus physiologiques et le double knockout murin des COX est létal tôt après la naissance (Reese, Paria et al. 2000; Loftin, Trivedi et al. 2001), démontrant qu'elles ont un impact vital au bon fonctionnement de l'organisme. Le role des PGs est décrit de manière plus détaillée à la section 1.3.

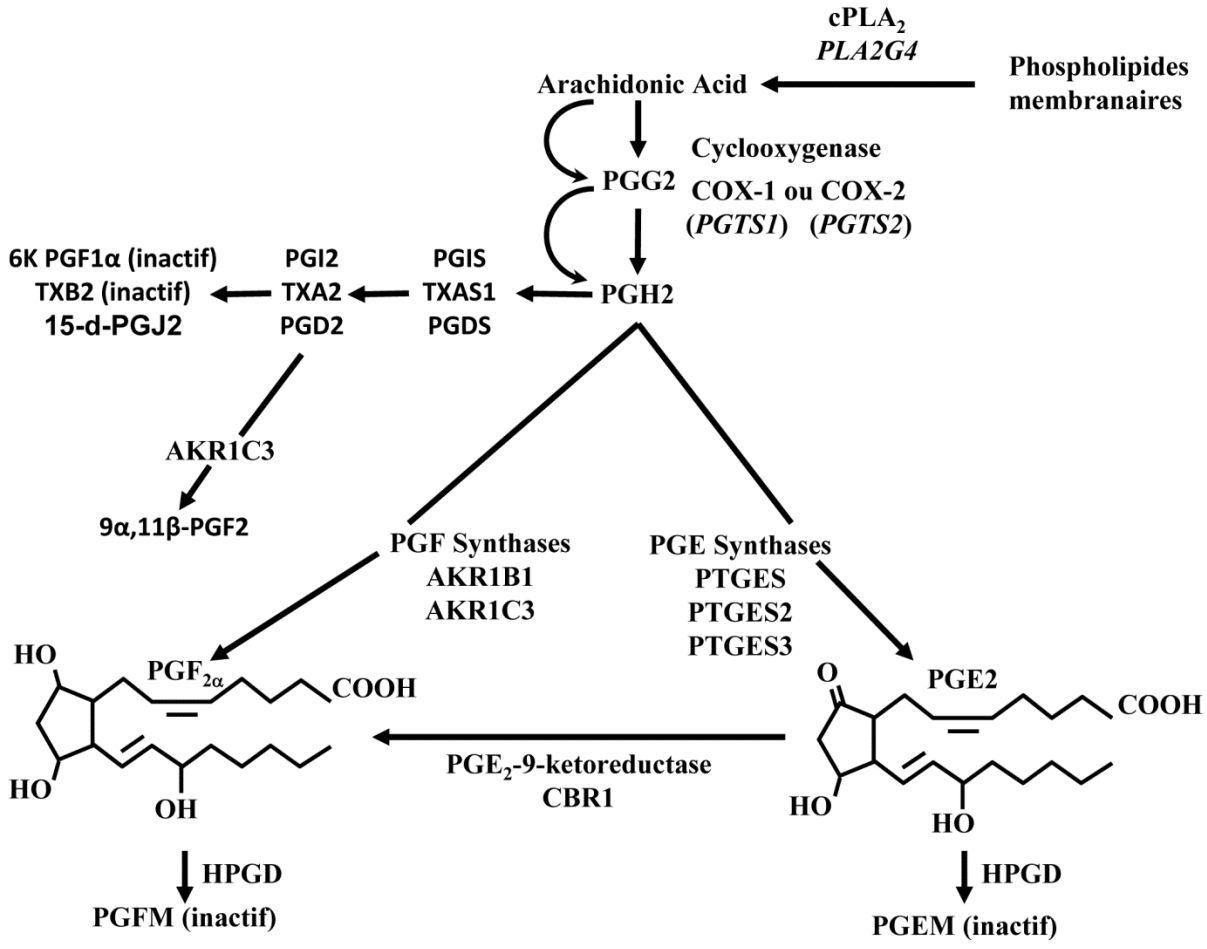


Figure 1: Schéma des enzymes impliquées dans la formation des prostaglandines.

1.1.2 Phospholipases

La phospholipase cytosolique A2α (cPLA2α) est la principale phospholipase responsable de libérer l'acide arachidonique des phospholipides membranaires qui constitue la première étape de la formation des prostaglandines (Kudo and Murakami 2002). La forme extracellulaire sécrétée (sPLA2) en est aussi capable mais semble avoir un rôle plus restreint (Grossman, Longo et al. 2000). Les deux isoformes sont dépendantes du calcium (Ca2+) et leur structure est très bien caractérisée (Dennis 1994). La phospholipase indépendante du calcium (iPLA2) est surtout impliquée dans le remodelage de la membrane plasmique, mais peut tout de même contribuer dans certains cas à la libération de l'acide arachidonique (Balsinde and Dennis 1997; Bingham and Austen 1999; Murakami and Kudo 2004). Une fois l'acide arachidonique libéré, ce sont les cyclooxygénases qui se chargent de l'étape suivante.

1.1.3 Cyclooxygénases

Les cycloxygénases sont responsables de la transformation de l'acide arachidonique en PGH2 (Figure 1). Cette réaction se fait en deux étapes. La première est la transformation de l'AA en PGG2 qui sera relâché et recapturé par l'enzyme pour être converti en PGH2 via deux sites catalytiques distincts (Simmons, Botting et al. 2004). C'est cette étape de la transformation de l'AA en PGH2 lors de la formation des prostaglandines qui est la cible des anti-inflammatoires non-stéroïdiens (NSAIDs) tel que l'aspirine et l'ibuprofène qui inhiberont de façon non sélective à la fois COX-1 et COX-2 (Priddy, Killick et al. 1990). Parmi les trois COX connues, COX-1 (PTGS-1) et COX-2 (PTGS-2) sont les principales isoformes (Arosh, Parent et al. 2002). COX-1 est constitutive et exprimée dans la plupart des types cellulaires. Elle est responsable des fonctions de base et de la réponse immédiate en prostaglandines à des niveaux élevés d'acide arachidonique (>10μM). COX-1 serait associée préférentiellement, mais pas exclusivement, à la thromboxane synthase, la prostaglandine E synthase cytosolique (cPGES) et la prostaglandine F synthase (PGFS). COX-2 quand à elle est inductible par divers facteurs pro-inflammatoires tels que les cytokines et les hormones et supporte la production prolongée à partir de niveaux plus faibles d'acide arachidonique (<10μM) (Sirois and Richards 1992 ; Arosh, Parent et al. 2002). Elle semble préférer la prostacycline synthase et la prostaglandine E synthase membranaire (mPGES). COX-3 quant à elle est dérivée du gène *PTGS-1* mais contrairement à celle du chien est non-fonctionnelle chez l'humain, , en raison de la rétention de son intron 1 qui fait 94 nucléotide et qui rend ainsi le reste de la protéine hors de son cadre de lecture {Kis, 2005 #3521}{Chandrasekharan, 2002 #2507;Kis, 2005 #3521}.

Suite à sa synthèse par COX-1 ou COX2, le PGH2 en lui-même est très instable. Il se convertira spontanément dans un délai de seulement 1 à 5 min en PGE2, PGD2 et PGF2α à la hauteur de 44%, 15% et

1,6% respectivement (Yu, Xiao et al. 2011). Afin de diriger leur formation, les synthases terminales (mPGES,-1, mPGES-2, cPGES, AKR1B1, AKR1C3, PGDS, PGIS, TXAS1) prendront rapidement en charge le PGH2 afin de le transformer en prostaglandines actives tel que décrit dans la section 1.1.4.

1.1.4 Synthases terminales

1.1.4.1 Prostaglandine E synthase

Jusqu'à ce jour, trois enzymes ont été caractérisées comme étant des PGE synthases fonctionnelles pouvant transformer le PGH2 en PGE2 (Figure 1). Elles sont respectivement prostaglandine E2 synthase-1 microsomale (mPGES-1/PTGES1) (Jakobsson, Thoren et al. 1999), prostaglandine E2 synthase-2 microsomale (mPGES-2/PTGES-2) (Tanikawa, Ohmiya et al. 2002) et prostaglandine E2 synthase cytosolique (cPGES/PTGES-3) (Tanioka, Nakatani et al. 2000). La mPGES-1 est inductible par les cytokines et le LPS (Jakobsson, Thoren et al. 1999) et est liée à l'inflammation, la fièvre et la douleur (Samuelsson, Morgenstern et al. 2007). Cette dernière contribue aussi à plusieurs maladies humaines dont l'athérosclérose (Wang, Song et al. 2008) et les cancers (Nakanishi, Gokhale et al. 2010). Elle fait partie de la famille des MAPEG (protéines associées à la membrane dans le métabolisme des eicosanoïdes et du glutathion) qui possède la capacité de conjuguer le glutathion. Autant la cPGES que la mPGES-1 nécessitent du GSH pour pouvoir catalyser la conversion du PGH2 en PGE2 (Smith, Urade et al. 2011). Il existe une association préférentielle entre mPGES-1 et COX-2 comparativement à COX-1, soutenant son rôle dans la réponse retardée et soutenue en PGE2 (Murakami, Naraba et al. 2000). La mPGES-2 et la cPGES sont exprimées constitutivement et semblent avoir un rôle dans l'homéostasie (Hara, Kamei et al. 2010). mPGES-2 a d'abord été décrite comme indépendante du GSH mais plus récemment démontrée comme liant à la fois l'hème et le GSH et possède une capacité de production de PGE2 *in vitro* mais pas *in vivo* due à sa liaison à l'hème (Takusagawa 2013). Cette découverte est cependant encore débattue (Watanabe, Ito et al. 2008; Takusagawa 2013). La mPGES-2 ne semble avoir aucune préférence particulière, s'associant autant à COX-1 qu'à COX-2 (Murakami, Nakashima et al. 2003). Le rôle de cPGES est cependant un peu controversé car elle semble pouvoir convertir le PGH2 à partir de COX-1 mais pas de COX-2 (Tanioka, Nakatani et al. 2000). Elle semblerait liée à la réponse immédiate en PGE2 en conjonction avec cPLA2 et COX-1 (Tanioka, Nakatani et al. 2000). Il existe aussi la forme μ de la glutathion s-transférase (GST-μ) qui semble capable de transformer le PGH2 en PGE2. Sa spécificité pour se coupler avec les COX reste encore à être déterminée (Murakami and Kudo 2004).

Dans l'endomètre humain et bovin, les trois PGE synthases sont exprimées durant le cycle en même temps que COX-1 et COX-2 (Fortier, Krishnaswamy et al. 2008). Chez le bovin, au niveau protéique, l'expression de cPGES est constante durant le cycle tandis que mPGES-1 est légèrement augmentée en fin

de cycle et mPGES-2 est plus fortement exprimée entre les jours 13 et 21 (Parent and Fortier 2005). Chez l'humain, au niveau du messager, *mPGES-1* est exprimée plus fortement autour des menstruations et diminuée au début de la phase sécrétoire, *mPGES-2* est constante durant le cycle et *cPGES* est augmentée durant les menstruations et diminuée durant la phase proliférative et à la fin de la phase sécrétoire (Catalano, Wilson et al. 2011).

Il serait particulièrement intéressant de pouvoir comparer les effets d'un inhibiteur de mPGES et de COX (Smith, Urade et al. 2011). Bien que plusieurs tentatives de développer un inhibiteur spécifique de mPGES-1 aient été entreprises, aucune n'est parvenue à un essai clinique. L'une des raisons principales semble être le manque d'homologie entre l'enzyme humaine et murine qui empêche les études d'efficacité pertinentes chez le rongeur (Xu, Rowland et al. 2008). Le développement d'une molécule efficace pouvant bloquer spécifiquement la synthèse de PGE2 reste donc encore à être fait.

1.1.4.2 Prostaglandine F synthase

Il y a deux sentiers principaux menant à la production de PGF2α impliquant respectivement la réduction de deux électrons du groupement 9,11-endopéroxide du PGH2 ou du groupement 9-keto du PGE2 (Figure 1). En plus, la réduction du groupement 11-keto du PGD2 donne un stéréo-isomère du PGF2α soit le 9α,11β-PGF2α (Watanabe 2002). La réaction la plus probable est cependant la réduction enzymatique directe du groupement 9,11-endopéroxide du PGH2, communément appelée activité PGF synthase (Fortier, Krishnaswamy et al. 2008). Plusieurs enzymes ayant cette capacité ont été identifiées. Chez le bovin, les premières furent la synthase pulmonaire (PGFS1, AKR1C7) (Watanabe, Yoshida et al. 1985), du foie (PGFS2, AKR1C11) (Kuchinke, Barski et al. 1992) et la DDBX (Suzuki, Fujii et al. 1999) tandis que chez l'humain AKR1C3 fut la première à se voir attribuer ce rôle (Suzuki-Yamamoto, Nishizawa et al. 1999). À ce jour, les enzymes capables de faire ces trois réactions appartiennent très majoritairement à la famille des aldo-kéto-réductases (AKR) (Smith, Urade et al. 2011). Les seules exceptions connues sont une enzyme de la famille "old yellow enzymes" chez *T. cruzi* (Kubata, Kabututu et al. 2002) et une synthase appartenant à la superfamille semblable aux thiorédoxines dans le cerveau de la souris et du porc (Moriuchi, Koda et al. 2008).

Les AKRs sont une famille d'enzymes monomériques solubles de 30-40 kDa et utilisant le NADPH plutôt que le NADH pour leur activité oxydoréductase, sans nécessiter de cofacteur métallique ou de flavine (Barski, Tipparaju et al. 2008). Dans les cellules métaboliquement actives, le NADP+ est le plus souvent trouvé sous sa forme réduite et en ce sens, la réduction est donc plus favorisée que l'oxydation (Pollak, Dolle et al. 2007). L'une des caractéristiques des AKRs est leur large éventail de substrats, allant de la réduction du

glucose, des glucocorticoïdes, de petits métabolites, des résidus carbonyles jusqu'à des conjugués glutathions et des aldéhydes de phospholipides (Barski, Tipparaju et al. 2008).

La PGE2 9-kétoréductase communément appelé carbonyle réductase (ou CBR1) catalyse la conversion du PGE2 en PGF2α et peut aussi faire la réaction inverse. Elle est dépendante du NADPH et a été purifiée dans le placenta chez le bovin et l'humain (Urade, Watanabe et al. 1995; Watanabe 2002). CBR1 ne présente pas qu'une activité PGE2 9-kétoréductase, c'est avant tout une carbonyle réductase ayant un vaste éventail de substrats. Chez le lapin, son équivalent (AKR1C5), possède une activité 20α-hydroxystéroïde déshydrogénase (20α-HSD) (Smith, Urade et al. 2011). En présence de NADP+, elle démontre aussi une activité 15-hydoxyprostaglandine déshydrogénase (HPGD de type II) (Chang, Sun et al. 1981; Wermuth 1981). Cependant, son k_m est beaucoup plus haut que la HPGD de type I (Tai, Ensor et al. 2002) et son rôle comme HPGD est donc probablement mineur. C'est son activité 20α-HSD qui a mené à la découverte d'AKR1B5 dans l'endomètre bovin (maintenant AKR1B1) (Madore, Harvey et al. 2003). Dans l'endomètre humain, la localisation de CBR1 est principalement dans l'épithélium glandulaire et luminal et son messager est augmenté durant la période menstruelle et diminue par la suite (Catalano, Wilson et al. 2011). Chez l'humain, AKR1C1 et AKR1C2 peuvent aussi présenter cette activité ainsi qu'une activité 11-kétoréductase (Nishizawa, Nakajima et al. 2000; Rizner 2012) mais aucune étude ne supporte leur implication dans ce processus *in vivo* (Catalano, Wilson et al. 2011). AKR1C1, AKR1C2 et AKR1C3 sont surtout reconnues *in vivo* pour leur implication dans le métabolisme des stéroïdes même si une implication dans le cas des prostaglandines n'est pas exclue (Rizner 2012).

L'enzyme responsable de la conversion du PGD2 en 9α,11β-PGF2α à été identifiée dans le poumon bovin (AKR1C7) (Watanabe, Yoshida et al. 1985) et humain (AKR1C3) (Suzuki-Yamamoto, Nishizawa et al. 1999). Le 9α,11β-PGF2α est équipotent au PGF2α pour la stimulation du récepteur FP (Sugimoto, Hasumoto et al. 1994) mais possède aussi des activités que la PGF2α n'a pas (Pugliese, Spokas et al. 1985). L'endomètre bovin (Ulbrich, Schulke et al. 2009) et humain (Rees and Kelly 1986) produisent du PGD2, cependant l'endomètre bovin est incapable de convertir la PGD2 en 9α,11β-PGF2α, ce qui s'explique par l'absence d'AKR1C7 (Madore, Harvey et al. 2003). L'endomètre humain possède AKR1C3 dans les cellules épithéliales mais pas dans les cellules stromales (Zakharov, Lin et al. 2010), malgré que les deux types cellulaires produisent du PGF2α (Schatz, Markiewicz et al. 1987). AKR1C3, en plus de sa capacité de transformer le PGD2 peut aussi faire la conversion directe du PGH2 en PGF2α selon le site actif qu'elle utilise (Watanabe, Yoshida et al. 1985) et possède aussi une activité 3α-hydroxystéroïde déshydrogénase de type II (3 alpha-HSD) (Komoto, Yamada et al. 2004).

La conversion du PGH2 en PGF2α sans passer par une autre prostaglandine active est le moyen le plus direct et fiable de produire ce métabolite. Cependant, pendant longtemps on a attribué l'activité PGF synthase à des enzymes convertissant une autre prostaglandine en PGF2α ou 9α,11β-PGF2α. Seul AKR1C3 (et ses homologues bovins AKR1C7 et AKR1C11) qui possédait une activité 11-kéto réductase avait la capacité de transformer directement le PGH2 (Watanabe 2011). Plus récemment, d'autres enzymes se sont montrées capables de convertir directement le PGH2 en PGF2α, plus particulièrement AKR5A2 et AKR5A1 dans les microorganismes *Trypasonoma brucei* et *Leishmania* respectivement (Kubata, Duszenko et al. 2000; Kabututu, Martin et al. 2003).

AKR1C3 a donc été longtemps reconnue comme la PGF synthase officielle chez l'humain faute d'en avoir identifié une autre capable de convertir le PGH2 plus efficacement. Cependant, l'utérus bovin qui produit de grandes quantités de prostaglandines est dépourvu d'AKR1C7 et d'AKR1C11 (Madore, Harvey et al. 2003). De plus, dans un système de coexpression utilisant les cellules HEK293, AKR1C7 n'a pas réussi à se coupler avec COX-1 ou COX-2, réduisant ses chances de contribuer à la production de PGF2α *in vivo* (Smith, Urade et al. 2011).

Cette absence de PGF synthase fonctionnelle dans l'utérus bovin a mené à la découverte d'AKR1B5, la 20α-hydroxystéroïde déshydrogénase (20α-HSD) bovine (maintenant rebaptisée AKR1B1), comme étant la PGF synthase prédominante dans l'endomètre bovin. Elle métabolise directement le PGH2 en PGF2α et l'on n'observe pas de conversion de PGE2 ou de PGD2 dans les cultures primaires épithéliales signifiant que son activité est directe (Madore, Harvey et al. 2003). Son homologue humain AKR1B1, mieux connue comme la principale enzyme du sentier des polyols, responsable de la conversion du glucose en sorbitol en conditions hyperglycémiques, possède aussi la capacité de convertir directement le PGH2 en PGF2α (Bresson, Boucher-Kovalik et al. 2011).

1.1.5 Transport des prostaglandines

1.1.5.1 PGT

Les prostaglandines sont des anions organiques au pH physiologique et existent donc sous forme de molécules chargées (Schuster 1998). Elles sont en conséquence pratiquement incapables de traverser les membranes cellulaires sans aide (Bito and Baroody 1975; Chan, Endo et al. 2002). Le premier et le plus caractérisé des transporteurs est le transporteur de prostaglandines (PGT). Il appartient à la famille des polypeptides transportant des anions organiques à 12 domaines transmembranaires (OATPs) (Schuster 1998). Il est exprimé dans plusieurs tissus mais en très forte quantité dans le poumon. Il est aussi fortement

augmenté dans l'endomètre vers la fin du cycle œstral chez le bovin (Banu, Arosh et al. 2003). Chez l'humain au cours du cycle menstruel, il est plus élevé dans les 2 premiers tiers du cycle (Jour 1-18) et chute en fin de cycle (Jour 19-30) (Kang, Chapdelaine et al. 2005). Son profil de préférence pour les substrats est PGE2 ≈ PGF2α > TXB2 > 6-Keto-PGF1α (Chan, Satriano et al. 1998; Schuster 1998). Le PGT transporte les prostaglandines indépendamment de la température ou de l'ATP mais catalyse l'échange prostaglandine-lactate et est donc dépendant du gradient de ce dernier comme antiporteur (Chan, Endo et al. 2002). Chez l'humain, une déficience en lactate déshydrogénase mène entre autre à une absence de contractions utérine et un échec du travail (Anai, Urata et al. 2002). Comme le gradient de lactate est majoritairement dirigé vers l'extérieur des cellules, PGT sert principalement à l'internalisation des prostaglandines plutôt que leur relâchement dans la plupart des types cellulaires (Chan, Endo et al. 2002).

1.1.5.2 MRP4

Puisque les prostaglandines ont besoin d'un transporteur d'influx pour pouvoir traverser les membranes et entrer dans la cellule, c'est aussi vrai pour l'efflux (Bito and Baroody 1975). La diffusion des prostaglandines et leur exocytose étant pratiquement inexistantes dans la plupart des types cellulaires, elles nécessitent donc un transporteur d'efflux pour pouvoir sortir des cellules et effectuer leur action (Chan, Satriano et al. 1998; Reid, Wielinga et al. 2003). Chez l'humain du moins, ce rôle semble être joué par un transporteur de type protéine associé à la résistance à plusieurs médicaments (MRP), membre de la sous-famille C de la famille des transporteurs avec casette liant l'ATP (ABCC) qui comporte à ce jour 9 membres (Russel, Koenderink et al. 2008). Il s'agit de MRP4 qui a été démontré comme étant un transporteur efficace des prostaglandines dans les cellules humaines, tandis que MRP1, MRP2, MRP3 et MRP5 n'en étaient pas capables dans des vésicules Sf9 les exprimant (Reid, Wielinga et al. 2003). MRP7 et MRP8 sont présents dans plusieurs tissus mais leur liste de substrats n'inclut pas les prostaglandines (Kruh, Guo et al. 2007). MRP9 quant à lui semble restreint aux seins et aux testicules. Il est fortement exprimé dans leurs cancers respectifs et semble incapable de transporter la majorité des substrats connus des autres MRP, incluant les prostaglandines (Bera, Iavarone et al. 2002). Comme la plupart des autres membres de sa famille, la liste de substrat transporté par MRP4 est très large, mais jusqu'à ce jour, il est considéré comme le seul membre qui transporte activement les prostaglandines et ce avec une assez grande affinité (Chen and Tiwari 2011). Il est vraisemblablement présent dans tous les tissus (Russel, Koenderink et al. 2008) et peut se retrouver préférentiellement sur l'une où l'autre des surfaces des cellules polarisées (Nies, Jedlitschky et al. 2004; Borst, de Wolf et al. 2007; Russel, Koenderink et al. 2008; Ming and Thakker 2010). Ce qui est d'autant plus intéressant est qu'il peut se localiser à la fois sur les membranes basolatérales et apicales, en fonction du tissu où il se trouve. Par exemple, dans les hépatocytes et la prostate, MRP4 se localise sur la membrane

basolatérale, alors que dans les tubules proximaux du rein, de l'intestin et des capillaires du cerveau, on l'a retrouvé à la membrane apicale (van Aubel, Smeets et al. 2002; Nies, Jedlitschky et al. 2004; Borst, de Wolf et al. 2007; Russel, Koenderink et al. 2008; Ming and Thakker 2010). Les mécanismes sous-tendant sa localisation spécifique n'ont été que partiellement élucidés (Hoque and Cole 2008; Hoque, Conseil et al. 2009). MRP4 est aussi un important transporteur de l'AMPc et contribue à la régulation de son niveau intracellulaire (van Aubel, Smeets et al. 2002). Aucune démonstration de l'impact d'un transporteur semblable n'a cependant été faite chez le bovin. Chez cette espèce on attribue encore la sortie des prostaglandines à PGT où à la diffusion facilitée (Schuster 2002).

Un des facteurs important de l'activité de MRP4 est que son transport des prostaglandines est inhibé par plusieurs anti-inflammatoires non-stéroïdiens tel que l'indométhacine et l'ibuprofène, s'ajoutant à leur rôle inhibiteur sur COX-1 et 2 (Reid, Wielinga et al. 2003). Étant donné que la grande majorité des inhibiteurs de ce transporteur sont aussi des inhibiteurs de la synthèse des prostaglandines, l'étude de son impact sur leur transport est complexe (Draper, Martell et al. 1997; Reid, Wielinga et al. 2003; Wu, Calcagno et al. 2005; Nozaki, Kusuhara et al. 2007).

Le MK-571 est un inhibiteur très efficace contre les transporteurs de type MRP (Gekeler, Ise et al. 1995). MRP4 est particulièrement sensible à cet analogue de LTD4 dans plusieurs espèces et types cellulaires (Reid, Wielinga et al. 2003; Wu, Calcagno et al. 2005; Wu, Klokouzas et al. 2005). Étant donné que MK-571 est capable d'inhiber le transport des nucléotides dans les plaquettes, il est reconnu que son action est majoritairement au niveau de l'activité du transporteur (Jedlitschky, Tirschmann et al. 2004). D'autres systèmes cellulaires ont aussi montré des effets inhibiteurs sur MRP4 sans affecter son expression protéique (Sinha, Ren et al. 2013). Ceci en fait un inhibiteur de choix pour l'étude de l'activité de MRP4.

1.1.6 Récepteurs

1.1.6.1 Récepteur EP

La PGE2 joue divers rôles importants dans plusieurs tissus. Dans le tractus gastro-intestinal elle joue un rôle protecteur et l'inhibition de sa synthèse par les NSAIDs induit de graves ulcères (Wallace, McKnight et al. 2000; Takeuchi 2012); il est essentiel à la reconnaissance de la gestation chez le bovin (Spencer and Bazer 2004; Lee, Banu et al. 2012); il est essentiel à la fonction rénale (Tang, Loutzenhiser et al. 2000); il est associé à la douleur et il a aussi été montré comme un modulateur du système immunitaire par la production d'IL-23 et d'IL-10 par les cellules dendritiques et l'inhibition de l'IL-2 dans les cellules Th1. Il induit normalement une inflammation locale forte mais possède aussi des effets anti-inflammatoires (Hata and

Breyer 2004; Kalinski 2012; Nakanishi and Rosenberg 2013). Il est un excellent vasodilatateur mais il a cependant un rôle contradictoire sur la contraction des muscles lisse tout comme dans le système immunitaire (Hata and Breyer 2004).

Les effets du PGE2 sont très bien caractérisés. Cependant, initialement, le fait qu'il exerce parfois des rôles opposés a mené à beaucoup de confusion. Ceci est dû au fait qu'il peut se lier à plus d'un récepteur et ainsi activer plusieurs voies de signalisation intracellulaire différentes (Figure 2). Il y a quatre sous-types de récepteur pour la PGE2 (EP1-4) encodés par 4 gènes distincts (Breyer, Bagdassarian et al. 2001). En plus, le récepteur EP3 possède 8 variantes produites par épissage alternatif et qui présentent des affinités distinctes pour PGE2. EP3 et 4 ont au moins 10 fois plus d'affinité pour la PGE2 que EP1 et 2 (Abramovitz, Adam et al. 2000) mais EP2 est capable de soutenir une stimulation prolongée contrairement à EP4 qui se désensibilise rapidement (Nishigaki, Negishi et al. 1996). Il faut donc caractériser l'effet du PGE2 selon le récepteur qu'il stimule. EP1 via sa sous-unité Gαq activera la PLC qui à son tour activera la PKC pour induire la mobilisation de calcium intracellulaire (Ca^{2+}) et induire la contraction des muscles lisses. Les récepteurs EP2 et EP4 via leur sous unité Gαs vont augmenter l'accumulation intracellulaire d'AMP cyclique (AMPc) et avoir un effet relaxant sur les muscles lisses. EP2 agira principalement via l'adénylate cyclase qui activera la PKA et la phosphorylation de la glycogène synthase kinase 3 (GSK-3). EP4 fera de même par un chemin plus complexe impliquant l'activation de PKA et de la kinase PI3 (PI3-K). EP2 peut induire la transactivation du récepteur du facteur de croissance épidermique (EGFR), menant à une activation de la voie des ERK1/2 (Sales, Maudsley et al. 2004). EP3 quant à lui induit plutôt un déclin de l'AMPc ou une augmentation et une mobilisation du calcium, selon le variant d'épissage activé (Breyer, Bagdassarian et al. 2001).

Des agonistes et antagonistes existent pour les différents récepteurs EP avec des sélectivités variables. Les agonistes les plus sélectifs sont ONO-DI-004 pour EP1, ONO-AE1-259, Butaprost et CAY-10399 pour EP2, ONO-AE-248 et SC-46275 pour EP3, ONO-AE1-329 et tetrazolo PGE1 pour EP4 (Sassone-Corsi ; Suzawa, Miyaura et al. 2000; Jones, Giembycz et al. 2009). EP1, EP3 et EP4 ont tous des antagonistes assez efficaces (Jones, Giembycz et al. 2009), cependant un antagoniste sélectif de EP2 est malheureusement non-disponible et son inhibition requiert donc l'utilisation d'antagonistes moins spécifiques à forte dose tel que le AH-6809 (Woodward, Pepperl et al. 1995). Il s'agit d'un antagoniste du récepteur EP et DP avec une affinité presque équivalente pour les récepteurs humain clonés EP1, EP2, EP3-III et DP1 (Abramovitz, Adam et al. 2000).

Dans les cellules endométriales bovines, les cellules stromales et épithéliales produisent toutes deux du PGE2 et possèdent le récepteur EP2, EP3 mais pas EP4 (Arosh, Banu et al. 2003; Arosh, Banu et al. 2004). *EP1* est détectable au niveau du messager dans les cellules endométriales mais pas dans l'oviducte

bovin (Gabler, Odau et al. 2008). Il est à peine détectable en protéines dans l'endomètre du mouton et ce seulement lors des premiers jours de grossesse puis n'est rapidement plus exprimé ensuite (Lee, Banu et al. 2012).

Dans l'utérus humain, le messager d'*EP1* est présent dans les cellules périphériques des glandes mais pas dans le stroma dans la période lutéale (Carson, Lagow et al. 2002) tandis que celui d'*EP2* et d'*EP4* sont présents à la fois dans le stroma et dans l'épithélium durant la phase proliférative et sécrétoire (Milne, Perchick et al. 2001). Quant à *EP3*, il est présent dans l'endomètre et le myomètre, mais deux isoformes (EP3-V et EP3-VI) sont présentes dans le myomètre et absentes de l'endomètre (Kotani, Tanaka et al. 2000). Les cellules épithéliales possèdent les 4 types de récepteurs contrairement au stroma qui en possède 3 étant donné l'absence d'*EP1* (Sun, Hsiao et al. 2003).

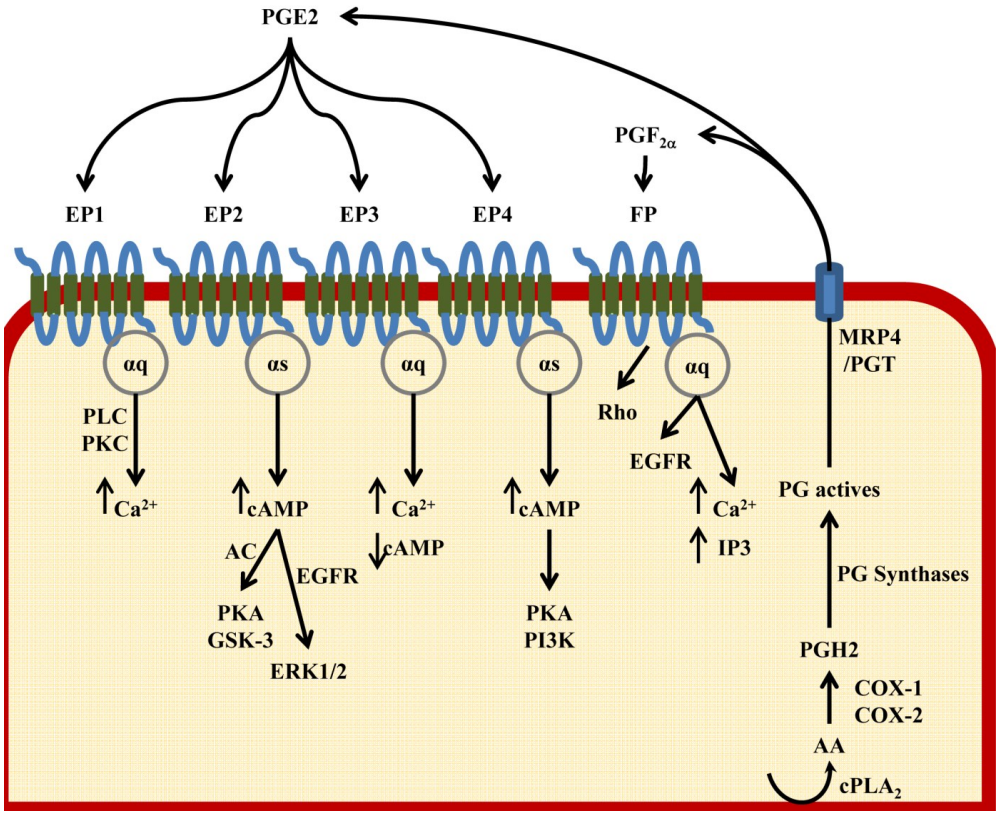


Figure 2: Schéma des voies de signalisation des récepteurs aux prostaglandines.

1.1.6.2 Récepteur FP

Contrairement au PGE2, le PGF2α se lie à un seul récepteur spécifique (FP). L'expression du récepteur FP à été démontrée dans le corps jaune, les myocytes ventriculaires, le rein et l'œil (Hata and Breyer 2004). Aucune expression n'a été observée dans la rate, le thymus ou les cellules immunitaires, signifiant que le récepteur FP ne semble pas avoir de rôle direct dans l'inflammation et les processus immunologiques (Tilley, Coffman et al. 2001). Il est produit dans l'endomètre majoritairement durant la phase sécrétoire du cycle chez l'humain (Abel and Baird 1980). Dans l'endomètre bovin, au niveau du messager, il est présent principalement dans le caroncule et plus faiblement dans l'inter caroncule (Arosh, Banu et al. 2004). Encore chez le bovin, le récepteur FP est localisé à la fin de la grossesse au niveau protéique dans les cellules épithéliales glandulaires et les cellules stromales (Wehbrink, Hassig et al. 2008). La souris knock-out pour le récepteur FP présente un taux élevé de progestérone durant la gestation menant à une réduction du récepteur à l'ocytocine et une parturition déficiente (Sugimoto, Yamasaki et al. 1997).

Comme pour le récepteur EP, le récepteur FP possède lui aussi un variant d'épissage qui à été mis à jour chez le mouton (Pierce, Bailey et al. 1997). FPA est la forme complète, tandis que FPB a une portion c-terminale tronquée. Ces deux variantes ont comme différence majeure leur taux de désensibilisation, FPA étant plus facilement désensibilisé par la PGF2α que FPB (Fujino, Srinivasan et al. 2000).

La PGF2α signale principalement via une sous-unité G_q, PLCβ et un relâchement de Ca^{2+} (Abramovitz, Boie et al. 1994; Watanabe, Nakao et al. 1994), stimulant la contractilité du myomètre et des vaisseaux sanguins (Lundstrom and Green 1978; Abramovitz, Boie et al. 1994; Bos, Richel et al. 2004; Jabbour and Sales 2004; Sales, List et al. 2005; Sales, Grant et al. 2008) (Figure 3). L'action du FP sur la phospholipase C-β (PLCβ) engendre la transformation du phosphatidylinositol 4,5-biphosphate (PIP_2) lié aux membranes en second messager inositol (1,4,5) trisphosphate (IP_3) et diacylglycerol (DAG). L'IP_3 engendre un relâchement de Ca^{2+} du réticulum endoplasmique tandis que le DAG active une sérine/thréonine kinase appelée PKC qui est souvent associée à la voie des MAP kinases via Raf et MEK. Comme plusieurs isoformes de PKC sont aussi activées par une hausse du Ca^{2+} intracellulaire, ces sentiers peuvent converger pour activer les mêmes effecteurs secondaires. Tout comme pour le récepteur EP2 qui peut stimuler EGFR, les cellules Ishikawa transfectées avec le récepteur FP et traitées avec du PGF2α ont rapidement montré une transphosphorylation du récepteur du facteur de croissance épidermique (EGFR) et la phosphorylation de la kinase régulée par signal extracellulaire ERK1/2 via PLCβ conduisant à une augmentation de la prolifération cellulaire (Sales, Milne et al. 2004; Sales, List et al. 2005). En plus de cette voie majeure, le récepteur FP peut aussi activer la voie de Rho sans activation de G_q et engendrer un réarrangement du cytosquelette (Pierce, Bailey et al. 1997). L'augmentation de COX-2 par le récepteur FP se fait via l'élément de réponse à l'AMPc

(CRE) sur le promoteur de COX-2 suite à une transactivation d'EGFR (Sales, Grant et al. 2008) et probablement une activation de la voie calcium-calmoduline (Abera, Sales et al. 2010).

Une fluctuation dans la production de PGF2α à été liée à plusieurs troubles reproductifs incluant des menstruations douloureuses prolongées avec des saignements importants (Smith, Abel et al. 1981), tandis qu'une augmentation du niveau du récepteur FP est impliquée dans les adénocarcinomes endométriaux (Sales, Milne et al. 2004). Le PGF2α joue aussi un rôle dans la fonction rénale (Breyer, Bagdassarian et al. 2001), l'hypertrophie cardiaque (Lai, Jin et al. 1996) et la pression intraoculaire (Alexander, Miller et al. 2002). D'ailleurs, les analogues de PGF2α sont utilisés pour traiter le glaucome et l'hypertension oculaire. Le récepteur FP est un peu moins spécifique que les autres récepteurs aux prostanoïdes, pouvant lier aussi le PGD2 et le PGE2 à des valeurs relativement proches du PGF2α. Dans les HEK293 le Km du récepteur FP est de 3.2, 6.7, et 119 nM pour le PGF2α, PGD2, et le PGE2 respectivement (Abramovitz, Adam et al. 2000). Plusieurs agonistes ont été tout de même produits et sont utilisés cliniquement, principalement dans la régulation de la pression oculaire tel que le fluprostenol, le latanoprost et le bimatoprost (Sharif, Williams et al. 2001; Alexander, Miller et al. 2002; Crowston, Lindsey et al. 2005). Le AL-8810 est un antagoniste de PGF2α peu efficace mais très sélectif vis-à-vis du récepteur FP. Il ne réagit pas avec le récepteur EP2 et 4, DP, TP et le récepteur à la vasopressine dans plusieurs types cellulaires à 10μM (Griffin, Klimko et al. 1999). Il est aussi important de noter que le PGF2α peut se lier avec une faible affinité à EP1 et EP3 et certains de ses effets pourraient en fait être induits via ces récepteurs (Breyer, Jacobson et al. 1993; Funk, Furci et al. 1993; Kiriyama, Ushikubi et al. 1997).

1.1.7 Catabolisme

La PGI2 et la TXA2 sont des PGs à action strictement locale car ces molécules sont instables et se dégradent spontanément en 6k-PGF1α et en TXB2 respectivement (Miller 2006). TXB2 est par la suite dégradée en 11-déhydo-TXB2 par la 11-TXB2-DH (Wu, Fritzo et al. 1990). Les autres prostaglandines sont plus stables mais rapidement métabolisées par oxydation de leur groupement 15(S)-hydroxyl par la 15-Hydroxyprostaglandin déshydrogénase (15-PGDH, HPGD) conduisant à des formes 15-cétoprostaglandines inactives (Anggard 1966). Il existe deux types de 15 PGDH, la type 1 est dépendante du NAD+ et métabolise les prostaglandines ainsi que le 9,10-phénanthrènequinone. Celle de type 2 utilise autant le NAD+ que le NADP+, possède un plus large éventail de substrats ainsi qu'une activité 9-cétoprostaglandine reductase (Tai, Ensor et al. 2002; Niesen, Schultz et al. 2010). Il s'agit de CBR1 et elle n'est pas réellement considérée comme spécifique aux prostaglandines pour cette activité contrairement à la 15-PGDH de type 1 qui est extrêmement efficace (Chang and Tai 1981; Tai, Ensor et al. 2002). La PGE2 et la PGF2α sont stables dans

le sang, cependant une fois dans la circulation, elles seront inactivées presque en totalité par la 15-PGDH au niveau du poumon après un seul passage (Piper, Vane et al. 1970; Tai, Ensor et al. 2002). Chez le bovin 65% sont transformées en un seul passage tandis qu'on parle de 99% chez le mouton et de 65-90% chez l'humain pour ces deux prostaglandines (Piper, Vane et al. 1970; Shrestha, Beg et al. 2012). Les PGD2 et TXB2 sont de mauvais substrats pour la 15-PGDH et PGD2 sera soit transformé spontanément en 15-d-PGJ2 ou enzymatiquement en 9α,11β-PGF2α par AKR1C3.

1.2 Modulation de la production des prostaglandines

1.2.1 Ocytocine

L'OT est une hormone neurohypophysaire de 9 acides aminés qui agit principalement via son propre récepteur (OTR) couplé aux protéines G (Gimpl and Fahrenholz 2001), lequel requiert du magnésium (Mg^{2+}) et du cholestérol pour son bon fonctionnement. OT fait partie des substances utilisées pour faciliter les accouchements (Dale 1906) depuis les années 1800 (Augustus Cory 1840) et est reconnue pour favoriser la lactation (Schafer and Mackenzie 1911). Son action ne se limite pas à ces deux rôles, mais inclut aussi la modulation de réflexes neuroendocriniens et l'établissement de comportements sociaux complexes reliés à la reproduction et aux soins de la progéniture (Gimpl and Fahrenholz 2001). OT peut même être utilisée pour traiter les comportements déficients liés à l'autisme (Bartz and Hollander 2008). Il faudra cependant attendre 1927 avant qu'elle ne soit purifiée et dissociée de la vasopressine (JAMA 1928). Son récepteur a quant à lui été séquencé en 1992 (Kimura, Tanizawa et al. 1992). Ce qui est clair, c'est que dans toutes les espèces étudiées, le récepteur à l'ocytocine subit une hausse de 10 à 100 fois dans l'utérus lors de la grossesse, démontrant son importance au niveau du système reproductif (Devost, Wrzal et al. 2008).

Dans les cellules humaines, l'OT induit la production de prostaglandines principalement via une sous-unité Gαq qui active la phospholipase Cβ (PLCβ) (Flint, Leat et al. 1986; Moore, Dubyak et al. 1988; Asselin, Drolet et al. 1998; Jeng, Liebenthal et al. 2000). Une fois activé, PLCβ réduit le phosphatidyl inositol 4,5-biphosphate (PIP_2) en inositol 1,4,5-triphosphate (IP_3) et 1,2-diacyl glycérol (DAG) qui sont des seconds messagers impliqués dans la libération du calcium intracellulaire et l'activation de la protéine kinase C (PKC), respectivement. Dans les cellules d'ovaires de hamster chinois (CHO), la transfection d'un récepteur à l'ocytocine du rat a montré que les voies des protéines kinases activées par des agents mitogènes (MAPK) et des kinases régulant des signaux extracellulaires ½ (ERK1/2) étaient les principales voies impliquées dans la production de PGE2 (Strakova, Copland et al. 1998). Ces voies de signalisation menant à une augmentation

de COX-2 a aussi été démontrée dans le myomètre humain (Molnar, Rigo et al. 1999). Il a aussi été rapporté que l'OT peut induire la phosphorylation de ERK1/2 via le récepteur de croissance épidermique (EGFR) dans les cellules myométriales et COSM6-OTR (Zhong, Yang et al. 2003). Chez le bovin, la voie de signalisation est présumée semblable mais n'a pas été étudiée en détail jusqu'à présent.

L'analyse du récepteur à l'ocytocine chez la brebis a démontré que durant le cycle l'OTR est abondant dans l'endomètre plutôt que dans le myomètre et que cette présence était corrélée avec la production de PGF2α en réponse à l'OT. Le récepteur commence à apparaitre dans l'épithélium luminal entre les jours 15-17 et induirait le relargage pulsatile de PGF2α responsable de la régression du corps jaune vers les jours 16-17. Chez l'humain, la concentration sanguine en ocytocine durant le cycle menstruel est relativement stable. On n'y observe aucune période de production accrue (Stock, Bremme et al. 1991) mais on peut observer une augmentation croissante durant la grossesse (de Geest, Thiery et al. 1985).

1.2.2 Interleukine-1α et β

L'IL-1α et l'IL-1β sont de puissants agents pro-inflammatoires et font partie de la famille des interleukines-1 qui comprend 11 cytokines encodées par 11 gènes différents. L'IL-1α et l'IL-1β se lient tous les deux au même récepteur IL-1RI qui possède un inhibiteur compétitif naturel nommé IL-1Ra. La fièvre est le principal symptôme résultant de l'action de l'IL-1, mais l'hyperalgésie, la vasodilatation, l'ovulation et l'hypotension sont d'autres exemples (Weber, Wasiliew et al. 2010). L'IL-1α peut agir via IL-1RI ou affecter directement la transcription alors que l'IL-1β agit exclusivement via le récepteur (Cohen, Rider et al. 2010). Les principales cellules produisant de l'IL-1 sont les cellules immunitaires mais sans être exclusives puisque les fibroblastes et les cellules épithéliales en sont aussi capables (Contassot, Beer et al. 2012). Les cellules répondent souvent à l'IL-1α par la production d'IL-1β. C'est notamment le cas des cellules endométriales stromales humaines (Semer, Reisler et al. 1991).

L'IL-1α et l'IL-1β sont de puissants stimulateurs des prostaglandines autant chez le bovin (Tanikawa, Acosta et al. 2005) que chez l'humain (Semer, Reisler et al. 1991), principalement par leur effet stimulateur sur COX-2 (Huang, Liu et al. 1998; Tanikawa, Lee et al. 2008). Au niveau de l'endomètre, chez le bovin, seules les cellules stromales produisent des prostaglandines suite aux stimulations par l'IL-1α et l'IL-1β (Tanikawa, Lee et al. 2008). Le messager de l'IL-1α ainsi que celui de l'IL-1β et de l'IL-1RI sont présents dans le corps jaune et l'endomètre bovin tout au long du cycle et au début de la gestation (Majewska, Woclawek-Potocka et al. 2010). In vivo chez le bovin, l'injection d'IL-1α agit comme agent lutéotropique et permet au corps jaune de prolonger le cycle au moins jusqu'au jour 30 (Majewska, Woclawek-Potocka et al. 2010). Chez l'humain,

l'expression de l'IL-1α est plus forte que celle de l'IL-1β et toutes deux sont exprimées tout au long du cycle (Tabibzadeh and Sun 1992). Autant les cellules stromales que les cellules épithéliales l'expriment et peuvent y répondre (Tabibzadeh and Sun 1992).

1.2.3 L'interféron-tau

Le facteur principal produit par l'embryon pour manifester sa présence une fois entré dans l'utérus (stade de 16 cellules) est l'interféron-tau (IFN-τ) (Roberts 2007; Bazer, Burghardt et al. 2008; Roberts, Chen et al. 2008). Cet interféron ne se retrouve que chez les ruminants et constitue le signal de reconnaissance de la gestation chez le bovin. Il appartient à la famille des interférons de type I et induit la cascade de signalisation JAK-STAT pour induire la production de gènes stimulés par l'interféron (ISG) (Roberts 2007; Bazer, Burghardt et al. 2008; Roberts, Chen et al. 2008; Ulbrich, Schulke et al. 2009). Il commence à être produit au stade blastocyste pour atteindre une production maximale au stade de trophoblaste vers le jour 14-15, pour finalement s'éteindre au moment de la placentation vers le jour 19-21 (Rodina, Cooke et al. 2009). Par la suite, il ne sera plus exprimé dans aucun tissu de l'animal (Roberts, Chen et al. 2008). Cette production de courte durée a lieu avant l'attachement à la paroi utérine et a pour but principal de stopper la régression du corps jaune et le maintien de la gestation. L'un des régulateurs important sa production est le facteur de croissance des fibroblastes (FGF) (Michael, Alvarez et al. 2006; Fields, Hansen et al. 2011) dont FGF1, 2 et 10 sont capables d'augmenter les niveaux (Cooke, Pennington et al. 2009).

Ses effets incluent la modulation des cellules immunitaires endométriales par la production de divers facteurs. Entre autres, il induit le facteur inhibant la migration des macrophages (MIF) produit par les cellules épithéliales (mais pas par les cellules stromales) (Wang and Goff 2003) ainsi que le GM-CSF produit par les leucocytes et les cellules stromales (mais pas par les cellules épithéliales) afin de stimuler la croissance et la survie de l'embryon (Emond, Asselin et al. 2000). Plusieurs autres classes de molécules sont aussi régulées par l'interféron-tau (Bazer, Burghardt et al. 2008). À faible dose, il inhibe la production de prostaglandines, tandis qu'à forte dose, il l'augmente (Asselin, Bazer et al. 1997; Asselin, Lacroix et al. 1997; Asselin, Drolet et al. 1998; Asselin and Fortier 2000; Guzeloglu, Michel et al. 2004). Son impact sur la production globale de prostaglandines est donc dépendant de sa concentration. Ceci a contribué à polariser la communauté scientifique entre deux principales hypothèses concernant le type de régulation produit par l'INF-tau. La première est que l'INF-τ empêche l'augmentation du récepteur aux œstrogènes ERα, empêchant une augmentation subséquente du récepteur à l'ocytocine (Spencer, Johnson et al. 2007). La deuxième hypothèse la plus reconnue est que l'INF-τ favorise une production de PGE2 au détriment du PGF2α tout en diminuant le récepteur à l'ocytocine (Asselin, Bazer et al. 1997; Arosh, Banu et al. 2004; Roberts 2007; Krishnaswamy,

Danyod et al. 2009). La lignée endométriale épithéliale bovine (bEEL) développée par le laboratoire de Michel A. Fortier a cependant conservé cette réponse à l'ocytocine, permettant de démontrer que l'INF-τ pouvait moduler la réponse à l'ocytocine en 3-6 hrs sans influencer l'expression de COX-2 ou le récepteur à l'ocytocine (Krishnaswamy, Danyod et al. 2009). C'est donc probablement le signal du récepteur qui est la cible de l'INF-τ dans son rôle de modulation des prostaglandines.

1.2.4 Les anti-inflammatoires non-stéroïdiens

Les anti-inflammatoires non-stéroïdiens (AINS ou NSAID) sont une classe de médicaments qui ont un fort potentiel analgésique, anti-inflammatoire et antipyrétique. Comme analgésiques, les AINS ont la particularité de ne pas être narcotiques et présentent donc peu de risque d'addiction. L'aspirine, l'ibuprofène et le naproxen sont les membres les plus connus (Warden 2010). Leur cible principale est la COX-1 et la COX-2. Bien que les effets désirés des AINS visent principalement COX-2, la plupart ne peuvent arriver à une réelle sélectivité et les deux isoformes sont inhibées *in vivo* surtout à cause des doses utilisées qui dépassent largement le spectre de sélectivité des différentes formulations (Warner, Giuliano et al. 1999). En pratique, toutes les prostaglandines seront donc inhibées par l'utilisation d'AINS. Plusieurs AINS dont l'ibuprofen et l'aspirine inhibent aussi l'activité de MRP4 qui chez l'humain est perçu comme le transporteur d'efflux des prostaglandines (Reid, Wielinga et al. 2003). L'aspirine est le seul AINS à inhiber les COX de façon irréversible (Toth, Muszbek et al. 2013). L'acétaminofène quant à lui, n'est pas considéré comme un AINS étant donné qu'il possède une faible activité anti-inflammatoire, même s'il s'agit d'un bloqueur probable de COX-2 ayant peut d'impact sur COX-1 (Hinz, Cheremina et al. 2008). Les effets secondaires des AINS sont principalement d'ordre gastrique et rénal mais peuvent aussi augmenter le risque de maladies cardiaques (à l'exception de l'aspirine à faible dose) (Gislason, Rasmussen et al. 2009; Trelle, Reichenbach et al. 2011). L'utilisation à long terme (plus de 3 mois) de AINS est aussi associée à une augmentation des troubles érectiles (Shiri, Koskimaki et al. 2006).

1.2.5 Les inhibiteurs d'aldose réductase ou ARIs

L'aldose réductase humaine ou AKR1B1 est présentement la meilleure PGF synthase connue à ce jour (Bresson, Boucher-Kovalik et al. 2011). Les ARIs ou inhibiteurs d'aldose réductase ont été développés pour bloquer la transformation du glucose en sorbitol (Yabe-Nishimura 1998; Petrash 2004). Plus particulièrement, dans les études cliniques, ils ont été testés pour leur efficacité à prévenir les neuropathies dues au diabète (Yabe-Nishimura 1998). Plusieurs ont échoué les études cliniques pour cause de trop faible

efficacité (Oates and Mylari 1999). Cependant, une seule activité dans un seul organe était étudiée. Un bon exemple est le ponalrestat (Statil) qui a été abandonné simplement par manque d'effet sur les neuropathies (Ziegler, Mayer et al. 1991) mais qui s'est montré un excellent inhibiteur de la production de PGF2α (Bresson, Boucher-Kovalik et al. 2011).

1.2.6 Méthylglyoxal

Le méthylglyoxal est une petite molécule fortement réactive, produite par le métabolisme même en conditions normales (Kalapos 2008). On le retrouve aussi en bonne quantité dans les aliments transformés (Thornalley 1993). Le méthylglyoxal est un électrophile réactif qui réagit spontanément avec les résidus arginine et arginyl des protéines pour créer un dérivé imidazole (Thornalley 1993) menant à la détérioration de sa structure et de ses fonctions (Kalapos 2008). On estime les niveaux normaux à 147 ± 78 nM dans le plasma de patients ou à 212 ± 73 nM lors de diabète de type 2 et pouvant aller jusqu'à 312 ± 135 lors de néphropathies diabétiques (Lu, Randell et al. 2011). Même si la concentration ne diffère que de 2-3 fois entre les patients normaux et diabétiques, on considère le méthylglyoxal comme un marqueur du diabète de type 2 (McLellan, Thornalley et al. 1994) qui peut être réduit par traitement à la metformine (Beisswenger, Howell et al. 1999). Plusieurs auteurs ont suggéré son rôle dans des maladies tel que le diabète de type 1 (Han, Randell et al. 2007), l'hypertension (Wang, Desai et al. 2004), l'alzheimer (Yu 2001; Kuhla, Luth et al. 2005), le parkinson (Oliveira, Lages et al. 2012) et le vieillissement (Ramasamy, Vannucci et al. 2005). La synthèse enzymatique du méthylglyoxal peut se produire de trois façons:

1- Par la méthylglyoxal synthase qui peut convertir le DHA-P en combinaison avec les isoenzymes du cytochrome P450 IIE1 et les amines oxydases participant au contournement glycolytique.

2- Le métabolisme de l'acétone.

3- La dégradation des acides aminés (Kalapos 1999).

Les produits terminaux de glycolation avancée (AGE) sont produits entre autres par la réaction entre le méthylglyoxal et diverses protéines. Chez les patients diabétiques, la formation des AGE se produit à grande échelle et se manifeste par des symptômes cliniques tel que les cataractes, l'athérosclérose, les néphropathies et les neuropathies (Calcutt, Cooper et al. 2009). Le méthylglyoxal est un substrat de l'aldose réductase placentaire (AKR1B1, EC: 1.1.1.21) (Thornalley 1993) et de l'aldéhyde réductase (AKR1A1, EC: 1.1.1.2) (Kalapos 1999) qui le convertissent en acétole (95%) et lactaldéhyde (5%) (Vander Jagt, Robinson et al. 1992). Un rôle protecteur de AKR1B1 sur les dommages au méthylglyoxal à des doses de 500 µM sur les

muscles lisse a déjà été étudié précédemment (Chang, Paek et al. 2002) et le ponalrestat augmente l'effet cytotoxique du méthylglyoxal (Yabe-Nishimura, Nishinaka et al. 2003). Un effet potentiel du méthylglyoxal sur la synthèse du PGF2α n'est donc pas exclu.

.

1.2.7 Glucose

Le glucose est un monosaccharide et une source d'énergie importante dans la plupart des organismes. En concentrations physiologiques, le glucose est majoritairement transformé en G-6-P, mais à fortes concentrations comme dans les cas de diabète, il peut être réduit en sorbitol par l'action de AKR1B1 dans le sentier des polyols chez l'humain (Yabe-Nishimura 1998; Pastel, Pointud et al. 2012). Ces voies métaboliques sont conservées dans la plupart des organismes. Le glucose est très stable comparativement à d'autres sucres, possédant un faible taux de réactions non-spécifique avec les protéines (Brownlee 1995). Par exemple, le fructose, dérivé du sorbitol, est 10 fois plus efficace pour former des AGE que le glucose (McPherson, Shilton et al. 1988).

Il n'y a que très peu d'études portant sur les influences mutuelles entre le glucose et les prostaglandines (Robertson 1983). Les taux de glucose circulant sont augmentés par la PGE et diminués par les NSAID, à l'exception de l'indométhacine qui induit une augmentation indépendante des prostaglandines (Robertson 1983). Cette nuance n'est probablement pas due à l'inhibition de l'activité cyclooxygénase, mais plutôt à un autre mécanisme. L'augmentation du PGE2 par le glucose est induite par PKC, du moins dans les cellules mésothéliales (Sitter, Haslinger et al. 1998). Le PGE peut stimuler la sécrétion d'insuline via l'AMPc, mais aussi inhiber le stimulus initial de la sécrétion d'insuline par le glucose (Robertson 1983). Chez le mouton, l'injection de prostaglandines ne fait pas varier le taux de glucose, mais en revanche, l'injection d'INF-τ induit une hausse du glucose dans le lumen utérin (Dorniak, Bazer et al. 2012).

1.3 Action des prostaglandines

1.3.1 Reconnaissance de la gestation chez le bovin

Le cycle œstral de la vache présenté à la Figure 2 a une durée de 21±3 jours où l'ovulation représente le jour 0 comparativement au jour 14 pendant le cycle menstruel humain (Ginther 1981; McCracken, Custer et al. 1999). La période de réceptivité au mâle précède l'ovulation de 24-48 heures et l'E2

descend abruptement suite à l'ovulation alors que le corps jaune commence à se former et à produire de la progestérone (P4). Vers la fin du cycle, l'ocytocine provenant de la neurohypophyse est libérée de façon pulsée et augmente la production de PGF2α par les cellules épithéliales de l'endomètre., Les cellules stromales étant dépourvues du récepteur (Asselin, Goff et al. 1996; Asselin, Drolet et al. 1998). Cette production initiale induit la production d'ocytocine par le corps jaune créant une boucle de rétroaction positive (Silvia, Lewis et al. 1991 ; Robinson, Mann et al. 1999). En absence de gestation la PGF2α agit directement sur le corps jaune pour induire sa régression vers les jours 16-17, (Figure 3) (Silvia, Lewis et al. 1991; Skarzynski, Ferreira-Dias et al. 2008). Une baisse du niveau de P4 et une augmentation des niveaux d'E2 se produira par la suite en raison de la croissance rapide des follicules jusqu'au jour 21. Par la suite, un nouveau cycle recommence.

La fenêtre de reconnaissance de la gestation est assez courte et se situe autour des jours 15 à 17 (Arosh, Banu et al. 2004), correspondant à l'expression maximale du récepteur à l'ocytocine entre les jours 13-15 (Robinson, Mann et al. 2001). Un signal suffisamment fort de PGE2 doit être émis par l'endomètre pour éviter la destruction du corps jaune (Okuda, Miyamoto et al. 2002) en plus d'exercer une action immuno-modulatrice qui favorise la tolérance immune envers la semi-allogreffe que représente l'embryon (Emond, Asselin et al. 2000). Plusieurs autres facteurs peuvent aussi influencer la production de PGE2 en réponse à l'embryon et le stade de réceptivité de l'endomètre à ce signal est crucial (Salilew-Wondim, Holker et al. 2010). L'un d'eux semble être l'IL-1α qui peut avoir un impact dans la régulation du corps jaune en évitant sa régression (Leung, Cheng et al. 2001; Nishimura, Bowolaksono et al. 2004; Majewska, Woclawek-Potocka et al. 2010).

L'IFN-τ est reconnu comme étant le facteur qui va induire la production de PGE2 essentielle à la reconnaissance de la gestation (Asselin, Lacroix et al. 1997) tout en favorisant une diminution de la production de PGF2α (Krishnaswamy, Danyod et al. 2009). Chez la vache gestante, les pulses de PGF2α sont tout de même présents mais beaucoup moins forts dû à l'impact de l'INF-τ (Leung, Cheng et al. 2001). Initialement, il a été proposé que l'interféron-tau exerçait un rôle inhibiteur sur la PGF2α en bloquant directement COX-2 ou encore en inhibant la hausse du récepteur à l'ocytocine (OTR) par la répression de la transcription du récepteur aux œstrogènes ERα (Spencer and Bazer 1996). Cependant, plusieurs études ont démontré que COX-2 est augmenté durant la fenêtre lutéolytique et la reconnaissance de la gestation chez la vache et le mouton et que l'INF-τ pouvait inhiber les prostaglandines en absence de baisse de COX-2 (Charpigny, Reinaud et al. 1997; Kim, Choi et al. 2003; Emond, MacLaren et al. 2004; Krishnaswamy, Danyod et al. 2009). Il a aussi été montré que les niveaux totaux de PGF2α et de son métabolite PGFM durant la fenêtre de reconnaissance de la gestation et le début de la grossesse sont élevés (Peterson, Tervit et al. 1976; Zarco, Stabenfeldt et al. 1988; Zarco, Stabenfeldt et al. 1988; Ulbrich, Schulke et al. 2009). L'INF-τ agit donc

probablement plus sur le rapport entre la PGF2α et la PGE2 que sur la quantité totale de PGF2α. Cette hypothèse est soutenue par le fait que de fortes concentrations d'INF-τ stimulent la production de PGE2 dans les cellules épithéliales primaires et les cellules épithéliales immortalisées BEND (Asselin, Bazer et al. 1997; Binelli, Guzeloglu et al. 2000). D'autres facteurs vont aussi influencer la production de PGE2 en réponse à l'embryon et le stade de réceptivité de l'endomètre à ce signal est crucial (Salilew-Wondim, Holker et al. 2010). Ceci fait en sorte que les mécanismes de communication embryo-maternelle dans la reconnaissance de la gestation sont beaucoup plus complexes qu'un simple signal binaire «oui/non».

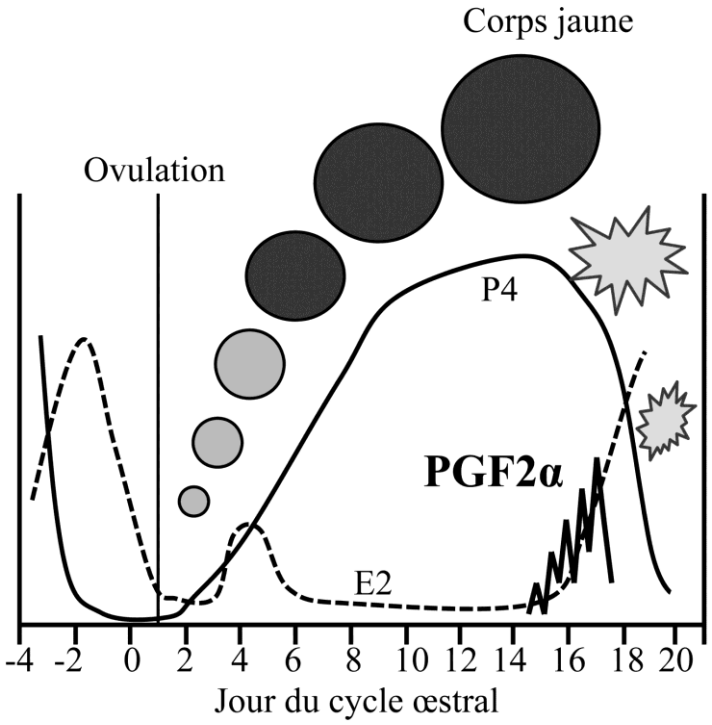


Figure 3: Schéma du cycle oestral bovin.

1.3.2 Implication des prostaglandines dans les maladies humaines

1.3.2.1 Désordres menstruels

Les désordres menstruels peuvent affecter les femmes tout au long de leur vie reproductive, mais sont plus souvent associés avec le début et la fin de la cyclicité de la femme. Il est postulé que les changements de mode de vie depuis la moitié du 20e siècle font en sorte que les femmes contemporaines ont environ 400 cycles comparativement à leurs aïeules qui en avaient environ 30-40 (Coulter, McPherson et al. 1988) et que cette augmentation est à l'origine de l'apparente prolifération des dérèglements menstruels

observés. Les désordres menstruels les plus fréquents sont la dysménorrhée et la ménorragie (Sales and Jabbour 2003; Jabbour, Kelly et al. 2006).

La dysménorrhée est caractérisée par des douleurs menstruelles excessives en absence d'anormalité pelvienne (Rees 1989). L'un des contributeurs majeurs est l'ischémie due à la réduction du flux sanguin lors d'une hypercontractilité utérine (Jabbour, Kelly et al. 2006). La PGF2α au sein de l'endomètre est à son plus haut niveau juste avant les menstruations et est responsable de la vasoconstriction, de l'ischémie, de la desquamation et des crampes dues à la contraction du myomètre (Jabbour, Kelly et al. 2006). La douleur associée à l'ischémie induite par la PGF2α peut être exacerbée par l'effet hyperalgésique de la PGE2 (Nishihara, Minami et al. 1995). Il a aussi été montré que les explants de l'endomètre de femmes souffrant de dysménorrhée produisaient plus de PGF2α en réponse à l'acide arachidonique que les femmes ne présentant pas la pathologie (Sales and Jabbour 2003).

On parle de ménorragie lorsqu'il y a perte de plus de 80 ml de sang durant les menstruations, résultant souvent en une anémie. Cette pathologie affecte 10-30% des femmes et jusqu'à 50% de celles en préménopause (Prentice 1999). Dans 90% des cas les femmes atteintes ne présentent aucune pathologie connue de l'endomètre (Farquhar, Lethaby et al. 1999). La pathologie serait caractérisée par un dérèglement dans l'homéostasie de l'endomètre menant à une perte 3 fois plus grande de sang dans les premiers jours des menstruations (Jabbour, Kelly et al. 2006). La quantité de PGE2 produite et l'abondance des récepteurs à la PGE2 tout comme la prostacycline et l'oxyde nitrique sont augmentés dans les cas de ménorragie (Hofmann, Rao et al. 1983). Des ratios élevés de PGE2 par rapport à la PGF2α caractérisent donc la ménorragie (Milne, Perchick et al. 2001).

Ces deux pathologies ont donc comme médiateur commun les prostaglandines et l'approche thérapeutique est l'utilisation de NSAIDs pour les inhiber via le blocage de la COX. Naturellement, les prostaglandines ne sont pas les seuls facteurs menant à la pathologie, mais sont tout de même suffisamment impliqués pour être la cible principale de traitement (Sales and Jabbour 2003; Jabbour, Kelly et al. 2006; Fortier, Krishnaswamy et al. 2008).

1.3.2.2 Diabète de type II

Le diabète de type 2 est caractérisé par une hyperglycémie et une résistance à l'insuline. L'hyperglycémie conduit à une augmentation du sorbitol par le sentier des polyols impliquant AKR1B1. La formation du sorbitol mène à une augmentation du stress oxydatif qui est l'un des aspects ayant le plus d'impact sur les complications précoces et à long terme du diabète (Obrosova 2005). L'aldose réductase

responsable de la conversion du glucose en sorbitol conduit aussi à une réaction inflammatoire (Ramana and Srivastava 2011).

Chez l'humain, le glucose aux concentrations normales ne constitue pas un bon substrat pour AKR1B1 (Srivastava, Ramana et al. 2005). En effet dans cette condition seulement 3% du glucose est transformé en sorbitol par AKR1B1 tandis qu'en concentrations plus élevées tel que retrouvé chez la patients diabétiques, jusqu'à 30% serait converti (Gonzalez, Barnett et al. 1984). AKR1B1 (Kasajima, Yamagishi et al. 2001) et la PGF2α (Helmersson, Vessby et al. 2005) sont augmentés lors de diabète de type II. Le lien entre la production de sorbitol, de PGF2α et les complications diabétiques devrait donc être étudiés étant donné leur rôle potentiel dans cette pathologie.

1.4 Problématique, hypothèse et objectifs

1.4.1 Problématique:

Les prostaglandines, avec les stéroïdes sexuels, sont d'importants régulateurs de la fonction reproductive. Chez le bovin, leur rôle est particulièrement important dans le contexte de reconnaissance de la gestation. La PGF2α et la PGE2 sont essentielles pour le déroulement normal du cycle œstral ainsi que pour la transition vers la reconnaissance et l'établissement de la gestation en présence d'un embryon viable. Chez l'humain, la contribution des prostaglandines à la condition médicale est documentée, mais se limite souvent au PGE2 ou à la dyade thromboxane / prostacycline. La PGF2α est peu étudiée dans le contexte des pathologies malgré l'évidence de son influence dans plusieurs dérèglements physiologiques. L'endomètre autant humain que bovin est un site intense de production de PGE2 et PGF2α par les cellules épithéliales et stromales. Ces prostaglandines sont les principales produites par l'utérus et jouent un rôle régulateur sur les fonctions reproductives dans les deux espèces. La production relative de ces deux prostaglandines représente un facteur clé de l'homéostasie dans l'endomètre tout comme dans plusieurs autres systèmes. Le système reproducteur féminin représente donc un modèle hautement pertinent pour l'étude des mécanismes associés aux prostaglandines tout en fournissant les bases pour comprendre leur action dans les autres systèmes. Malheureusement, alors que la biosynthèse du PGE2 a été étudiée en profondeur en relation avec la douleur, l'inflammation et le cancer, le rôle du PGF2α et sa biosynthèse ont souvent été négligés. Jusqu'à récemment AKR1C3 était la seule synthase de PGF2α identifiée chez l'homme. AKR1B1, mieux connue comme l'aldose réductase de la voie des polyols et présumée responsable de plusieurs complications du diabète, a été

démontrée comme ayant une forte activité PGF synthase. Elle est donc devenue un incontournable pour expliquer la régulation de cette prostaglandine. Son importance relative vis-à-vis d'AKR1C3 et son implication dans plusieurs pathologies où la PGF2α pourrait avoir un impact font l'objet de controverses. Chacune de ces enzymes ayant des caractéristiques distinctes, leur sensibilité aux inhibiteurs et compétiteurs métaboliques doit être évaluée séparément. Dans l'endomètre, beaucoup d'attention a été accordée à la PGE2 : en contrepartie, la biosynthèse et la fonction de la PGF2α, ainsi que l'implication des deux synthases AKR1B1 et AKR1C3 dans ces processus, sont mal comprises. Sachant que de nombreux médiateurs cellulaires ou solubles associés au cycle menstruel, à la grossesse, à la présence d'inflammation ou lors d'un désordre métabolique (diabète) peuvent influencer la production et la fonction des prostaglandines en général, il est primordial d'investiguer les mécanismes entourant la synthèse et la localisation de la PGF2α dans l'endomètre, en lien avec ces éléments. Ceci implique d'identifier des enzymes situées en amont dans la voie de synthèse de la PGF2α et les différents modulateurs de leur activité, ainsi que l'étude des processus entourant son transport à travers la paroi cellulaire; de plus, les facteurs régulant son activité (notamment ceux dictant sa liaison à ses récepteurs spécifiques) doivent être identifiés.

1.4.2 Hypothèse:

Les prostaglandines sont d'importants modulateurs de la fonction endométriale. Bien que l'importance globale des prostaglandines soit reconnue, celle de PGF2α en particulier est souvent négligée et considérée comme accessoire comparée à PGE2. **L'hypothèse de ce travail est qu'une régulation fine du PGF2α autant au niveau de la biosynthèse que de son action est essentielle et exerce plusieurs rôles physiologiques et pathologiques autant chez l'humain que chez le bovin.** Cette régulation peut se manifester au niveau de la biosynthèse, du transport, de la signalisation des récepteurs et des interactions avec d'autres substrats des enzymes catabolisant le PGF2α.

1.4.3 Objectifs:

1-Étude de la biosynthèse et du transport des prostaglandines chez le bovin.

Le premier objectif est de mieux caractériser les phénomènes entourant la production de PGF2α chez le bovin. Ceci, en étudiant les mécanismes stimulant la production de PGF2α, son transport et sa synthèse.

2- Étude de la biosynthèse de la prostaglandine F2α chez l'humain.

Le deuxième objectif est d'étudier chez l'humain la régulation des deux synthases connues soit AKR1B1 et AKR1C3 et leur impact relatif dans la production de PGF2α et dans les maladies humaines.

3-Implication des prostaglandines dans la communication stromale-épithéliale.

Le troisième objectif est de définir comment la communication via les récepteurs EP/FP influence les réponses subséquentes en prostaglandines ainsi que la communication entre les deux types cellulaires composant l'endomètre.

Chapitre 2: Epidermal Growth Factor Receptor Is an Obligatory Intermediate for Oxytocin-Induced Cyclooxygenase 2 Expression and Prostaglandin F2α Production in Bovine Endometrial Epithelial Cells.

Narayanan Krishnaswamy, Nicolas Lacroix-Pepin, Pierre Chapdelaine, Hiroaki Taniguchi, Gilles Kauffenstein, Arpita Chakravarti, Ghislain Danyod and Michel A. Fortier

Ce chapitre est présenté sous forme d'article et a été publié dans la revue :
Endocrinology. 2010 Mar;151(3):1367-74

Centre de Recherche du CHUQ, Département d'Obstétrique et Gynécologie, Unité d'Ontogénie et Reproduction, Centre de Recherche en Biologie de la Reproduction,
Université Laval, Ste-Foy, Québec, Canada GIV 4G2

RÉSUMÉ

L'ocytocine (OT) déclenche les impulsions lutéolytiques de la prostaglandine F2α (PGF2α) à partir des cellules épithéliales de l'endomètre chez les ruminants. Nous avons proposé que le signal embryonnaire interféron-τ exerce son effet antilutéolytique en perturbant l'axe de signalisation de l'OT. En conséquence, nous avons essayé de définir la voie de signalisation de la production PGF2α induite par l'OT dans l'endomètre bovin en utilisant notre lignée de cellules épithéliales nouvellement caractérisée (bEEL). Le récepteur OT est couplé à la voie Gaq classique comme en témoigne la libération de calcium et l'activation de la phospholipase C. De même, la production de PGF2α par l'OT a été médiée par la voie canonique ERK1/2. En raison de l'importance des récepteurs et enzymes cytoplasmiques de type tyrosine kinases dans la signalisation des récepteurs couplés à la protéine G, nous avons étudié le rôle du récepteur épidermique de facteur de croissance (EGFR), c-Src, et phosphoinositide 3-kinase (PI3-K) sur la production de PGF2α induite par l'OT en association avec la cyclooxygénase 2 (COX-2), ERK1/2 et la phosphorylation de Akt. L'inhibiteur de EGFR AG1478 (10 μm) a aboli la production de PGF2α basale et induite par l'OT et induit une diminution de COX2 et de la phosphorylation d'ERK1/2. Parce que l'EGFR transactivé peut servir de ligand pour les protéines de signalisation avec un domaine d'homologie à Src (SH2), nous avons supposé un rôle de c- Src et PI3K dans la production PGF2α induite par l'OT. Les inhibiteurs de c-Src (PP2, 10 μm) et PI3-K (LY294002, 25 μm) ont entraîné une diminution significative de la production PGF2α induite par l'OT et réduit l'expression de COX2. Aussi, PP2, mais pas LY294002, a diminué la phosphorylation de ERK1 / 2 par l'OT. Parce que LY294002 n'a pas affecté la phosphorylation de ERK1 / 2, mais a inhibé la production de PGF2α et l'expression de COX2, il est probable que la voie Akt est également impliquée dans la production de PGF2α. Ainsi, EGFR peut activer simultanément c -Src et PI3K pour amplifier la signalisation à l'OT afin d'augmenter la production de PGF2α dans les cellules bEEL.

ABSTRACT

Oxytocin (OT) triggers the luteolytic pulses of prostaglandin F2α (PGF2α) from the endometrial epithelial cells in ruminants. We have proposed that the embryonic signal interferon-τ exerts its antiluteolytic effect by disrupting the OT signaling axis. Accordingly, we have attempted to define the signaling pathway of OT-induced PGF2α production in the bovine endometrium using our newly characterized epithelial cell line (bEEL). OT receptor was coupled to the classical Gαq pathway as evidenced by calcium release and activation of phospholipase C. Similarly, OT-induced PGF2α production was mediated through the canonical ERK1/2 pathway. Because of the importance of receptor and nonreceptor tyrosine kinases in G protein-coupled receptor signaling, we studied the role of epidermal growth factor receptor (EGFR), c-Src, and phosphoinositide 3-kinase (PI3K) on OT-induced PGF2α production in association with cyclooxygenase 2 (COX2) expression and ERK1/2 and Akt phosphorylation. The EGFR inhibitor AG1478 (10 μm) nearly abolished basal and OT-induced PGF2α production and down-regulated COX2 expression and ERK1/2 phosphorylation. Because the transactivated EGFR can serve as a ligand for the signaling proteins with Src homology 2 (SH2) domain, we hypothesized a role for c-Src and PI3K in OT-induced PGF2α production. Inhibitors of c-Src (PP2, 10 μm) and PI3K (LY294002, 25 μm) produced a significant decrease in OT-induced PGF2α production and reduced COX2 expression. Also, PP2, but not LY294002, decreased OT-induced ERK1/2 phosphorylation. Because LY294002 did not affect ERK1/2 phosphorylation, but inhibited PGF2α production and down-regulated COX2 expression, it is likely that the Akt pathway is also involved in PGF2α production. Thus, EGFR may simultaneously activate c-Src and PI3K to amplify the OT signaling to increase the output of PGF2α in bEEL cells.

INTRODUCTION

Prostaglandins (PG), notorious mediators of pain and inflammation, also regulate a wide range of physiologically important female reproductive functions including ovulation, luteolysis, implantation, cervical ripening, parturition, and postpartum involution of the uterus in mammals (Weems, Weems et al. 2006). In ruminants, oxytocin (OT) triggers the pulsatile release of PGF2α by the endometrium at the end of an infertile estrous cycle to induce luteolysis (McCracken, Custer et al. 1999). OT receptor (OTR), which is up-regulated during the late luteal phase and at estrus, is hypothesized to be down-regulated during early pregnancy by the conceptus-derived trophoblastic interferon-τ (IFNτ) (Spencer, Burghardt et al. 2004). However, our prima facie evidence suggests an alternative hypothesis whereby IFNτ impairs the signaling axis of OT-induced PGF2α to exert its antiluteolytic effect in bovine endometrial epithelial cells (bEEL) (Krishnaswamy, Danyod et al. 2009). Understanding the signal transduction pathway of OT-induced PGF2α production will provide insight into the mechanisms underlying maternal recognition of pregnancy in ruminants.

OT signals through its cognate receptor that belongs to the large family of membrane-bound heptahelical guanine nucleotide-binding protein (G protein)-coupled receptors (GPCR) (Gimpl and Fahrenholz 2001). In the ovine endometrium, OT stimulated the release of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) presumably through the activation of phospholipase C (PLC) (Flint, Leat et al. 1986) and DAG-induced PGF2α production (Silvia, Lee et al. 1994). In the bovine, OT stimulated the release of IP3 (Asselin, Drolet et al. 1997), and Ca^{2+} was essential for PGF2α release (Burns, Hayes et al. 1998). OT was shown to induce PGE2 production through a PLCβ-DAG-protein kinase C (PKC)-ERK1/2 pathway in an OTR overexpression model (Strakova, Copland et al. 1998). In addition, OT activated the ERK1/2 (also called p42/44 MAPK) pathway in the ovine endometrium (Burns, Hayes et al. 1998) and human breast Hs578T cells (Copland, Jeng et al. 1999). In addition to the Gαq subunit (Gimpl and Fahrenholz 2001), it has been shown that pertussis toxin-sensitive Gαi was also involved in ERK1/2 activation in human myometrial (Ohmichi, Koike et al. 1995) and OTR-transfected CHO cells (Strakova, Copland et al. 1998). Thus, the available evidence suggests that both pertussis toxin-insensitive Gαq and pertussis toxin-sensitive Gα subunits are functionally linked to OT-induced ERK1/2 phosphorylation depending on the cell type.

Since 1996, many GPCR agonists like endothelin-1, lysophosphatidic acid, and thrombin were shown to activate the ERK1/2 pathway by a novel mechanism involving epidermal growth factor receptor (EGFR) as a signaling intermediate through receptor transactivation (Daub, Weiss et al. 1996; Ohtsu, Dempsey et al. 2006). This process required the activation of a metalloprotease by the agonist activated GPCR and the subsequent release of 'soluble EGF-related peptides' that serve as a ligand for EGFR (Prenzel, Zwick et al.

1999). Furthermore, EGFR was shown to mediate the effects of OT in human myometrial and COSM6-OTR cells (Zhong, Yang et al. 2003).

Recently, we have reported the establishment of the bEEL cell line with functional OTR coupled to PGF2α production (Krishnaswamy, Danyod et al. 2009). In this paper, we first tested the role of the Gαq-PLC pathway, ERK1/2 activation, and involvement of EGFR in OT-induced PGF2α production bEEL cells. Second, we studied the modulation of OT-induced cyclooxygenase 2 (COX2) expression and ERK1/2 and Akt phosphorylation by EGFR, c-Src, and phosphoinositide 3-kinase (PI3K). Third, we demonstrated that EGFR transactivation might lead to the recruitment of c-Src and PI3K and propose a role for the Akt (protein kinase B) pathway in OT-induced PGF2α production. Finally, we propose that EGFR plays a central role in OT-induced PGF2α production and ERK1/2 activation in bEEL cells.

MATERIALS AND METHODS

Materials

RPMI 1640 medium was purchased from Invitrogen (Burlington, Ontario, Canada); fetal bovine serum, Hanks' balanced salt solution (HBSS), penicillin, and streptomycin were from Multicell, Wisent Inc. (Quebec, Canada); and six- and 24-well format culture plates and other culture-wares like flasks, filters, and pipettes were from Sarstedt (St. Leonard, Quebec, Canada). Biochemicals like fura-2 AM, GW5074, phorbol myristate acetate (PMA) and OT were sourced from Sigma (Oakville, Ontario, Canada); AG1478, LY294002, manumycin, PD98059, and PP2 were from Calbiochem, Canada. Protein marker and nitrocellulose membrane were from Bio-Rad (Mississauga, Ontario, Canada); Western enhanced chemiluminescent kit was from PerkinElmer (Norwalk, CT); Biomax x-ray film was from Kodak Corp. (Rochester, NY); laboratory chemicals like glycine, sodium dodecyl sulfate, Tris, Tween 20, glycerol, dimethylsulfoxide (DMSO), sodium and potassium phosphates, and sodium chloride were from Fisher Chemicals (Mississauga, Ontario, Canada); ELISA plates were from Nunc Corp. (Roskilde, Denmark); rabbit antisheep antibody (Ab) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); $PGF_{2\alpha}$ Ab was from BioQuant (Ann Arbor, MI); and acetyl choline linked tracer and U73122 were from Cayman Chemicals (Ann Arbor, MI).

Cell culture

Stock bEEL cells were thawed and seeded in 75-cm^2 culture flasks containing RPMI 1640 supplemented with fetal bovine serum (10%) and strepto-penicillin (1%), and the medium was replaced every 48 h. Confluent cultures were trypsinized and seeded in 24-well format culture plates (for experiments

involving $PGF_{2\alpha}$ assay) or six-well plates (for Western blotting experiments) at 4×10^4 cells/ml so that confluency was seen by 96 h. On d 5, culture medium was replaced with fresh medium supplemented with 10% dextran-charcoal-extracted (steroid-free) serum for overnight preconditioning.

Treatment protocol

Serum-free RPMI 1640 served as the solvent for the reagents. On d 6, each 24-well plate was preincubated for 1 h with vehicle (DMSO or dichloromethane) or inhibitor (except for Fig. 2C). After aspirating the medium, the cells were treated in the presence or absence of OT (500 nM) and/or inhibitor for 6 h. Supernatant was harvested and stored at −20°C until assay for $PGF_{2\alpha}$, and the lysate was used for COX2 and β-actin immunoblots. The concentration of OT (500 nM) used in the study corresponds to the EC50 described for bEEL and primary bovine endometrial epithelial cells (Asselin, Goff et al. 1996; Krishnaswamy, Danyod et al. 2009). For immunoblotting of phospho-ERK1/2 and phospho-Akt (six-well dish), cells were serum starved 24 h before preincubation to minimize background phosphorylation caused by the serum growth factors and then treated with or without OT for 5 min. The stimulation was stopped by the addition of ice-cold PBS. Cell lysate preparation, protein extraction, and estimation were done as described previously (Chapdelaine, Vignola et al. 2001).

Enzyme immunoassay

$PGF_{2\alpha}$ was assayed by custom-made competitive ELISA as described previously (Asselin, Goff et al. 1996).

Western blotting

An aliquot of 10–20 μg protein was loaded in each lane, resolved in 10% SDS-PAGE, and electrotransferred onto 0.45-μm nitrocellulose membrane. The membrane was blocked in 5% (wt/vol) nonfat dry milk resuspended in PBS containing 0.05% Tween 20 for 1 h and incubated overnight at 4 C with primary Ab such as phospho-ERK1/2 Ab (Cell Signaling Biotechnology, Beverly, MA; 1/1000 dilution in 5% BSA-PBS-Tween 20), phospho-Akt Ab (Cell Signaling; 1/1000 dilution in 5% BSA-PBS-Tween 20), COX2 (a gift from Dr. K. S. Kargman, Merck Frosst, Montreal, Canada; 1/3000 in fat-free milk-PBS-Tween 20) or β-actin (Sigma, Canada; 1/5000 in fat-free milk-PBS-Tween 20). The membrane was washed 10 min in PBS-Tween three times and incubated with the appropriate secondary Ab such as goat antirabbit or goat antimouse (1/10,000) for 1 h. After three washes of 10 min each, the membranes were exposed to enhanced chemiluminescent substrate. The membrane was stripped and reprobed for total ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1/1000 dilution) or pan-Akt (cell Signaling; 1/1000). Total cell lysate from three different experiments was used for immunoblotting of all the proteins tested, and a representative blot is presented.

Intracellular Ca^{2+} mobilization

Intracellular Ca^{2+} mobilization was done as reported elsewhere (Ambler and Taylor 1986). Briefly, bEEL cells were seeded and grown as described above onto rectangular glass coverslips (10 × 20 mm) in a six-well culture plate. Confluent coverslips were washed briefly in HBSS and incubated with Ca^{2+}-sensitive fluorescent dye fura-2 AM (2.5 μM) for 45 min at room temperature in the dark. After three washes in HBSS, two coverslips were placed back to back into a 1-cm^2 quartz cuvette fitted with a nonreactive support stage containing 3 ml HBSS using a holder. Coverslips fitted snugly into a horizontal diagonal slot in the stage, and the bathing medium was permanently stirred with a magnetic bar to allow fast agonist mixing. Fluorescence was measured using a spectroflurometer (Fluorolog; Horiba Jobin Yvon Inc., Edison, NJ). Cells were excited alternately at 340 and 380 nm, and the signal emitted at 510 nm was collected. Peak excitation of Ca^{2+}-bound fura-2 was at 340 nm, whereas it was 380 nm for free-fura-2. Results are expressed as 340/380 ratio. Basal tracings were recorded for 45 sec before the addition of OT (500 nM) or ATP (100 μM).

Statistical analysis

Completely randomized design with equal sample size was used for the experiments, where treatment with inhibitors represented the main effect. Because a maximum of six experimental units (replicates) can be allotted per group for four treatments in a 24-well format culture, each experiment was repeated thrice. The resulting data on $PGF_{2\alpha}$ (nanograms per milliliter) is expressed as mean ± SEM (n = 18) and used for data analysis. The group mean of different treatments was tested by one-variable ANOVA using GraphPad Prism 5. If the null hypothesis was rejected, Tukey's multiple comparison was used as a *post hoc* test to find the critical difference between pairs of treatment means. In all the experiments, confidence level was set at 95% to determine the significance of difference ($P < 0.05$).

RESULTS

Effect of OT on the release of intracellular $Ca^{2+}$and ERK1/2 phosphorylation and the role of PLC and PKC on OT-mediated $PGF_{2\alpha}$ production in bEEL cells

Because OT stimulates intracellular Ca^{2+} release in most cell systems studied so far (Gimpl and Fahrenholz 2001), it was studied first in bEEL cells. As expected, Ca^{2+} mobilization was observed after the addition of OT (500 nm). ATP (100 μm), a standard stimulator of Ca^{2+} release in other models, was used as a positive control (Fig. 1A). Mobilization of Ca^{2+} suggested the activation of PLC with the subsequent production of IP3 and DAG through the hydrolysis of phosphatidylinositol 4,5-biphosphate. Accordingly, we pretreated bEEL cells with PLC inhibitor U73122 (10 μm) and found that it reduced OT-induced PGF2α production by

50–60% (Fig. 1B; $P < 0.05$). We then sought to explore the other arm of the PLC pathway using PMA as a surrogate of DAG to activate PKC. PMA-induced PGF2α production was comparable to that of OT (Fig. 1C; $P > 0.05$). It is well known that OT induces ERK1/2 phosphorylation (Gimpl and Fahrenholz 2001). Accordingly, we studied the phosphorylation of ERK1/2 at different time points for 30 min after OT stimulation and found a visible increase at 5 min (Fig. 1D).

Involvement of ERK1/2 pathway on OT-induced COX2 expression and $PGF_{2\alpha}$ production in bEEL cells

To determine the association between OT-induced PGF2α production and ERK1/2 phosphorylation, we sought to inhibit the kinases of ERK1/2 module (Ras/Raf-1/MEK1/2) with specific inhibitors. Pretreatment of bEEL cells with the Ras inhibitor manumycin (2 μm) decreased OT-induced PGF2α production by 70% (Fig. 2B; $P < 0.05$); however, it also had an intrinsic PGF2α-stimulating ability (Fig. 2A; $P < 0.05$). Inhibition of Raf-1 (also called C-Raf) with GW5074 (1 μm) practically abolished OT-induced PGF2α production (Fig. 2B; $P < 0.05$). As expected, pretreatment with the MAPK kinase (MEK) inhibitor PD98059 (10 μm) reduced OT-induced PGF2α production by 80% (Fig. 2C; $P < 0.05$). Because MEK1/2 is the immediate upstream kinase of ERK1/2, we elected to study the effect of PD98059 on OT-induced ERK1/2 phosphorylation and COX2 up-regulation. Inhibition of MEK by PD98059 (10 μm) visibly inhibited OT-induced ERK1/2 phosphorylation and COX2 expression (Fig. 2D).

Role of c-Src, EGFR, and PI3K on OT-induced $PGF_{2\alpha}$ production in bEEL cells

Because OT was shown to transactivate the EGFR tyrosine kinases (TK) in other systems (Zhong, Yang et al. 2003), this was tested in bEEL cells. After the pretreatment of cells with the EGFR inhibitor AG1478 (10 μm, also known as tyrphostin), OT-induced PGF2α production was reduced by at least 80% (Fig. 3A; $P < 0.05$). Furthermore, AG1478 greatly diminished OT-induced ERK phosphorylation. It down-regulated OT-induced as well as basal COX2 expression (Fig. 3B). It is worth mentioning that COX2 expression could be found under basal conditions in bEEL cells. Similarly, the basal phosphorylation of ERK1/2 was also present at an appreciable level in 24-h serum-starved bEEL cells. We also studied the effect of AG1478 on the phosphorylation of Akt (also called protein kinase B) because it is a common downstream target of receptor TK (RTK). Surprisingly, bEEL cells exhibited constitutive phosphorylation of Akt at Ser473 even after 24 h serum starvation, and this was not modulated by OT (Fig. 3B). On the other hand, AG1478 reduced phosphorylation of Akt (Fig. 3B) both in the presence and absence of OT. Then we sought to study the role of nonreceptor TK c-Src, which is often activated by RTK. Pretreatment of bEEL cells with c-Src inhibitor PP2 (10 μm, also known as AG1879) significantly reduced OT-induced PGF2α output (Fig. 3C; $P < 0.05$) and inhibited OT-induced ERK1/2 phosphorylation (Fig. 3D). It down-regulated OT-induced COX2 expression comparably with the effect of PD98059 on COX2. As with AG1478, phosphorylation of Akt was inhibited by

PP2 but was not modulated by OT (Fig. 3D). Because PI3K is known to be activated by RTK and nonreceptor TK, we preincubated the cells with the reversible inhibitor of PI3K, LY294002 (25 μm), and found that it significantly inhibited OT-induced PGF2α production in bEEL cells (Fig. 3E; $P < 0.05$). However, LY294002 showed no inhibitory effect on OT-induced ERK1/2 phosphorylation. It down-regulated OT-induced COX2 expression. As expected, it visibly inhibited phosphorylated Akt, like AG1478 and PP2 (Fig. 3F). It is interesting to note that AG1478 (10 μm), PP2 (10 μm), and LY294002 (25 μm) inhibited PMA-induced PGF2α production, and the degree and magnitude of decrease was comparable with that of OT (results not shown).

DISCUSSION

The cellular and molecular mechanism of luteolysis and antiluteolysis in ruminants has been investigated using endometrial explants, primary culture, and immortalized cells. Although the major limitation of explants and primary cultures is heterogeneity in cell types and OT response, the first bovine endometrial cell line developed, bovine endometrial cell (BEND), lacked OT response, and phorbol ester was used as a surrogate to mimic OT (Binelli, Guzeloglu et al. 2000). Similarly, OT responsiveness of the one ovine endometrial epithelial cell line was not described (Johnson, Burghardt et al. 1999). In other words, lack of an OT-sensitive endometrial cell line partly hampered the understanding of the biochemical and molecular targets of OT and IFNτ. Using bEEL cells, we have recently shown that IFNτ inhibited OT-induced PGF2α production within 3–6 h, suggesting that it may impair the intracellular effectors of OT-mediated signal in the bovine (Krishnaswamy, Danyod et al. 2009). This observation prompted us to dissect the signaling pathway of OT-induced PGF2α production. Toward this end, we first tested and reproduced the known cellular effects of OT such as calcium release. We then investigated ERK1/2 activation and involvement of EGFR in bEEL cells. In addition, we provide preliminary evidence for the involvement of c-Src, PI3K, and Akt on OT-induced COX2 expression and PGF2α production.

Release of intracellular Ca^{2+} in bEEL cells by OT (Fig. 1A) is observed in the same concentration range as primary bovine endometrial epithelial cells (Asselin, Drolet et al. 1997) and in line with the reported essential role of Ca^{2+} for $PGF_{2\alpha}$ production in the bovine (Burns, Hayes et al. 1998). It has also been shown that OT stimulated IP3 release from ovine endometrial explants, suggesting the hydrolysis of phosphoinositides by PLC (Flint, Leat et al. 1986). Mobilization of Ca^{2+} (Fig. 1A), involvement of PLC (Fig. 1B; $P < 0.05$), and activation of $PGF_{2\alpha}$ production by PMA (Fig. 1C; $P < 0.05$) suggest that OT-induced $PGF_{2\alpha}$ production is coupled to $G\alpha_q$ subunit in bEEL cells. This is in agreement with the observations made in cells stably transfected with OTR (Strakova, Copland et al. 1998) and rabbit amnion (Jeng, Liebenthal et al. 2000) ovine endometrial (Burns, Mendes et al. 2001), and human myometrial cells (Zhong, Yang et al. 2003). It should be noted that the inhibition of PLC by U73122 reduced PG output by 50–60%, whereas inhibition of the

ERK1/2 module, c-Src, EGFR, and PI3K, produced over 80% inhibition. Suramin, which uncouples OTR from the underlying G proteins induced 50–60% inhibition (similar to U73122) on OT-stimulated $PGF_{2\alpha}$ production (results not shown). Thus, it is likely that other Gα subunits (Ohmichi, Koike et al. 1995; Strakova and Soloff 1997; Strakova, Copland et al. 1998) or GPCR-independent mechanisms (Blumer, Smrcka et al. 2007) may also contribute to the net production of $PGF_{2\alpha}$ after OT stimulation in bEEL cells.

Rapid phosphorylation of ERK1/2 after OT stimulation (Fig. 1D) suggested that $PGF_{2\alpha}$ production might be mediated through this pathway in bEEL cells. To test this observation, we inhibited Ras, Raf-1, and MEK1/2 kinases and found a significant reduction (>80%) in OT-induced $PGF_{2\alpha}$ production (Fig. 2, A–C; $P < 0.05$). As expected, the MEK inhibitor PD98059 down-regulated OT-induced ERK1/2 phosphorylation (Fig. 2D). Our results confirm and complement previous studies on ERK activation in the ovine endometrium (Burns, Mendes et al. 2001) and on OTR-transfected CHO cells (Strakova, Copland et al. 1998) and myometrium (Zhong, Yang et al. 2003). OT up-regulated COX2 expression at 6 h as shown previously (Krishnaswamy, Danyod et al. 2009), and PD98059 had a moderate inhibitory effect on OT-induced COX2 expression. Although it has been shown that PD98059 can disturb PG production directly at the level of COX1 and COX2 (Borsch-Haubold, Pasquet et al. 1998), the minimal effect on COX-2 relative to $PGF_{2\alpha}$ production supports a direct action of PD998059 on MEK1/2. Considering the high basal expression of COX2 and rapid production of $PGF_{2\alpha}$ after OT treatment in bEEL cells, the release of arachidonic acid after activation of phospholipase A2 would appear as a logical limiting factor. It is also possible that OT regulate the coupling of COX2 to PGF synthase. The immediate response is consistent with the pulsatile secretory pattern of $PGF_{2\alpha}$ from the bovine endometrium observed *in vivo* (McCracken, Custer et al. 1999), whereas activation of cytosolic phospholipase A2 by phosphorylated ERK1/2 has been shown in a rat sertoli cell line (Ulisse, Cinque et al. 2000).

Some GPCR agonists induce receptor transactivation, a process of concerted signaling events that culminate in the shedding of the ectodomain of cell surface proteins that then act as ligands for RTK (Ohtsu, Dempsey et al. 2006). Such transactivation of EGFR was shown to be essential for ERK1/2 phosphorylation by OT in myometrial and OTR-transfected COSM6 cells (Zhong, Yang et al. 2003). Accordingly, we inhibited EGFR with AG1478 and found that it nearly abolished OT-induced $PGF_{2\alpha}$ production (Fig. 3A; $P < 0.05$). Of all reagents tested, AG1478 was the most evident inhibitor of basal and OT-induced ERK1/2 phosphorylation (Fig. 3, B, D, and F; Fig. 2D). The effect of EGFR on Akt was investigated because of the documented RTK-PI3K-Akt pathway by growth factors (Liu, Cheng et al. 2009). The relatively high basal phosphorylation of Akt (Fig. 3, B, D, and F) may be associated with the active proliferation and growth of bEEL cells. It has been reported that concomitant activation of the PI3K-Akt and Ras-ERK signaling pathways is essential for the oncogenic transformation by the avian erythroblastosis virus S13-encoded oncogene V-SEA TK (Agazie,

Ischenko et al. 2002), and the transcription factor Myc has been proposed to integrate ERK and PI3K signals (Lee, Yao et al. 2008). In recent years, signaling pathways different from the classical OT-induced ERK1/2 activation have been reported. For instance, location of OTR inside or the outside caveolae/lipid raft has been shown to modulate the temporal pattern of EGFR/ERK activation with opposing effects on cell growth (Rimoldi, Reversi et al. 2003), and ERK5 has been shown to be activated by OT (Devost, Wrzal et al. 2008).

Src represents a family of nonreceptor TK that are activated by ligands that bind to their Src homology 2 (SH2) or SH3 domain. The activated Src then either phosphorylates its substrates in the cytosol and inner-face of plasma membrane or serves as a docking site for the proteins with SH2 domain. We hypothesized a role for c-Src in OT-induced $PGF_{2\alpha}$ production because of its documented association with EGFR (Martin 2001). Interestingly, the Src inhibitor PP2 reduced OT-induced $PGF_{2\alpha}$ production by 80% (Fig. 3, C and E; $P < 0.05$). We believe that the effect of PP2 on ERK1/2 and Akt phosphorylation (Fig. 3D) may be due to the close association of c-Src with the activated EGFR. Indeed, it has been reported that the activation of Ras requires phosphorylation by different TK including c-Src for the activation of the ERK1/2 pathway (Marais, Light et al. 1997). Moreover, in glioblastoma cells, PMA has been shown to signal through the Src-EGFR-ERK1/2 pathway by activating PKCδ, a novel Ca^{2+}-independent isoform of PKC (Amos, Martin et al. 2005).

PI3K are heterodimeric proteins activated by RTK and GPCR. Upon activation, they generate phosphatidylinositol-3,4,5-trisphosphate, providing docking sites for signaling proteins with pleckstrin homology domains such as Akt (Liu, Cheng et al. 2009). Because chorionic gonadotropin activates PI3K-ERK1/2 pathway to stimulate PGE synthase in human endometrial epithelial cell line, we theorized a similar role for OT-induced $PGF_{2\alpha}$ production in the bovine endometrium (Banerjee, Sapru et al. 2009). Indeed, the phosphotyrosine residues of the transactivated EGFR can activate the p85 regulatory subunit of PI3K that has the SH2 domain. Accordingly, the PI3K inhibitor LY294002 significantly inhibited OT-induced $PGF_{2\alpha}$ production by 80% (Fig. 3E; $P < 0.05$). It also had a moderate inhibitory effect on basal and OT-induced COX2 expression. However, these effects do not appear to be mediated through ERK1/2 phosphorylation (Fig. 3F).

The TK inhibitors AG1478, PP2, and LY294002 had the following two effects in common. First, they achieved a level of inhibition of OT-induced $PGF_{2\alpha}$ production that was comparable with that of ERK1/2 inhibitors (Fig. 2, A–C; $P < 0.05$). Second, they reduced OT-induced COX2 expression and inhibited basal phospho-Akt expression (Fig. 3, B, D, and F). The lack of effect of OT on Akt phosphorylation may be attributed to the high constitutive levels (Fig. 3, B, D, and F). Taken together, the results suggest that EGFR is central for the amplification of OT signal because of the simultaneous activation of c-Src and PI3K, both involved in induced COX2 expression and increased $PGF_{2\alpha}$ production (Fig. 4). In this respect, OT-induced

prostacyclin production and COX2 expression was shown to be inhibited most effectively by a TK inhibitor in human myometrial cells (Molnar, Rigo et al. 1999). Interestingly, $PGF_{2\alpha}$ production in bEEL cells involves activation of most of the classical protooncogenes such as Ras, c-Src, and PI3K. Considering the known antiproliferative effects of type I IFN, it is tempting to speculate that protooncogenes can be a target of IFNτ. Identification of the isoforms of PKC, PLC, c-Src, and PI3K and the type of membrane-anchored EGF ligand released after OT treatment represent logical future studies.

REFERENCES

Agazie, Y., I. Ischenko, et al. (2002). "Concomitant activation of the PI3K-Akt and the Ras-ERK signaling pathways is essential for transformation by the V-SEA tyrosine kinase oncogene." Oncogene **21**(5): 697-707.

Ambler, S. K. and P. Taylor (1986). "Mobilization of intracellular calcium by alpha 1-adrenergic receptor activation in muscle cell monolayers." J Biol Chem **261**(13): 5866-71.

Amos, S., P. M. Martin, et al. (2005). "Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase Cdelta/c-Src pathways in glioblastoma cells." J Biol Chem **280**(9): 7729-38.

Asselin, E., P. Drolet, et al. (1997). "Cellular mechanisms involved during oxytocin-induced prostaglandin F2alpha production in endometrial epithelial cells in vitro: role of cyclooxygenase-2." Endocrinology **138**(11): 4798-805.

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod **54**(2): 371-9.

Banerjee, P., K. Sapru, et al. (2009). "Chorionic gonadotropin regulates prostaglandin E synthase via a phosphatidylinositol 3-kinase-extracellular regulatory kinase pathway in a human endometrial epithelial cell line: implications for endometrial responses for embryo implantation." Endocrinology **150**(9): 4326-37.

Binelli, M., A. Guzeloglu, et al. (2000). "Interferon-tau modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-A(2) from bovine endometrial cells." Biol Reprod **63**(2): 417-24.

Blumer, J. B., A. V. Smrcka, et al. (2007). "Mechanistic pathways and biological roles for receptor-independent activators of G-protein signaling." Pharmacol Ther **113**(3): 488-506.

Borsch-Haubold, A. G., S. Pasquet, et al. (1998). "Direct inhibition of cyclooxygenase-1 and -2 by the kinase inhibitors SB 203580 and PD 98059. SB 203580 also inhibits thromboxane synthase." J Biol Chem **273**(44): 28766-72.

Burns, P. D., S. H. Hayes, et al. (1998). "Cellular mechanisms by which oxytocin mediates uterine prostaglandin F2 alpha synthesis in bovine endometrium: role of calcium." Domest Anim Endocrinol **15**(6): 477-87.

Burns, P. D., J. O. Mendes, Jr., et al. (2001). "Cellular mechanisms by which oxytocin mediates ovine endometrial prostaglandin F2alpha synthesis: role of G(i) proteins and mitogen-activated protein kinases." Biol Reprod **65**(4): 1150-5.

Chapdelaine, P., K. Vignola, et al. (2001). "Protein estimation directly from SDS-PAGE loading buffer for standardization of samples from cell lysates or tissue homogenates before Western blot analysis." Biotechniques **31**(3): 478, 480, 482.

Copland, J. A., Y. J. Jeng, et al. (1999). "Demonstration of functional oxytocin receptors in human breast Hs578T cells and their up-regulation through a protein kinase C-dependent pathway." Endocrinology **140**(5): 2258-67.

Daub, H., F. U. Weiss, et al. (1996). "Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors." Nature **379**(6565): 557-60.

Devost, D., P. Wrzal, et al. (2008). "Oxytocin receptor signalling." Prog Brain Res **170**: 167-76.

Flint, A. P., W. M. Leat, et al. (1986). "Stimulation of phosphoinositide hydrolysis by oxytocin and the mechanism by which oxytocin controls prostaglandin synthesis in the ovine endometrium." Biochem J **237**(3): 797-805.

Gimpl, G. and F. Fahrenholz (2001). "The oxytocin receptor system: structure, function, and regulation." Physiol Rev **81**(2): 629-83.

Jeng, Y. J., D. Liebenthal, et al. (2000). "Complementary mechanisms of enhanced oxytocin-stimulated prostaglandin E2 synthesis in rabbit amnion at the end of gestation." Endocrinology **141**(11): 4136-45.

Johnson, G. A., R. C. Burghardt, et al. (1999). "Development and characterization of immortalized ovine endometrial cell lines." Biol Reprod **61**(5): 1324-30.

Krishnaswamy, N., G. Danyod, et al. (2009). "Oxytocin receptor down-regulation is not necessary for reducing oxytocin-induced prostaglandin F(2alpha) accumulation by interferon-tau in a bovine endometrial epithelial cell line." Endocrinology **150**(2): 897-905.

Lee, T., G. Yao, et al. (2008). "Sensing and integration of Erk and PI3K signals by Myc." PLoS Comput Biol **4**(2): e1000013.

Liu, P., H. Cheng, et al. (2009). "Targeting the phosphoinositide 3-kinase pathway in cancer." Nat Rev Drug Discov **8**(8): 627-44.

Marais, R., Y. Light, et al. (1997). "Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases." J Biol Chem **272**(7): 4378-83.

Martin, G. S. (2001). "The hunting of the Src." Nat Rev Mol Cell Biol **2**(6): 467-75.

McCracken, J. A., E. E. Custer, et al. (1999). "Luteolysis: a neuroendocrine-mediated event." Physiol Rev **79**(2): 263-323.

Molnar, M., J. Rigo, Jr., et al. (1999). "Oxytocin activates mitogen-activated protein kinase and up-regulates cyclooxygenase-2 and prostaglandin production in human myometrial cells." Am J Obstet Gynecol **181**(1): 42-9.

Ohmichi, M., K. Koike, et al. (1995). "Oxytocin stimulates mitogen-activated protein kinase activity in cultured human puerperal uterine myometrial cells." Endocrinology **136**(5): 2082-7.

Ohtsu, H., P. J. Dempsey, et al. (2006). "ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors." Am J Physiol Cell Physiol **291**(1): C1-10.

Prenzel, N., E. Zwick, et al. (1999). "EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF." Nature **402**(6764): 884-8.

Rimoldi, V., A. Reversi, et al. (2003). "Oxytocin receptor elicits different EGFR/MAPK activation patterns depending on its localization in caveolin-1 enriched domains." Oncogene **22**(38): 6054-60.

Silvia, W. J., J. S. Lee, et al. (1994). "Cellular mechanisms mediating the stimulation of ovine endometrial secretion of prostaglandin F2 alpha in response to oxytocin: role of phospholipase C and diacylglycerol." J Endocrinol **141**(3): 481-90.

Spencer, T. E., R. C. Burghardt, et al. (2004). "Conceptus signals for establishment and maintenance of pregnancy." Anim Reprod Sci **82-83**: 537-50.

Strakova, Z., J. A. Copland, et al. (1998). "ERK2 mediates oxytocin-stimulated PGE2 synthesis." Am J Physiol **274**(4 Pt 1): E634-41.

Strakova, Z. and M. S. Soloff (1997). "Coupling of oxytocin receptor to G proteins in rat myometrium during labor: Gi receptor interaction." Am J Physiol **272**(5 Pt 1): E870-6.

Ulisse, S., B. Cinque, et al. (2000). "Erk-dependent cytosolic phospholipase A2 activity is induced by CD95 ligand cross-linking in the mouse derived Sertoli cell line TM4 and is required to trigger apoptosis in CD95 bearing cells." Cell Death Differ **7**(10): 916-24.

Weems, C. W., Y. S. Weems, et al. (2006). "Prostaglandins and reproduction in female farm animals." Vet J **171**(2): 206-28.

Zhong, M., M. Yang, et al. (2003). "Extracellular signal-regulated kinase 1/2 activation by myometrial oxytocin receptor involves Galpha(q)Gbetagamma and epidermal growth factor receptor tyrosine kinase activation." Endocrinology **144**(7): 2947-56.

FIGURES

Figure 1

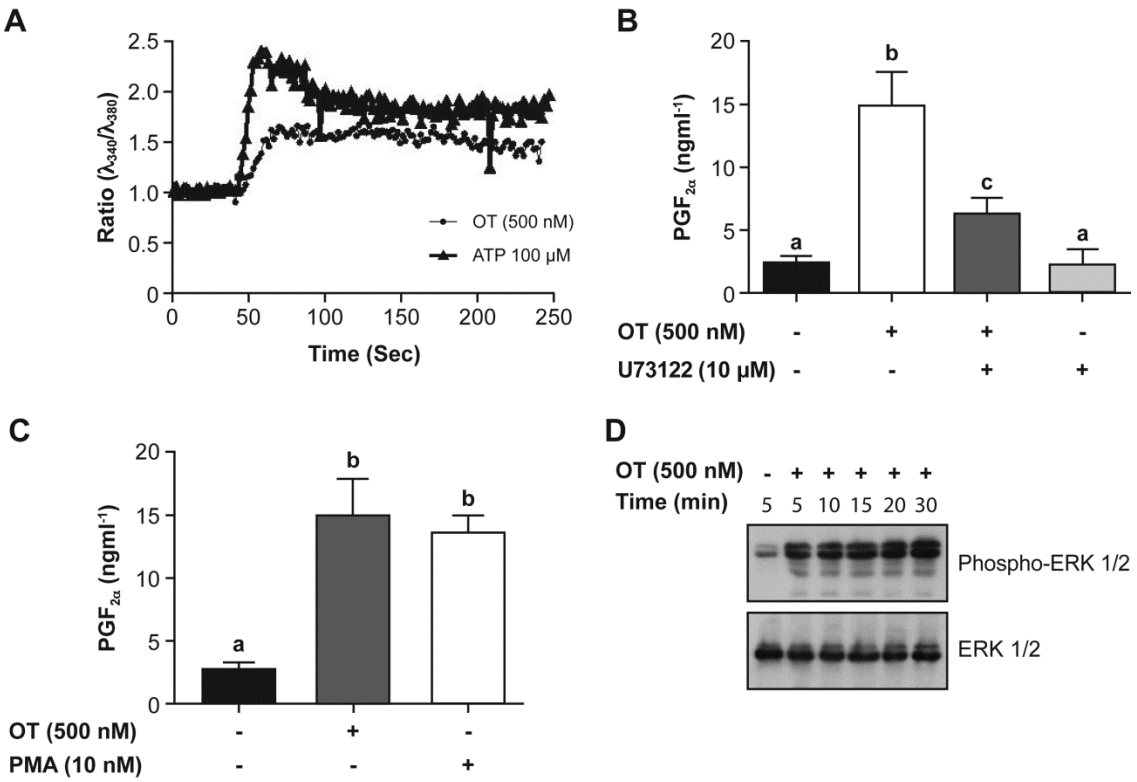


Figure 4 - Article 1: Figure 1 Effect of OT on the release of intracellular Ca2+and ERK1/2 phosphorylation and the role of PLC and PKC on OT-mediated PGF2α production in bEEL cells.

A, Coverslip preparation of bEEL cells was incubated with fura-2 AM (2.5 µm) for 45 min and then stimulated with OT (500 nm), and the Ca^{2+} mobilizations were recorded for 3–4 min in a spectrofluorometer. ATP (100 µm) served as positive control. **B**, Confluent bEEL cells were preincubated with DMSO or U73122 (10 µm) for 1 h and coincubated in the presence or absence of OT (500 nm) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium, and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data were analyzed by one-way ANOVA with Tukey's multiple-comparison *post hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$). **C**, Confluent bEEL cells were treated with OT (500 nM) or PMA (10 nM) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium, and the values represent mean ± SEM (18) of three different experiments run in hexaplicate. Data were analyzed as mentioned in B. **D**, Confluent bEEL cells were serum starved 24 h and stimulated with OT (500 nM) for 5, 10, 15, 20, and 30 min. Protein extracted from the cell lysate was probed for phosphorylated or total ERK1/2. Representative immunoblots of phosphorylated and total ERK1/2 are shown.

Figure 2

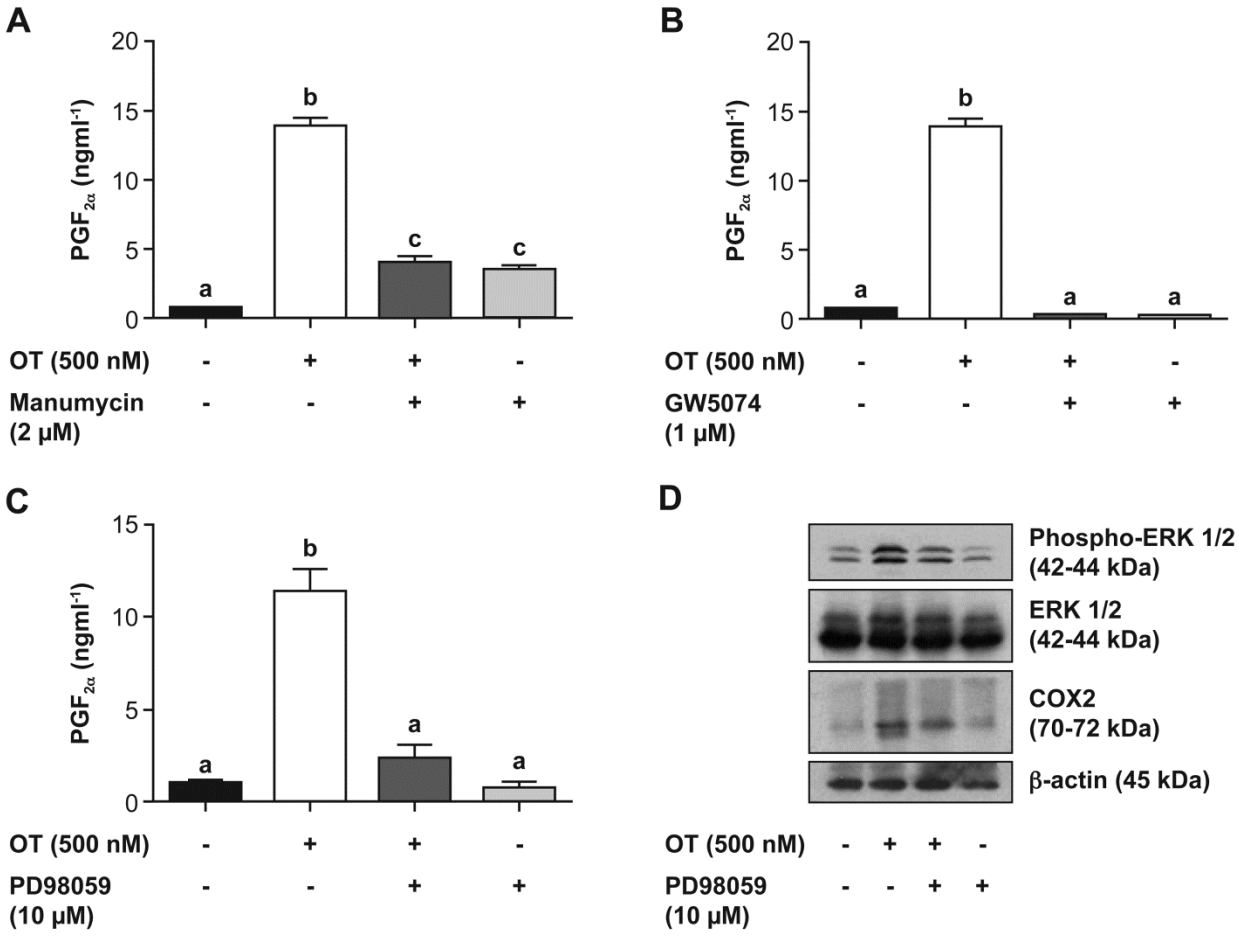


Figure 5 - Article 1: Figure 2 Involvement of ERK1/2 pathway on OT-induced COX2 expression and PGF2α production in bEEL cells.

A, Effect of Ras inhibitor (manumycin 2 μm) on OT-induced $PGF_{2\alpha}$ production. **B**, Effect of Raf-1 inhibitor (GW5074 1 μm) on OT-induced $PGF_{2\alpha}$ production. **C**, Effect of MEK1/2 inhibitor (PD98059 10 μm) on OT-induced $PGF_{2\alpha}$ production. Protein extracted from the cell lysate was used for immunoblotting of COX2 and β-actin. **A**–**C**, Confluent bEEL cells were preincubated with DMSO or appropriate inhibitor for 1 h and coincubated in the presence or absence of OT (500 nm) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium, and the values represent mean ± sem (18) of three different experiments run in hexaplicate. Data were analyzed by one-way ANOVA with Tukey's multiple-comparison *post hoc* test to find the critical difference between pairs of treatment means. Bars with different superscripts differ significantly ($P < 0.05$). **D**, Confluent bEEL cells were serum starved 24 h and preincubated with DMSO or PD98059 (10 μM) for 1 h. The medium was aspirated, and the cells were stimulated with OT for 5 min in the presence or absence of PD98059. Protein extracted from the cell lysate was probed for phosphorylated or total ERK1/2.

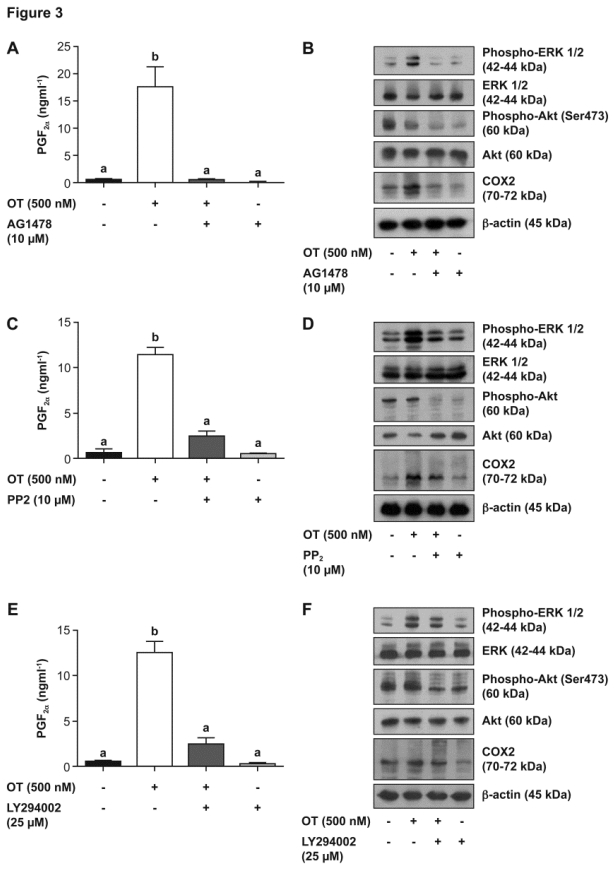


Figure 6 - Article 1: Figure 3 Role of EGFR, c-Src, and PI3K on OT-induced PGF2α production in bEEL cells.

A, Effect of AG1478 (10 μm) on OT-induced $PGF_{2\alpha}$ production; **B**, effect of AG1478 (10 μm) on OT-induced phosphorylation of ERK1/2 and Akt as well as expression of COX2; **C**, effect of PP2 (10 μm) on OT-induced $PGF_{2\alpha}$ production; **D**, effect of PP2 (10 μm) on OT-induced phosphorylation of ERK1/2 and Akt as well as expression of COX2; **E**, effect of LY294002 (25 μm) on OT-induced $PGF_{2\alpha}$ production; **F**, effect of LY294002 (25 μm) on OT-induced phosphorylation of ERK1/2 and Akt as well as expression of COX2 For the experiments in **A**, **C**, and **E**, bEEL cells were preincubated with DMSO or respective inhibitors for 1 h and coincubated in the presence or absence of OT (500 nm) for 6 h. $PGF_{2\alpha}$ was measured in the culture medium, and the values represent mean ± sem (18) of three experiments run in hexaplicate. Data were analyzed by one-way ANOVA with Tukey's *post hoc* test to find the critical difference between pairs of treatment means. *Bars with different superscripts* differ significantly ($P < 0.05$). Protein extracted from the cell lysate was used for immunoblotting of COX2. β-Actin served as a loading control. For the experiments in **B**, **D**, and **F**, Confluent bEEL cells were serum starved for 24 h and preincubated with DMSO or respective inhibitors for 1 h. The media was aspirated and the cells were stimulated with OT for 5 min in the presence or absence of the inhibitor. Protein extracted from the cell lysate was probed for phosphorylated ERK1/2 and Akt. Total ERK1/2 and Akt served as internal control.

Figure 4

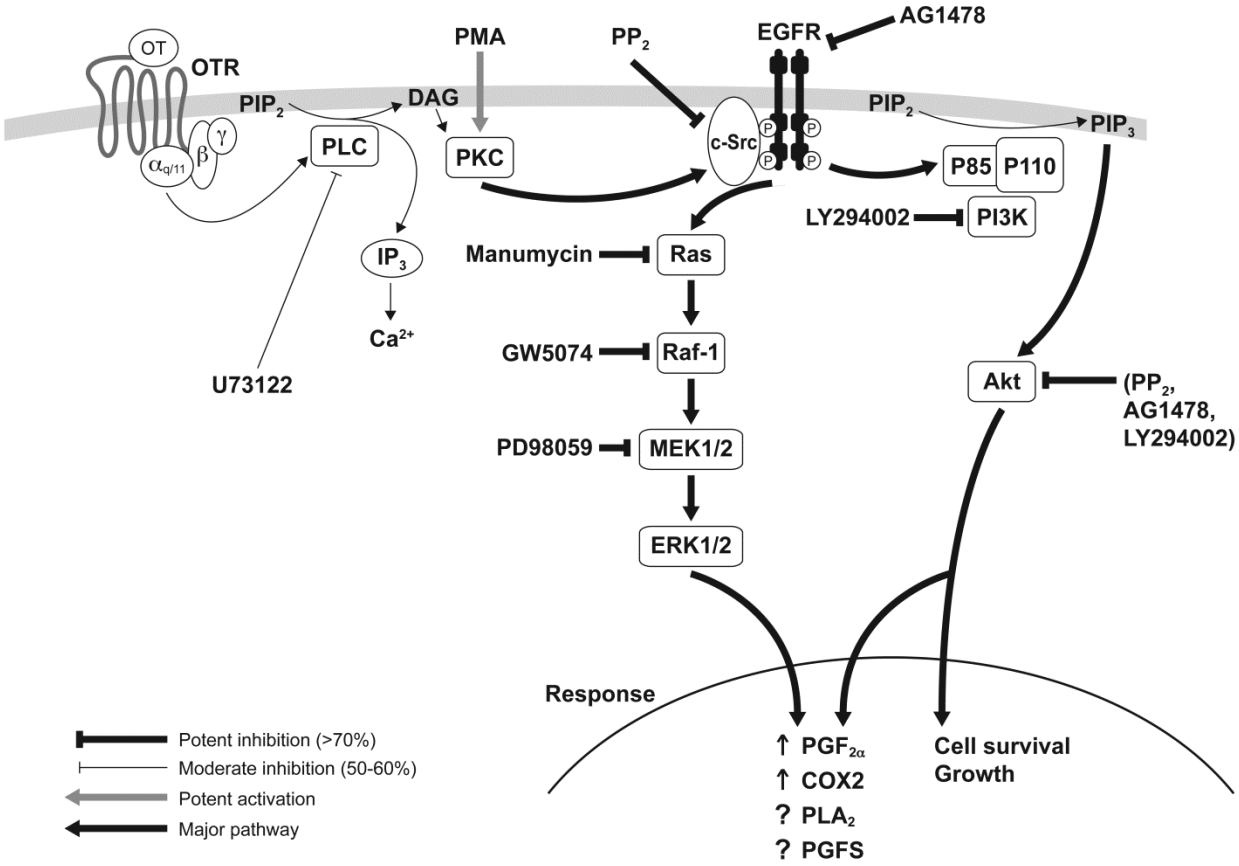


Figure 7 - Article 1: Figure 4 A proposed signaling pathway of OT-induced $PGF_{2\alpha}$ production in bEEL cells.

Binding of OT to its cognate receptor activates the PLC-DAG-PKC pathway. As shown in other models, activated PKC may also activate EGFR. Transactivated EGFR serves as a docking site for the signaling proteins with an SH2 domain, such as c-Src. The c-Src-EGFR in turn initiates the sequential phosphorylation of Ras-Raf-1-MEK-ERK1/2 pathway to induce COX2 expression and $PGF_{2\alpha}$ production. In parallel, phosphotyrosine residues of EGFR can also activate the PI3K-Akt pathway to augment COX2 expression and $PGF_{2\alpha}$ production. High levels of basal phospho-Akt may be associated with the growth and proliferation of bEEL cells. Signaling intermediates shown in *gray* [phosphatidylinositol 4,5-biphosphate (PIP_2), IP_3, and DAG] were not investigated but are included for clarity

Chapitre 3: The Multidrug Resistance associated Protein 4 (MRP4) appears as a functional carrier of prostaglandins regulated by Oxytocin in the Bovine endometrium.

Nicolas Lacroix Pépin, Ghislain Danyod, Narayanan Krishnaswamy, Sukanta Mondal, Pei-Min Rong, Pierre Chapdelaine, Michel A Fortier

Ce chapitre est présenté sous forme d'article et a été publié dans la revue :
Endocrinology. 2011 Dec;152(12):4993-5004

Centre de Recherche du CHUQ, Département d'Obstétrique et Gynécologie, Unité d'Ontogénie et Reproduction, Centre de Recherche en Biologie de la Reproduction, Université Laval, Ste-Foy, Québec, Canada GIV 4G2

RÉSUMÉ

Les prostaglandines (PGs) sont impliquées dans plusieurs processus au niveau reproductif et leur action est régulée à l'étape de la biosynthèse, du catabolisme et de la transduction du signal. Le transport facilité à travers les membranes cellulaires se révèle être un point de contrôle supplémentaire pour la régulation de leur action. Nous avons déjà documenté l'action du transporteur SLCO2A1 (PGT) en relation avec l'efflux de prostaglandines dans l'endomètre bovin. Dans cette étude, nous présentons l'expression fonctionnelle et la régulation de MRP4/ABCC4, un transporteur alternatif appartenant à la famille des transporteurs ayant une cassette liant l'ATP. Nous avons trouvé que la protéine MRP4 est présente tout au long du cycle œstral et possède un patron d'expression similaire à PGT avec une expression maximale pendant la phase lutéale dans l'endomètre bovin. L'expression fonctionnelle et la régulation de MRP4 ont été étudiées *in vitro* en utilisant nos lignées cellulaires épithéliales (bEEL) et stromales (CSC) bovines nouvellement développées. L'ocytocin (OT) stimule la production de PGF2α et de l'ARNm de *MRP4* ainsi que de la protéine de façon dose- et temps-dépendante mais sans effet sur PGT. L'OT induit une accumulation préférentielle de PG à l'extérieur des cellules et une sécrétion dirigée vers le côté basolatéral des cellules bEEL polarisées lorsque cultivées sur la membrane d'un insère de culture. MK-571 et l'indométacine, deux inhibiteurs documentés de l'activité de MRP4, ont bloqué préférentiellement l'accumulation des PGs mais l'interféron-tau et le NS-398 n'ont pas eu d'effet sur MRP4 au niveau de son expression ou de la direction du transport. Nos résultats suggèrent donc que MRP4 est un transporteur fonctionnel des PGs sous la régulation de l'OT dans l'endomètre bovin.

ABSTRACT

Prostaglandins (PGs) are involved in several female reproductive processes and their action is regulated at the levels of biosynthesis, catabolism and signal transduction. Facilitated transport across cell membranes emerges as an additional check point regulating prostaglandin action. We have already reported on the influx transporter SLCO2A1 (PGT) in relation with PG action in the bovine endometrium. In the present study, we report on the functional expression and regulation of MRP4/ABCC4 an alternate PG transporter belonging to the ATP binding cassette carrier (ABC) family. We have found that MRP4 protein was present throughout the estrous cycle and exhibited a pattern of expression similar to that of PGT with maximal expression during early-mid luteal phase in the bovine endometrium. Functional expression and regulation of MRP4 was studied in vitro using the newly developed bovine endometrial epithelial bEEL and stromal CSC cell lines. Oxytocin (OT) stimulated PGF2α production and *MRP4* mRNA and protein in a time and dose dependent manner but had no effect on PGT. OT induced preferred accumulation of PG outside the cells and secretion toward the basolateral side of polarised bEEL cells grown on membrane inserts. MK-571 and indomethacin two documented inhibitors of MRP4 activity blocked preferred accumulation of PGs but Interferon-tau and NS-398 had no effect on MRP4 expression or the direction of PG transport. Our results suggest that MRP4 is a functional PG carrier under the regulation of OT in the bovine endometrium.

INTRODUCTION

Prostaglandins (PGs) are key regulators of female reproductive function in mammals and are involved in ovulation, luteolysis, implantation, cervical ripening, parturition and postpartum involution of the uterus (Poyser 1995; Lim, Paria et al. 1997; Thatcher, Guzeloglu et al. 2001; Jabbour and Sales 2004; Spencer, Johnson et al. 2007). In ruminants, endometrial epithelial cells are the primary source of PGF2α whereas stromal cells release more PGE2 (Krishnaswamy, Chapdelaine et al. 2009; Krishnaswamy, Danyod et al. 2009). In bovine uterine flushing, PGF2α levels are higher than PGE2 even during early pregnancy (Ulbrich, Schulke et al. 2009). The different PGs exert distinct and often opposite actions on many tissues and systems and are tightly regulated at the biosynthetic, catabolic, signal transduction and more recently at the selective transport levels (Fortier, Krishnaswamy et al. 2008). In the reproductive system, PGF2α is known to be a luteolytic and vasoconstrictive agent (McCracken, Custer et al. 1999) whereas PGE2 is a luteoprotective and vasorelaxant mediator (Pratt, Butcher et al. 1979). Around day 16 of the bovine estrous cycle, in absence of a viable embryo, luteolytic pulses of PGF2α are released by endometrial epithelial cells in response to Oxytocin (OT). PGF2α from the endometrium is transferred from the uterine vein to the ovarian artery through the utero-ovarian plexus (UOP) (Ginther 1981). We have shown that PGT/SLCO2A1 probably contributes to this transfer and is upregulated both in the endometrium and the UOP during the luteolytic window in the bovine (Banu, Arosh et al. 2003). We also showed an upregulation of COX2, a rate limiting enzyme of PGs biosynthesis during the same period (Arosh, Parent et al. 2002). In ruminants, interferon tau (IFNτ) is the embryonic signal produced by the trophoblast to repress PGF2α production by endometrial epithelial cells, and prevent luteolysis thus allowing sustained production of progesterone (P4) to maintain pregnancy. Using a newly developed bovine endometrial epithelial cell line (bEEL), we have been able to reproduce in vitro the responses to OT and IFNτ and generate a model ideally suited to study the underlying molecular and cellular mechanisms (Krishnaswamy, Danyod et al. 2009).

As mentioned above the PG transporter PGT is expressed in the cow reproductive tract at critical periods of PG action. Such transporters are necessary for PG action because PGs exist as organic anions and diffuse poorly through the plasma membrane in spite of their lipid nature. Therefore facilitated transport is necessary to cross the different cell layers in order to allow PG transfer from their site of biosynthesis to their site of action. It is proposed that the cells expressing these carriers create a path used by PGs to penetrate tissues and reach their site of action. It has recently been shown that functional transporters are necessary for luteolysis in the ewe (McCracken, Custer et al. 1999; Banu, Arosh et al. 2003) as proposed for the cow (Banu, Arosh et al. 2003; Lee, McCracken et al. 2010). PGT was the first specialized carrier associated with prostaglandin transport (PGT) and preferentially involved in PG influx inside cells (Kanai, Lu et al. 1995; Schuster 1998; Chan, Endo et al. 2002). PGT/SLCO2A1, also called OATP2A1, belongs to the Solute Carrier

Organic Anion Transporting Polypeptide family (SLCO2A1) and is highly expressed in several tissues including the uterus and ovary (Banu, Arosh et al. 2003; Banu, Arosh et al. 2005; Banu, Lee et al. 2008). More recently, the Multidrug Resistance associated Protein 4 (MRP4) a member of ATP binding cassette carriers also identified as ABCC4 (Reid, Wielinga et al. 2003; Russel, Koenderink et al. 2008) has been shown to mediate the efflux of PGs outside the producing cells in mammals (Dallas, Miller et al. 2006; Russel, Koenderink et al. 2008) and to be the most effective among the ABC family for PGE2 and PGF2α transport (Reid, Wielinga et al. 2003). The carrier activity of MRP4 was shown to be blocked by the non steroidal anti inflammatory drug indomethacin and by MK-571. Although the role of PGT to facilitate entry of PGs in cells is well accepted, efflux of PGs through PGT, passive transport or facilitated transport is still debated (Schuster 2002; Chi, Khersonsky et al. 2006; Banu, Lee et al. 2008; Banu, Lee et al. 2010). To our knowledge, no data is available on the expression and impact of ABC transporters in the female reproductive system which appears ideally suited to study the physiological regulation of PG action. Given the demonstrated importance of PGs and the potential regulatory function of their transport for luteolysis and recognition of pregnancy, we have investigated the expression, regulation and function of MRP4 gene and protein using our newly generated endometrial epithelial bEEL (Krishnaswamy, Danyod et al. 2009) and stromal CSC (Krishnaswamy, Chapdelaine et al. 2009) cell lines.

MATERIALS AND METHODS

Materials

The reagents were purchased from the following suppliers: Superscript III reverse transcriptase, DNA ladder, dithiothreitol, 5x first strand buffer, TRIzol and RPMI 1640 (without phenol) from Invitrogen Life Technologies Inc. (Burlington, ON, Canada). Random primer-pd(N)6, deoxy-NTPs, RNA Guard, rTaq DNA polymerase, PCR 10x buffer, from GE healthcare Canada (Baie d'Urfé, QC, Canada). Plasmid pDrive (TA cloning kit), DNA purification kits and QuantiTect SYBR Green PCR Kit for quantitative real time PCR were from Qiagen (Mississauga, ON, Canada) using LightCycler® System (Roche Diagnostics, Laval, QC, Canada). Tissue culture plates from Sarstedt (St Leonard, QC, Canada); fetal bovine serum and antibiotics from Wisent Inc. (Montréal, QC, Canada). All oligonucleotide primers were chemically synthesized using ABT 394 synthase (Perkin-Elmer, Foster City, CA). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). Oxytocin, 8-bromo-cAMP, Indomethacin and PMA was from (Sigma-Aldrich, Oakville, ONT, Canada) and recombinant ovine IFNτ was donated by Drs. F. W. Bazer and T. E. Spencer (Animal Biotechnology Laboratory, Texas A&M University, College Station, TX). NS-398 and MK-571 were from Cayman (Chemicals, Ann Arbor, MI).

Preparation of endometrial tissues

Bovine uteri were collected at a local abattoir immediately after exsanguinations, placed on ice and brought to the laboratory within 1.5 h. The endometrial samples were classified as described previously based on gross morphology and examination of ovaries (Arosh, Parent et al. 2002). Endometrial strips were cut into small pieces, snap-frozen in liquid nitrogen, and stored at -80 C until used for analysis.

Analysis of PGT and MRP4 by Western Blot

Protein extraction, quantification and western blot analysis were performed as described previously (Arosh, Parent et al. 2002). For each sample, aliquots of 10-20 µg protein were separated on 12% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (10% for PGT). Proteins were then transferred onto nitrocellulose membranes (Bio-RaD Laboratories (Canada Ltd, Mississauga, Ontario). The membranes were blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 5% fat-free dry milk (BLOTTO) and 0.05% Tween 20. For the detection of bovine PGT (bPGT), the membrane was probed with a rabbit anti-bPGT (1:1000) targeting 14 amino acids (RVKKNKEYNVQEKA) found in positions 627 to 640 of the C-terminal bPGT (GI: 282468 SLCO2A1, NP 777254.1). The bPGT antibody was produced by Genescript (GenScript USA Inc., Piscataway, NJ) and tested in our laboratory. To detect bovine MRP4 protein (GI: 515333 ABCC4), we used a mouse monoclonal antibody (Mab) against human MRP4 (GI: 10257 ABCC4) targeting N-terminal amino acids 1-110 (ab56675,ABCAM, Cambridge, MA, USA). The predicted bovine N-terminal MRP4 amino acid sequence (XP_593336.2) exhibits 87% identity with the corresponding portion of the human protein (NP_001098985.1). The MRP4 Mab was used at 2.5 µg/ml in PBS-T containing 5% BLOTTO overnight at 4°C. A monoclonal β-Actin antibody (Sigma-Aldrich Canada Ltd, Oakville, ONT, Canada) was used at 1:5000 in PBS-T containing 5% BLOTTO for one hour at room temperature and served as a loading and quantification control. Following the first antibody incubation, membranes were washed in PBS-T and secondary antibodies, a goat anti-rabbit (for bPGT) or anti-mouse (for MRP4 and β-Actin) horseradish peroxidase-conjugated IgG (Jackson laboratories, West Grove, PA, USA) were used at 1:10000 for 1 h at room temperature in PBS-T containing 5% BLOTTO followed by several washes in PBS-T. The chemiluminescence signal was analyzed with an autoradiography film after treatment of the membrane with Renaissance reagent (NEN, Perkins Elmer, Boston, MA, USA). The signal intensity of the immunoreaction was quantified by densitometry using a Multimage Light Cabinet equipped with AlphaImager 2000 software (Cell Biosciences, Santa Clara, California,)

Cell culture

The bovine endometrial epithelial cell line (bEEL) and bovine caruncular stromal cells (CSC) were cultured as reported previously (Krishnaswamy, Lacroix-Pepin et al. 2010). Briefly, a frozen aliquot was grown

in RPMI-1640 medium without phenol red (Gibco-BRL (Invitrogen), Mississauga, ON, Canada) supplemented with 10% FBS and 50 IU penicillin-streptomycin (Wisent Inc, QUE); confluent cultures were trypsinized and seeded at $4X10^4$ cells per milliliter. Confluent cells were conditioned overnight with fresh RPMI medium before treatment with OT, IFNτ, PMA and NS-398.

Alternatively, bEEL cells were grown on coated inserts (Millicell-PCF, Millipore Products Corporation, Bedford, Mass., USA) to allow differentiation into polarized cultures. Briefly, 30 mm collagen coated membrane inserts with 0.4 µm pore size were coated with 800µl of a 1:4 dilution (70% ethanol) of Type 1 calf skin collagen (Sigma-Aldrich Canada, Oakville, ON) and allowed to dry overnight under a laminar flow hood. Cells were seeded at $1.5X10^5$ cells per cm^2 and transepithelial electric resistance (TEER) was used to evaluate confluency and polarization. Polarized cells were conditioned overnight with fresh RPMI medium before treatment with OT, PMA, 8-bromo-cAMP, IFNτ, Indomethacin, MK-571 or NS-398.

Measurement of Trans-epithelial electric resistance (TEER)

TEER was monitored as an indication of tight junction formation and epithelial monolayer integrity using a set of Ag:AgCl electrodes and a Millicell-ERS (Electrical Resistance System) (Millipore Products Corporation, Bedford, Mass., USA). Electrical resistance measurements (expressed in ohms, Ω) were taken daily after alcohol sterilization of the probes. TEER of at least 6 inserts for each experience were assessed daily to follow progression. TEER was measured for each insert before experimentation.

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) reactions were performed using a Roche Light Cycler (Roche) and QuantiTect™ Syber® Green PCR kit (Qiagen). Samples from three independent experiments were run in duplicate. Primers were designed in such a way that the amplified region spanned over intron–exon boundaries to avoid contamination by coamplification with genomic DNA. *ABCC4/MRP4* sequence ID XM_593336 with primers 5'ATATAGCCTAGATGGGCCTCTG3'f (3147) and 5'GAACTTTTTCCAGCTCCTGTTC3'r (3248). *PGT/SLCO2A1* sequence ID AY134618 with primers 5'GTGGAGACGATGGGATTGAATA3'f (1376) and 5'AAGGAGATGAGGAAGATGGTTG3'r (1571). *18S* RNA Sequence ID identical to human DQ222453 with primers 5'GTAACCCGTTGAACCCCATT3'f (1579) and 5'CCATCCAATCGGTAGTAGCG3'r (1731). Total RNA (3.0 µg) was reverse-transcribed by SuperScript™ III RT (Invitrogen) in a 20-µl reaction mixture according to the manufacturer's instructions. A RT-minus control (no enzyme) was included as a negative control. Two µl cDNA were used as the template in a final PCR reaction volume of 20 µl; a two µl aliquot of diluted cDNA *18s* RNA (1 in 10) was used to normalize the level of each sample analyzed. Thermocycling was initiated by a 15-min incubation at 95°C, followed by 40 cycles (*MRP4*), 45 cycles (*COX-2*) and 50 cycles (*PGT*) at 95°C for 15 s; 55°C (*MRP4* and *COX-2*), 60°C (*PGT*) for

30 s; and 72°C for 20 s, and a single fluorescence reading was taken at the end of each cycle at 77°C (*MRP4* and *COX-2*), 82°C (*PGT*). Each run was monitored with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. Comparative threshold cycle (C_t) values were determined by software using a fluorescence threshold automatically and further analysis was done. The amplified products were verified by agarose gel electrophoresis and showed single bands of predicted sizes for each sample and no product for the negative controls (water instead of cDNA). *18S* rRNA gene served as an internal control to normalize the expression of each gene.

Treatment of bEEL cell cultures

bEEL cells were treated with increasing concentrations of OT (5 to 500 nM) or IFNτ (0.01 to 10 µg/ml) for 6 or 24 h to study the expression of MRP4 and PGT transcripts and protein levels as well as PGF2α and PGE_2 accumulation in the culture medium. Oxytocin stimulation was performed in the presence or not of NS-398 (1 µM), a selective inhibitor of COX-2. BEEL cells were also treated with OT (500 nM) with increasing concentration of MK-571 (1,6 to 25 µM) for 6h to study the impact on PGF2α and PGE_2 accumulation in the culture medium.

Treatment of bEEL cells on collagen coated inserts

bEEL cells were treated with OT (500 nM), the smallest concentration eliciting maximal PG production (Krishnaswamy, Danyod et al. 2009), IFNτ (0.01 and 10 µg/ml), PMA (10 nM), 8-bromo-cAMP (500µM), NS-398 (1 µM), Indomethacin (25 µM) or MK-571 (12.5 µM) for 6 h with equal volumes on each side of the insert to evaluate PGF2α and PGE2 accumulation in the apical or basolateral compartment of the culture system in relation with MRP4 and PGT protein. Accumulation on each side is presented as ng/ml whereas the relative accumulation on each side is presented as the % of total release.

Downregulation of MRP4 in endometrial stromal cells using siRNA.

Three specific siRNAs for MRP4 were designed according to the T7 RNAi Oligo Designer program (TROD v1.1.2) (Donze and Picard 2002) according to its mRNA sequence (XM_593336.3). The siRNAs were then synthesized with T7 RiboMAX™ Express RNAi System (Promega). The resulting products (250 ng/well) were used to transfect CSC cells (75% confluency) in 24-well plates with Lipofectamine™ 2000 (Invitrogen) using manufacturer's instructions. After overnight transfection, the culture medium was replaced with RPMI with 10% FBS and incubated at 37°C for 24 h. The culture was then treated with PMA (10nM) for 6h in RPMI without serum. The efficiency of the different siRNAs to reduce *MRP4* mRNA expression in CSC was analysed by qRT-PCR. The most efficient siRNA targeting the *MRP4* nucleotide sequence corresponding to positions 1418-1438 (5'-AGGAGGATCGCGTATGTTTCTC-3') was selected. A mismatch (scramble) was also generated with a sequence of (5'-GTGACTAGGCCTTGGTGTTAC-3'). Downregulation of MRP4 protein was

analysed for the specific siRNA and scramble by Western blot as described above with β-actin as an internal standard. The impact on PGE2 and PGF2α release in the culture medium was measured by ELISA.

Enzyme Immunoassays (EIAs) of PGE_2 and PGF2α

Prostaglandins were assayed by competitive EIA using acetylcholinesterase–linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996).

Statistical analysis

Data were analyzed by One way ANOVA with Bonferroni as post-hoc test with 95% confidence intervals using GraphPad Prism 5 program. For data with less than 3 columns a two-tailed unpaired t test with 95% confidence intervals was performed using GraphPad Prism 5 program. Data are presented as the Mean ± SEM. Each experiment was repeated at least three times.

RESULTS

Expression and regulation of MRP4 and PGT proteins during the estrous cycle.

As described in the materials and methods section, a commercial antibody raised against the human MRP4 was validated to cross-react with a protein of expected MW (over 175 kDa) present in the bovine endometrium and used for Western blot analysis of bovine MRP4 (Fig. 1A). Bovine PGT protein was estimated using a custom made polyclonal antibody raised against the C-terminal portion as a replacement for our former in house antibody that was no longer working. The new antibody detected selectively a protein of 70 kDa (MW) corresponding to bovine PGT (Fig. 1B). MRP4 protein was present throughout the cycle with maximal levels between days 7 to 15 (Fig.1C) while maximal protein expression for PGT was observed between days 10 to 18 (Fig. 1D).

Effect of OT and IFNτ on MRP4 and PGT mRNA expression in bEEL cells

The potential regulation of *PGT/SLCO2A1* and *MRP4/ABCC4* genes was studied in response to OT and IFNτ in our recently developed bovine endometrial epithelial cell line identified as bEEL (Krishnaswamy, Danyod et al. 2009; Krishnaswamy, Lacroix-Pepin et al. 2010) (Fig. 2). Treatment of bEEL cells with IFNτ at 10 ng/ml or OT at 500 nM for 24 h induced contrasting responses for each treatment and transporter. *MRP4* mRNA was upregulated almost three fold by OT but IFNτ had no effect (Fig 2A). By contrast, *PGT* mRNA was increased two fold by IFNτ but was not influenced by OT (Fig. 2B). As expected, OT strongly stimulated PGF2α production but, at the concentration used, IFNτ alone did not have any effect (Fig. 2C).

Effect of increasing concentrations of OT and IFNτ on MRP4 and PGT protein expression in bEEL cells

As previously shown, the treatment of bEEL cells with OT induced a dose dependent increase in PGF2α production (Fig. 3A) whereas IFNτ elevated PGs production only at the highest (10 µg/ml) concentration tested (Fig. 3B). At the protein level, a significant increase in MRP4 was observed in response to OT at 500 nM (3C and E). No significant change was observed in response to IFNτ. No increase in PGT protein level could be seen with either oxytocin or INFτ (3D and F).

Effects of OT and IFNτ on PGF2α and PGE2 efflux in polarized bEEL cells

bEEL cells were seeded on collagen inserts to allow polarization and differentiation into apical and basolateral sides (Munson, Wilkinson et al. 1990). Trans-epithelial electric resistance (TEER) resulting from formation of tight junctions between adjacent cells was measured daily. TEER increased progressively with cell growth and reached a maximum of 600 to 800 ohms (Ω) after 7 to 9 days which corresponds to confluence and optimal polarization of the culture (Fig. 4A) (Munson, Wilkinson et al. 1990; Bowen, Newton et al. 1996; Delabarre, Claudon et al. 1997). Intra-assay variation was quite low remaining within 100 Ω. Inter-assay variation was higher during exponential growth but maximal resistance was similar at confluency. The following experiments were conducted in confluent cultures with TEER between 600 to 800 Ω. PGF2α was assayed in samples collected from the apical (Fig. 4B) or basolateral (Fig. 4C) sides of polarized bEEL cells following 6 h treatment with OT (500 nM), INFτ (10 ng/ml) or both (Fig. 4). OT stimulated the overall release of PGF2α, but interestingly, also induced preferred accumulation in the baso-lateral compartment (Fig. 4C and D) relative to the apical side (Fig. 4B and D). In presence of OT approximately 80% of PGs accumulated on the basolateral side compared to 50% under control conditions (Fig. 4D). Moreover, even though IFNτ inhibited OT stimulation of PG release, it did not reverse the preferred accumulation on the basolateral side or increase in MRP4 protein (Fig. 4E and F). Preferred accumulation in the basolateral compartment was observed no matter OT was added on the apical or basolateral side (Fig. 5A). Consistent with our previous characterization of OT response in bEEL cells (Krishnaswamy, Lacroix-Pepin et al. 2010), the effect of OT could be mimicked by the PKC activator PMA (10 nM) but not following activation of the PKA pathway by 8–bromo-cAMP (500 µM) (Fig. 5B).

Effect of COX-2 inhibition on MRP4 regulation by OT.

The possibility that OT action on MRP4 levels was mediated indirectly by increased PG production was tested with NS-398 (1 µM), a selective inhibitor of COX-2 activity that has no effect on MRP4 at the concentration used (Morioka, Kumagai et al. 2004). Treatment of bEEL cells with OT (500 nM) increased PGF2α production (Fig 6B), *COX-2* mRNA (Fig. 6A) as well as *MRP4* mRNA and protein (Fig. 6C, E and F).

While NS-398 completely blocked OT stimulation of PGF2α release, it had no effect on *COX-2* or *MRP4* (Fig. 6A, B, C and F) or preferential accumulation of prostaglandins on the basolateral side (Fig. 6D). These results together with those shown in fig. 4 E and F demonstrate that increased PG production is not necessary for OT regulation of MRP4 level or action.

Effect of MRP4 inhibition on PGF2α efflux in polarized bEEL cells

The specificity of MRP4 action on preferred basolateral accumulation of PGs was tested using two known inhibitors of MRP4 action. PGF2α was assayed in samples collected from the apical or basolateral sides of polarized bEEL cells following 6 h treatment with OT alone or in combination with Indomethacin (25 μM) previously shown to inhibit MRP4 activity (Reid, Wielinga et al. 2003; Morioka, Kumagai et al. 2004; Nozaki, Kusuhara et al. 2007; Russel, Koenderink et al. 2008) (Fig. 7). Indomethacin was able to inhibit prostaglandin production but by contrast with NS-398 it also inhibited basolateral accumulation (Fig. 7A) without affecting the level of MRP4 (Fig. 7B and C).

An additional MRP4 inhibitor (MK-571) was used to confirm the role of this transporter on PG accumulation on the basolateral side. PGF2α was assayed in samples collected from the apical or basolateral sides of polarized bEEL cells following 6 h treatment with OT in combination with MK-571 (12.5 μM) the reported EC50 for this inhibitor in other systems (Jedlitschky, Tirschmann et al. 2004; Morioka, Kumagai et al. 2004; Russel, Koenderink et al. 2008). MK-571 inhibited the basolateral accumulation of prostaglandins (Fig. 7D) like Indomethacin, but in contrast with NS-398 that did not have this effect. MK-571 also acts on OT induced increase in MRP4 protein (Fig. 7E and F). Globally, experiments with COX and MRP4 inhibitors demonstrate that OT induces MRP4 protein and activity thus favoring basolateral accumulation of PGF2α and that this effect is independent of stimulation of PG production.

Effect of MRP4 downregulation on PGs accumulation outside endometrial stromal CSC cells.

Invalidation of MRP4 to confirm unequivocally its contribution to PG transport using siRNA could not be performed on epithelial cells because these cannot be transfected or nucleofected efficiently. However, we have found that endometrial stromal cells do express MRP4 constitutively and can be transiently transfected with specific siRNA to reduce its expression. Unfortunately stromal cells do not express OT receptors and do not form a tight polarized monolayer, comparable to epithelial cells to study PG transit. Figure 8 illustrates the effect of MRP4 downregulation on PGE2 and PGF2α accumulation outside CSC cells. siRNA transfection reduced MRP4 protein by 50% (C, D) in CSC cells and inhibited PGE2 accumulation 15% (A) and PGF2a 35% (B) thus confirming in bovine endometrial cells the preferred transport of PGE2 reported in other species and cell types (Russel, Koenderink et al. 2008).

DISCUSSION

Prostaglandin transport is emerging as an additional check point in the regulation of PG action (Schuster 1998; Reid, Wielinga et al. 2003). We were the first to propose a role for PGT in the regulation of bovine reproductive function (Banu, Arosh et al. 2003). Additional studies in vivo and in vitro support the involvement of PGT and PG transport in recognition and establishment of pregnancy in ewes (Banu, Lee et al. 2008) as well as in the menstrual cycle in human (Kang, Chapdelaine et al. 2005). PGs are lipid mediators that cannot freely cross cell membranes because of their negative charge and thus need facilitated transport in both direction (Schuster 2002). PGT has been demonstrated to primarily mediate influx of PGs inside cells (Chan, Endo et al. 2002), but it is still debated if efflux occurs via passive diffusion or facilitated transport (Schuster 2002; Chi, Khersonsky et al. 2006; Banu, Lee et al. 2008; Banu, Lee et al. 2010). PG transit kinetics in various study models suggest that physiological action requires facilitated transport (Reid, Wielinga et al. 2003) and that MRP4 is a serious candidate to fulfill this task in mammals (Russel, Koenderink et al. 2008). In the present study, we took the initiative to investigate the expression of MRP4 (ABCC4) a member of the ABC carriers, as the potential efflux carrier of endometrial PGs in the bovine. We have also included PGT/SLCO2A1 (Banu, Arosh et al. 2003), in the study to test reproducibility as well as specificity of the regulation of transport mechanisms. PGT (OATP family) and MRP4 (ABC protein) are the best candidate proteins associated with PGs transport (Kanai, Lu et al. 1995; Reid, Wielinga et al. 2003). The protein expression profiles of MRP4 (ABC family) and PGT (OATP family) (Fig. 1) exhibit some similarities, with higher expression in early to mid luteal phases. This could suggest regulation by steroid hormones during the estrous cycle, as was reported for other ABC carriers such as MDR1 upregulated by P_4 and repressed by estrogen (E_2) in mice endometrium (Schiengold, Schwantes et al. 2006). However, in the present study we have concentrated on possible regulation by OT and IFNτ known to regulate PG biosynthesis and action at the time of recognition of pregnancy (Poyser 1995; Asselin, Drolet et al. 1998; McCracken, Custer et al. 1999).

We have used our newly developed bEEL cell line (Krishnaswamy, Danyod et al. 2009; Krishnaswamy, Lacroix-Pepin et al. 2010) to investigate the modulation of MRP4 and PGT in response to OT or IFNτ. We have used IFNτ at 10 ng/ml, a concentration in the low physiological range shown to inhibit OT stimulation of PG production and induce Stat phosphorylation, without direct action on COX-2 or PG output when added alone (Krishnaswamy, Danyod et al. 2009) (Fig. 2). Oxytocin was used at a concentration of 500nM demonstrated as the minimal concentration inducing maximal PGF2α production during our characterization of bEEL cells (Krishnaswamy, Danyod et al. 2009; Krishnaswamy, Lacroix-Pepin et al. 2010).

This is higher than normal circulating concentrations (10 to 30 nM) found *in vivo* (Ginther, Shrestha et al. 2010) but it compares with local concentrations in luteal cells (100 nM) (Mlynarczuk, Wrobel et al. 2010). We have shown previously that the same OT concentration allows stimulation over a period up to 48hrs without apparent receptor desensitization and can be blocked by low doses of interferon-τ (Kim and Fortier 1995; Krishnaswamy, Danyod et al. 2009).

The hypothesis that OT could regulate PG transit through MRP4 was tested in bEEL cells grown on membrane inserts to allow the formation of tight junctions thus isolating the apical and the basolateral side of the monolayer into distinct compartments. Indeed we see that as the cells grow and form a dense monolayer, the electrical resistance increases to a stable (tight) level (fig. 4A). Under those conditions, we could reproduce the regulation of PG secretion observed on cells grown on plastic with additional advantages. We see that OT stimulated the production of PGF2α but additionally it favors accumulation of PGs on the basolateral side (Fig. 4D), corresponding in vivo to the stromal endometrium and uterine vascular system. First they confirm that IFNτ at the concentration tested is able to block OT stimulation of PG release (Fig 4B and C), while not preventing the increase in MRP4 protein (Fig. 4E and F). Second, it shows that OT favors efflux of PGs and extracellular basolateral accumulation, possibly a result of increased MRP4 expression (Fig. 4F). The side of the stimulation did not affect the basolateral accumulation of prostaglandins (Fig. 5A). Finally, while upregulation of *COX-2* and *MRP4* in response to OT (Fig 2) suggested possible common mechanisms, the absence of downregulation of MRP4 in presence of IFNτ (figure 4E and F) is in clear contrast with what was observed for COX-2 in a previous study (Krishnaswamy, Danyod et al. 2009). Again, while IFNτ inhibits OT stimulated PG production, it does not influence the distribution across polarized epithelial cell cultures. *PGT* mRNA expression was not affected by OT but significantly upregulated by IFNτ (Fig. 2B). On the other hand, OT (500 nM) induced an increase in PGs production (Fig. 2C), and upregulation of *MRP4* mRNA (Fig. 2A). At the protein level, OT induced a dose dependent increase in MRP4 protein that correlated with increased PG production (Fig 3 A, C, E) whereas there was no effect on PGT or in response to IFNτ (Fig. 3 B, D, F). The lack of correspondence in PGT expression at the protein and the mRNA level may depend on co-factors needed to effect translation, require a longer period for apparition or need more replicates to reach statistical significance in which case it may bare minimal physiological relevance. We have shown that PKC was part of the OT signaling cascade in bEEL cells (Krishnaswamy, Lacroix-Pepin et al. 2010) and accordingly, the PKC activator PMA was used to mimic OT action. Interestingly, PKC activation reproduced the effect of OT on PG production, MRP4 expression and preferred accumulation on the basolateral side. By contrast, PKA activation with 8-bromo-cAMP had no effect (Fig. 5B). This indicates that basolateral accumulation of prostaglandin is a result of oxytocin stimulation of the PKC pathway. To investigate whether OT induced MRP4 upregulation involved PG production, bEEL cells were treated with NS-398 (1 μM), a

specific inhibitor of COX2 activity that has no reported effect on MRP4 activity in this concentration range (Morioka, Kumagai et al. 2004). NS-398 completely inhibited the OT induced PG production (Fig. 6B), but this inhibition had no effect on *MRP4* mRNA (Fig. 6C), protein (Fig. 6E and F) or *COX2* expression (Fig 6A). NS-398 alone or in combination with OT did not affect *PGT* mRNA expression (data not shown). The oxytocin effect on basoleteral accumulation was not affected by NS-398 (Fig. 6D). To further confirm MRP4 action on prostaglandin transport, we used two known inhibitors of this transporter, Indomethacin and MK-571 (Fig. 7). Indomethacin has been demonstrated as a potent inhibitor of MRP4 activity in HEK 293 cells or platelets (Nozaki, Kusuhara et al. 2007; Russel, Koenderink et al. 2008) or in transport assays with vesicles (Reid, Wielinga et al. 2003). This tends to demonstrate that its effect on MRP4 is on the activity level more than the regulation of the protein level since it can inhibit the transporter in vesicular system and platelets (Reid, Wielinga et al. 2003; Morioka, Kumagai et al. 2004; Russel, Koenderink et al. 2008). Indomethacin is best known as a potent inhibitor of PG production by non selective inhibition of COX enzymes. By contrast with NS-398 a COX-2 selective inhibitor without action on MRP4 (Morioka, Kumagai et al. 2004), Indomethacin was able to inhibit the OT induced increase of basolateral PGs (Fig. 7A). As expected, the protein levels of MRP4 were not affected by Indomethacin (Fig. 7C). MK-571 has already been demonstrated as a very potent MRP4 inhibitor in various cells (Reid, Wielinga et al. 2003; Wu, Calcagno et al. 2005; Wu, Klokouzas et al. 2005) and it was able to inhibit oxytocin basolateral accumulation of PGF2α in our polarized cell system (Fig. 7D). The effect of MK-571 was associated with partial inhibition of MRP4 protein (Fig. 7E and F). Unequivoqual demonstration of the contribution of MRP4 in PG transit would require gene inactivation and downregulation of *MRP4* in our polarized cell model. Unfortunately bEEL cells cannot be transfected efficiently. For this reason we had to rely on our endometrial stromal cell line CSC which was recently developed and characterized and shown to be amenable for transient transfection (Krishnaswamy, Chapdelaine et al. 2009). Interestingly, Fig 8 shows that downregulation of MRP4 protein using siRNA inactivation, reduced significantly PG accumulation outside CSC cells. The inhibition of MRP4 averaged 50% resulting in a 35% reduction in PGF2α accumulation in the culture medium whereas PGE2 transit was reduced by 15%. The difference observed for the two PGs reflects the reported higher affinity of MRP4 for PGE2 relative to PGF2α (Reid, Wielinga et al. 2003; Russel, Koenderink et al. 2008) and confers to this transporter a rate limiting action on PGF2α release outside producing epithelial cells. Globally, our observations on the direction of PGs secretion may represent an important physiological function and support the exocrine-endocrine theory for recognition of pregnancy in the pig (Bazer and Thatcher 1977; Gross, Mirando et al. 1990). Our results also suggest that intraluminal PGs represent only a minute fraction of total PGs in presence of OT. Given that the bovine endometrium may be exposed to OT as early as day 10 (Schams 1983; Parkinson, Wathes et al. 1992) luminal secretion of PGs may be reduced during most of the luteal period and minimize the predictive value of luminal PGs (Ulbrich, Schulke et al. 2009). Indeed in

presence of OT, most of epithelial PG should be secreted towards the stroma whereas the basolateral membrane would prevent PGs from the stroma to reach the uterine lumen. Together, these results suggest a functional association between MRP4 and PG efflux toward the basolateral (circulation) side. Interestingly MRP4 was also reported as a basolateral efflux transporter in Caco-2 cells (Ming and Thakker 2010). Additional OT mediated effect on PG transit through MRP4 may include improved affinity for PGs or ATP reactivity (Sauna, Nandigama et al. 2004). IFNτ is known to inhibit the ability of OT to induce PGF2α production through different mechanisms (Roberts, Chen et al. 2008) but we have shown recently that this response could be driven quickly without alteration of OT receptor expression (Krishnaswamy, Danyod et al. 2009). The present study confirms that in presence of IFNτ some components of OT response other than increased PGF2α production remain functional in cultured endometrial cells. The present study shows functional evidence that MRP4 is under the regulation of OT and alters the direction of PG secretion out of endometrial epithelial cells and towards the basolateral side of the basement membrane. In this respect, MRP4 may be one important factor linking OT and PGs during activation of the uterus at the time of parturition in different species including the human where OT appears to play a prevailing contribution (Blanks and Thornton 2003). We propose that MRP4 complements the influx transporter function of PGT by favoring PG transport in the opposite direction. The combined action of these transporters would favor rapid transit of newly synthesized PGs from their site of production in the endometrium to a remote site of action in the ovary. OT promoted secretion of PGs towards the circulation would suggest that in presence of a viable embryo it would synergize with embryonic signals to favor recognition of pregnancy by allowing PGE2 from the stromal compartment to reach the CL. The combined and complementary action of PGT and MRP4 strongly militate in favor of PG transit as an important regulatory step in PG action and clearance in all species and systems where they exert physiological and pathologic actions.

REFERENCES

Arosh, J. A., J. Parent, et al. (2002). "Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle." Biol Reprod **67**(1): 161-9.

Asselin, E., P. Drolet, et al. (1998). "In vitro response to oxytocin and interferon-Tau in bovine endometrial cells from caruncular and inter-caruncular areas." Biol Reprod **59**(2): 241-7.

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod **54**(2): 371-9.

Banu, S. K., J. A. Arosh, et al. (2003). "Molecular cloning and spatio-temporal expression of the prostaglandin transporter: a basis for the action of prostaglandins in the bovine reproductive system." Proc Natl Acad Sci U S A **100**(20): 11747-52.

Banu, S. K., J. A. Arosh, et al. (2005). "Expression of prostaglandin transporter in the bovine uterus and fetal membranes during pregnancy." Biol Reprod **73**(2): 230-6.

Banu, S. K., J. Lee, et al. (2008). "Molecular cloning and characterization of prostaglandin (PG) transporter in ovine endometrium: role for multiple cell signaling pathways in transport of PGF2alpha." Endocrinology **149**(1): 219-31.

Banu, S. K., J. Lee, et al. (2010). "Interferon tau regulates PGF2alpha release from the ovine endometrial epithelial cells via activation of novel JAK/EGFR/ERK/EGR-1 pathways." Mol Endocrinol **24**(12): 2315-30.

Bazer, F. W. and W. W. Thatcher (1977). "Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2alpha by the uterine endometrium." Prostaglandins **14**(2): 397-400.

Blanks, A. M. and S. Thornton (2003). "The role of oxytocin in parturition." BJOG **110 Suppl 20**: 46-51.

Bowen, J. A., G. R. Newton, et al. (1996). "Characterization of a polarized porcine uterine epithelial model system." Biol Reprod **55**(3): 613-9.

Chan, B. S., S. Endo, et al. (2002). "Identification of lactate as a driving force for prostanoid transport by prostaglandin transporter PGT." Am J Physiol Renal Physiol **282**(6): F1097-102.

Chi, Y., S. M. Khersonsky, et al. (2006). "Identification of a new class of prostaglandin transporter inhibitors and characterization of their biological effects on prostaglandin E2 transport." J Pharmacol Exp Ther **316**(3): 1346-50.

Dallas, S., D. S. Miller, et al. (2006). "Multidrug resistance-associated proteins: expression and function in the central nervous system." Pharmacol Rev **58**(2): 140-61.

Delabarre, S., C. Claudon, et al. (1997). "Influence of several extracellular matrix components in primary cultures of bovine mammary epithelial cells." Tissue Cell **29**(1): 99-106.

Donze, O. and D. Picard (2002). "RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase." Nucleic Acids Res **30**(10): e46.

Fortier, M. A., K. Krishnaswamy, et al. (2008). "A postgenomic integrated view of prostaglandins in reproduction: implications for other body systems." J Physiol Pharmacol **59 Suppl 1**: 65-89.

Ginther, O. J. (1981). "Local versus systemic uteroovarian relationships in farm animals." Acta Vet Scand Suppl **77**: 103-15.

Ginther, O. J., H. K. Shrestha, et al. (2010). "Circulating hormone concentrations within a pulse of a metabolite of prostaglandin F2alpha during preluteolysis and early luteolysis in heifers." Anim Reprod Sci **122**(3-4): 253-8.

Gross, T. S., M. A. Mirando, et al. (1990). "Reorientation of prostaglandin F secretion by calcium ionophore, estradiol, and prolactin in perifused porcine endometrium." Endocrinology **127**(2): 637-42.

Jabbour, H. N. and K. J. Sales (2004). "Prostaglandin receptor signalling and function in human endometrial pathology." Trends Endocrinol Metab **15**(8): 398-404.

Jedlitschky, G., K. Tirschmann, et al. (2004). "The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage." Blood **104**(12): 3603-10.

Kanai, N., R. Lu, et al. (1995). "Identification and characterization of a prostaglandin transporter." Science **268**(5212): 866-9.

Kang, J., P. Chapdelaine, et al. (2005). "Expression of human prostaglandin transporter in the human endometrium across the menstrual cycle." J Clin Endocrinol Metab **90**(4): 2308-13.

Kim, J. J. and M. A. Fortier (1995). "Cell type specificity and protein kinase C dependency on the stimulation of prostaglandin E2 and prostaglandin F2 alpha production by oxytocin and platelet-activating factor in bovine endometrial cells." J Reprod Fertil **103**(2): 239-47.

Krishnaswamy, N., P. Chapdelaine, et al. (2009). "Development and characterization of a simian virus 40 immortalized bovine endometrial stromal cell line." Endocrinology **150**(1): 485-91.

Krishnaswamy, N., G. Danyod, et al. (2009). "Oxytocin receptor down-regulation is not necessary for reducing oxytocin-induced prostaglandin F(2alpha) accumulation by interferon-tau in a bovine endometrial epithelial cell line." Endocrinology **150**(2): 897-905.

Krishnaswamy, N., N. Lacroix-Pepin, et al. (2010). "Epidermal growth factor receptor is an obligatory intermediate for oxytocin-induced cyclooxygenase 2 expression and prostaglandin F2 alpha production in bovine endometrial epithelial cells." Endocrinology **151**(3): 1367-74.

Lee, J., J. A. McCracken, et al. (2010). "Transport of Prostaglandin F2{alpha} Pulses from the Uterus to the Ovary at the Time of Luteolysis in Ruminants Is Regulated by Prostaglandin Transporter-Mediated Mechanisms." Endocrinology.

Lim, H., B. C. Paria, et al. (1997). "Multiple female reproductive failures in cyclooxygenase 2-deficient mice." Cell **91**(2): 197-208.

McCracken, J. A., E. E. Custer, et al. (1999). "Luteolysis: a neuroendocrine-mediated event." Physiol Rev **79**(2): 263-323.

Ming, X. and D. R. Thakker (2010). "Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil." Biochem Pharmacol **79**(3): 455-62.

Mlynarczuk, J., M. H. Wrobel, et al. (2010). "Effect of environmental pollutants on oxytocin synthesis and secretion from corpus luteum and on contractions of uterus from pregnant cows." Toxicol Appl Pharmacol **247**(3): 243-9.

Morioka, N., K. Kumagai, et al. (2004). "Nonsteroidal anti-inflammatory drugs potentiate 1-methyl-4-phenylpyridinium (MPP+)-induced cell death by promoting the intracellular accumulation of MPP+ in PC12 cells." J Pharmacol Exp Ther **310**(2): 800-7.

Munson, L., J. E. Wilkinson, et al. (1990). "Effects of substrata on the polarization of bovine endometrial epithelial cells in vitro." Cell Tissue Res **261**(1): 155-61.

Nozaki, Y., H. Kusuhara, et al. (2007). "Species difference in the inhibitory effect of nonsteroidal anti-inflammatory drugs on the uptake of methotrexate by human kidney slices." J Pharmacol Exp Ther **322**(3): 1162-70.

Parkinson, T. J., D. C. Wathes, et al. (1992). "Plasma and luteal concentrations of oxytocin in cyclic and early-pregnant cattle." J Reprod Fertil **94**(1): 161-7.

Poyser, N. L. (1995). "The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation." Prostaglandins Leukot Essent Fatty Acids **53**(3): 147-95.

Pratt, B. R., R. L. Butcher, et al. (1979). "Effect of continuous intrauterine administration of prostaglandin E2 on life span of corpora lutea of nonpregnant ewes." J Anim Sci **48**(6): 1441-6.

Reid, G., P. Wielinga, et al. (2003). "Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5." Mol Pharmacol **63**(5): 1094-103.

Reid, G., P. Wielinga, et al. (2003). "The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs." Proc Natl Acad Sci U S A **100**(16): 9244-9.

Roberts, R. M., Y. Chen, et al. (2008). "Interferons and the maternal-conceptus dialog in mammals." Semin Cell Dev Biol **19**(2): 170-7.

Russel, F. G., J. B. Koenderink, et al. (2008). "Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules." Trends Pharmacol Sci **29**(4): 200-7.

Sauna, Z. E., K. Nandigama, et al. (2004). "Multidrug resistance protein 4 (ABCC4)-mediated ATP hydrolysis: effect of transport substrates and characterization of the post-hydrolysis transition state." J Biol Chem **279**(47): 48855-64.

Schams, D. (1983). "Oxytocin determination by radioimmunoassay. III. Improvement to subpicogram sensitivity and application to blood levels in cyclic cattle." Acta Endocrinol (Copenh) **103**(2): 180-3.

Schiengold, M., L. Schwantes, et al. (2006). "Expression of mdr isoforms in mice during estrous cycle and under hormone stimulation." Genetics and Molecular Biology **29**(4): 755-761.

Schuster, V. L. (1998). "Molecular mechanisms of prostaglandin transport." Annu Rev Physiol **60**: 221-42.

Schuster, V. L. (2002). "Prostaglandin transport." Prostaglandins Other Lipid Mediat **68-69**: 633-47.

Spencer, T. E., G. A. Johnson, et al. (2007). "Fetal-maternal interactions during the establishment of pregnancy in ruminants." Soc Reprod Fertil Suppl **64**: 379-96.

Thatcher, W. W., A. Guzeloglu, et al. (2001). "Uterine-conceptus interactions and reproductive failure in cattle." Theriogenology **56**(9): 1435-50.

Ulbrich, S. E., K. Schulke, et al. (2009). "Quantitative characterization of prostaglandins in the uterus of early pregnant cattle." Reproduction **138**(2): 371-82.

Wu, C. P., A. M. Calcagno, et al. (2005). "Modulatory effects of plant phenols on human multidrug-resistance proteins 1, 4 and 5 (ABCC1, 4 and 5)." FEBS J **272**(18): 4725-40.

Wu, C. P., A. Klokouzas, et al. (2005). "Interactions of mefloquine with ABC proteins, MRP1 (ABCC1) and MRP4 (ABCC4) that are present in human red cell membranes." Biochem Pharmacol **70**(4): 500-10.

FIGURES

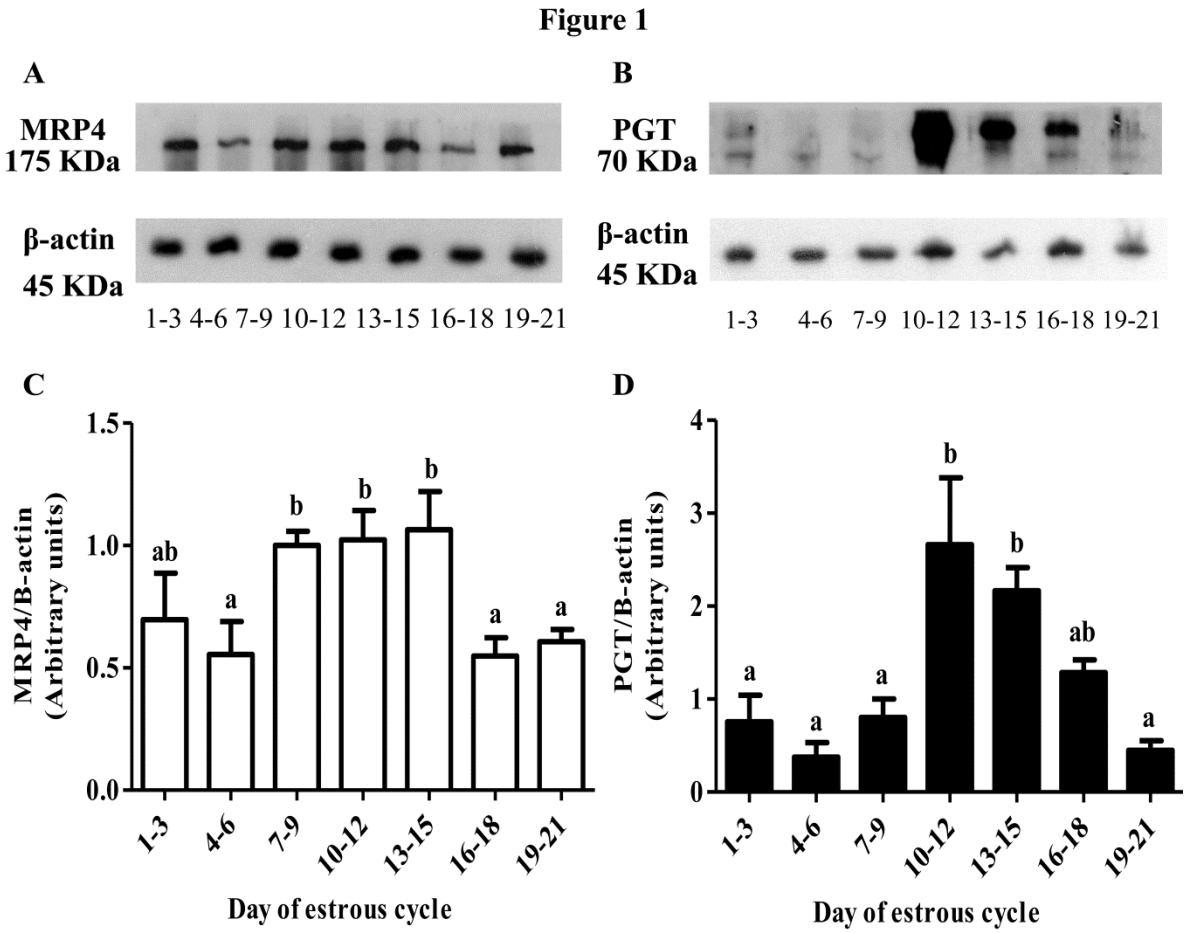


Figure 8 - Article 2: Figure 1 Evaluation of MRP4/ABCC4 and PGT/SLCO2A1 levels in the bovine endometrium during the estrous cycle.

Endometrial tissues were collected at the slaughter house from cows at different periods of the estrous cycle. For the purpose of this study, three samples each were taken for each of the following periods: days 1-3, 4-6, 7-8, 10-12, 13-15, 16-18 and 19-21. Samples were processed for analysis by Western blotting using validated polyclonal antibodies targeting MRP4 or PGT as described in material and methods. **A)** Representative blot of MRP4/ABCC4 and β-actin levels across the estrous cycle. **C)** Corresponding bar graphs showing the average levels of MRP4 following quantitation of three blots using image analysis. **B)** Representative blots of PGT/SLCO2A1 and β-actin across the estrous cycle. **D)** Corresponding bar graphs showing the average levels of PGT following quantitation of three blots using image analysis Quantification (arbitrary units) was performed with Alpha Imager using spot denso program. Results were expressed as the mean ± SEM of MRP4 or PGT relative to β-actin levels. Bars with different superscript are significantly different ($P<0.05$).

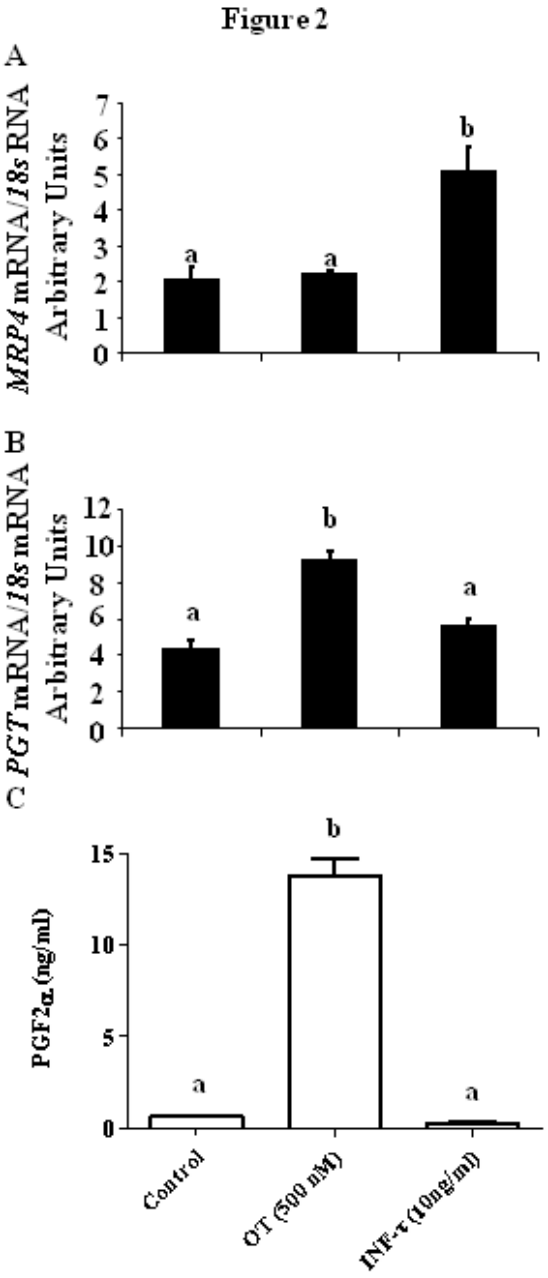


Figure 9 - Article 2: Figure 2 Effects of OT and IFNτ on gene expression of MRP4/ABCC4 and PGT/SLCO2A1 in the bovine endometrial epithelial cell line bEEL.

bEEL cells were grown to confluency, the culture medium was replaced with fresh medium without serum and cells were treated for 24 h with OT (500 nM) or IFNτ (10 ng/ml). The culture medium was collected for measurement of PGs and the cell fraction processed for mRNA extraction and qRT-PCR analysis. **A)** and **B)** illustrate respectively *MRP4* and *PGT* mRNA levels relative to *18S* rRNA and represent the mean ± SEM of 3 different experiments. **C)** Corresponding PGF2α accumulation over the 24 h treatment period. Bars with different superscript are significantly different (P<0.05).

Figure 3

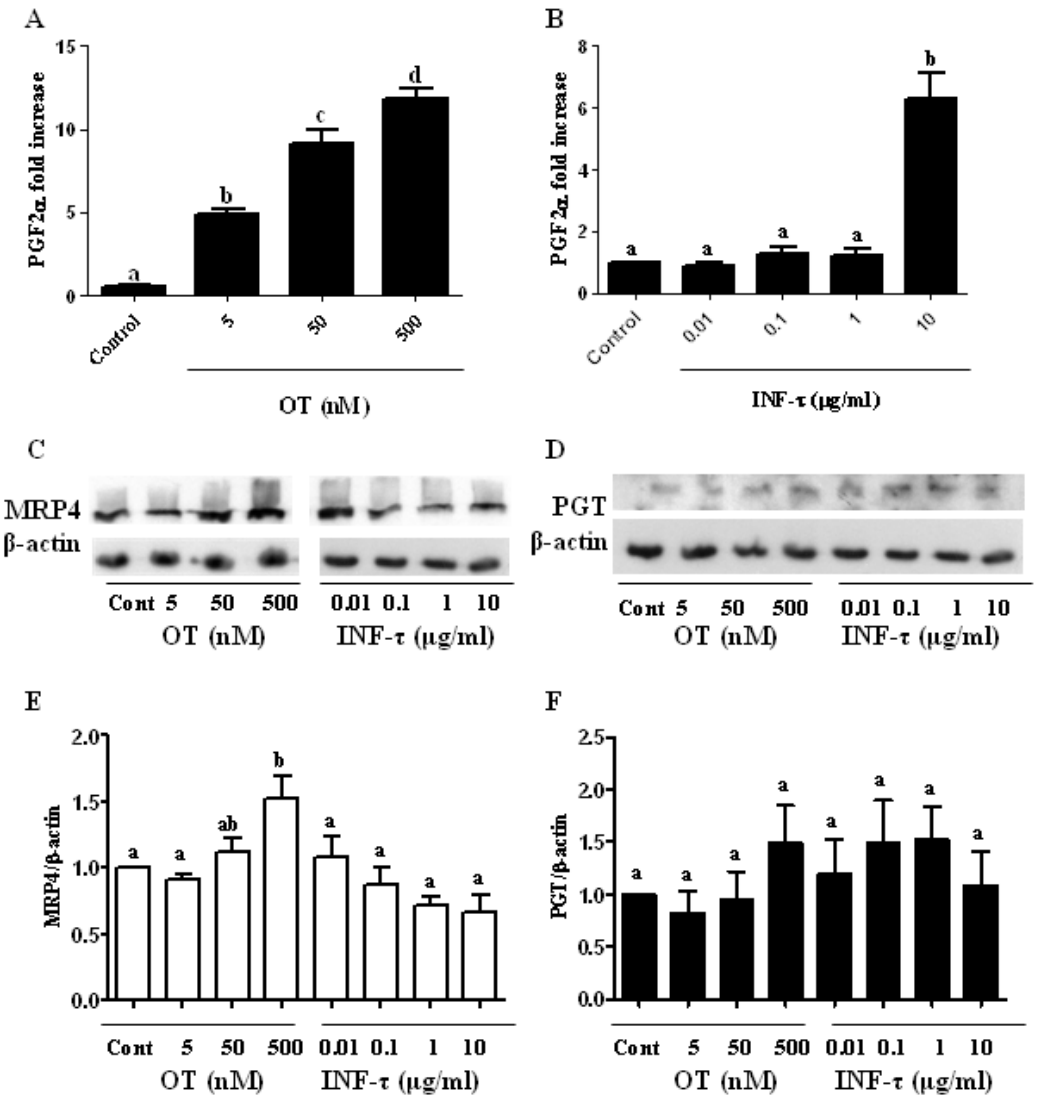


Figure 10 - Article 2: Figure 3 Effects of increasing concentrations of OT and IFNτ on MRP4 and PGT protein levels in bEEL cells.

bEEL cells were grown to confluency, the culture medium was replaced with fresh medium without serum and cells were treated for 6 h with increasing doses of OT (5 to 500 nM) or IFNτ (0.01 to 10 µM). A, B) PGF2α production following treatment of bEEL cells with increasing concentrations of respectively OT or IFNτ. Similar results were obtained for PGE2 (not shown). Values represent the mean ± SEM of three independent experiments run in hexaplicate. C, D) Western blot analysis of MRP4 and PGT following treatment with increasing doses of OT and IFNτ. β-actin shown as loading and quantitation control. E, F) Quantification (arbitrary units) of MRP4 and PGT levels relative to β-actin with Alpha Imager using spot denso program. Results represent the mean ± SEM of 3 different blots for each transporter.

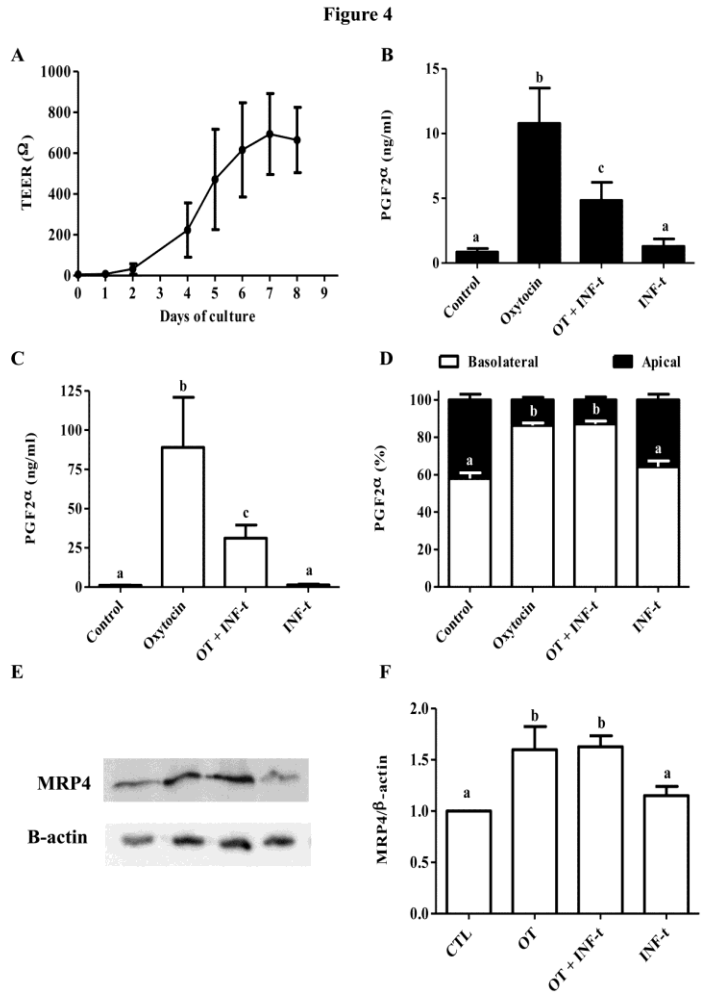


Figure 11 - Article 2: Figure 4 Regulation of directional secretion of PGs in polarized bEEL cells.

Polarized bEEL cells were produced as described in material and methods. Formation of tight junctions between adjacent cells was monitored by measurement of transepithelial resistance (TEER) daily with an EVOM Epithelial Voltohmmeter. A) TEER increased progressively with cell growth and reached a plateau at 600 to 800 Ω (ohms) after 7 to 9 days in culture, values represent the mean ± SEM of 12 separate experiments. bEEL cells were cultured for 6 to7 days until TEER reached at least 600 Ω and treated with OT (500 nM), IFNτ (10 ng/ml) or both for 6 h. At the end of the treatment period, the culture medium was recovered from the top (Apical, B) or the bottom (Basolateral, C) compartment of the transwell culture system and PGF2α content was assayed by EIA. The relative distribution of PGE2 or PGF2α on the apical vs basolateral side is illustrated in D). Values represent the mean ± SEM of three independent experiments run in triplicate. Note that OT favors basolateral secretion of both PGs and that this is not affected by the presence of IFNτ. Western blot analysis of MRP4 following treatment is shown in E). β-actin shown as loading and quantification control. F) Quantification (arbitrary units) of MRP4 levels relative to β-actin with Alpha Imager using spot denso program. Results represent the mean ± SEM of 3 different blots.

Figure 5

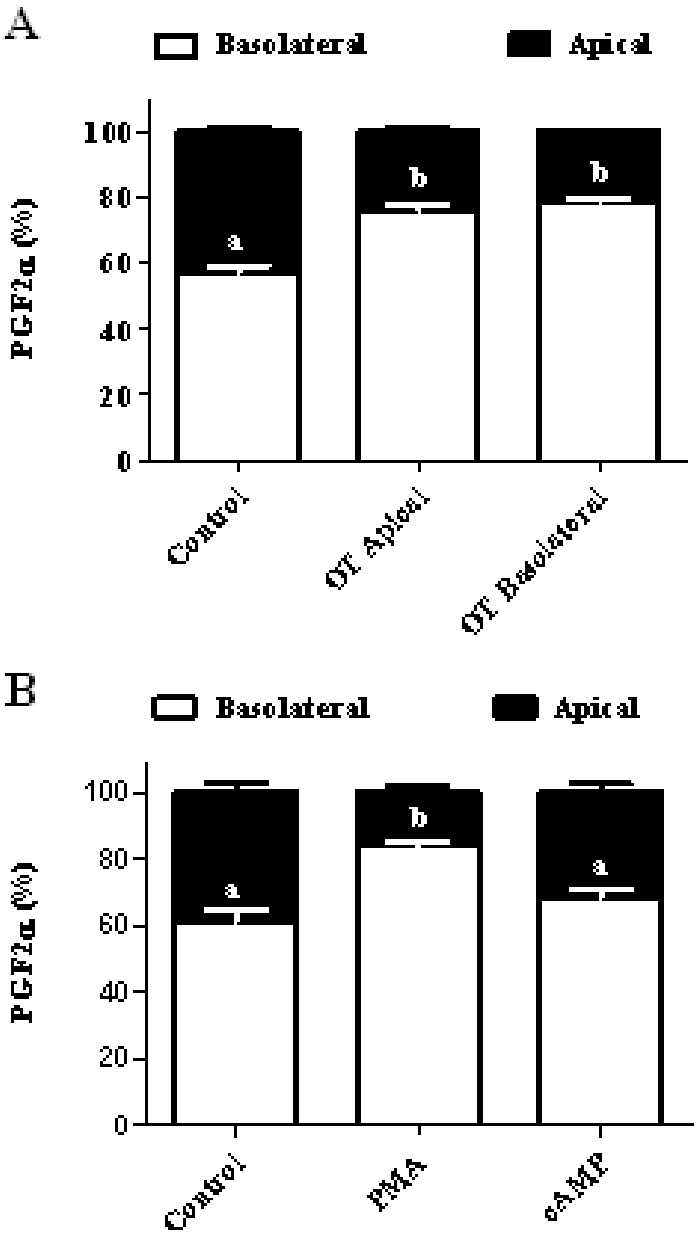


Figure 12 - Article 2: Figure 5 Evaluation of compartments and pathways involved in OT regulation of directional secretion of PGF2α.

bEEL cells were cultured on collagen coated inserts as described in Fig 4 and treated with OT (500 nM) on the basolateral or the apical side of the insert for 6 h (**A**). Cells were also treated with PMA (10 nM) or cAMP (500µM) to evaluate the contribution of the PKC and PKA pathways on PGF2α accumulation in the basolateral and apical compartments of the transwell plate (**B**). The relative distribution on each side is represented as a % of total PGF2α. Results represent the mean ± SEM of three independent experiments run in triplicate.

Figure 6

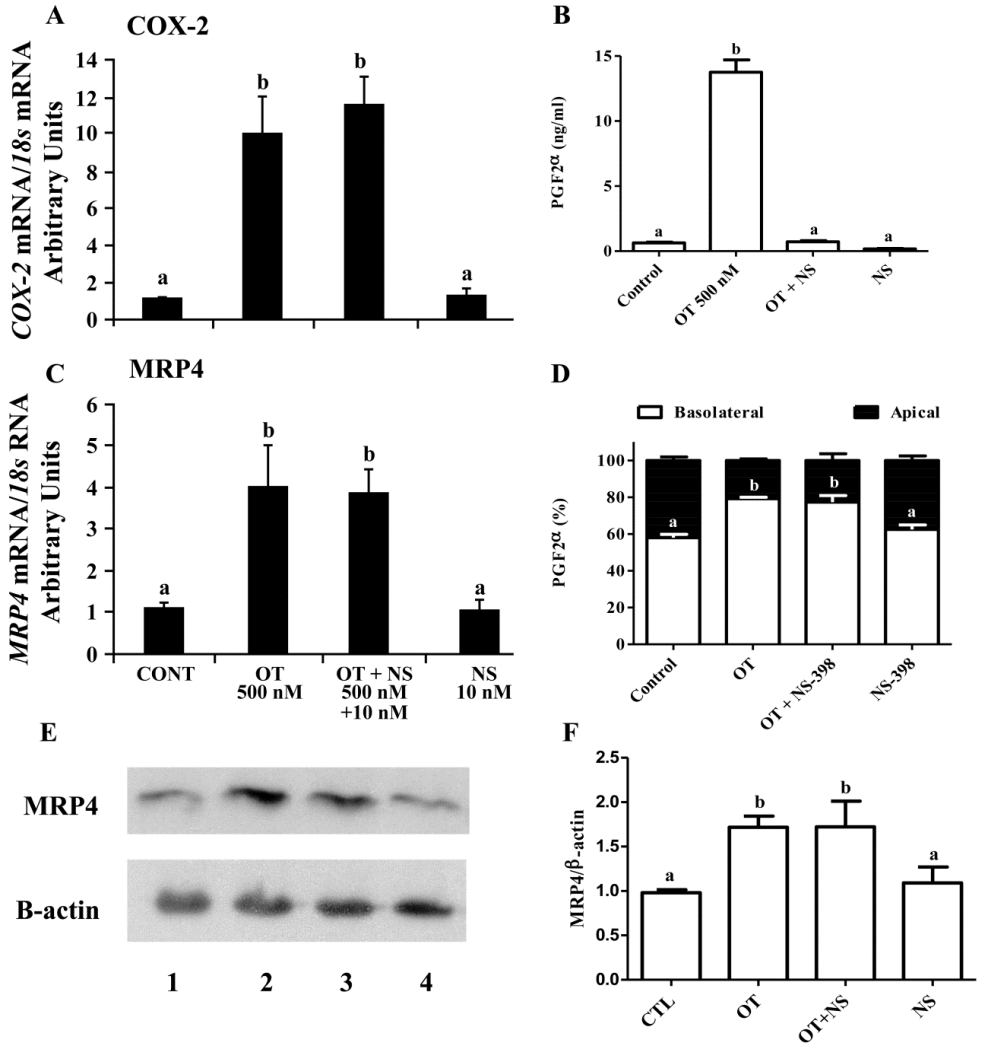


Figure 13 - Article 2: Figure 6 Evaluation of the contribution of PGs on the effect of OT on MRP4 expression and function.

bEEL cells were grown to confluency, the culture medium was replaced with fresh medium without serum and cells were treated for 6 h with OT (500 nM) in presence or absence of the COX-2 inhibitor NS-398 (1µM) At the end of the treatment period, the culture medium was recovered for PG measurement and the cell fraction processed for mRNA or protein analysis. mRNA levels respectively for COX-2 A) and MRP4 C) were analyzed by qRT-PCR and represent the mean ± SEM of expression relative to 18S rRNA for 3 different experiments. E, F) Representative blot and quantitation of MRP4 levels relative to β-actin mean ± SEM of three independent experiments. Line 1 control, 2 OT, 3 OT+NS-398 and 4 NS-398 alone. B) PGF2α accumulation over the 6h treatment period, mean ± SEM of three independent experiments run in hexaplicate. Similar results were obtained for PGE2 (not shown). bEEL cells were cultured on collagen coated inserts as described in material and methods. The relative amount on apical and basolateral side of insert culture is presented in D).

Figure 7

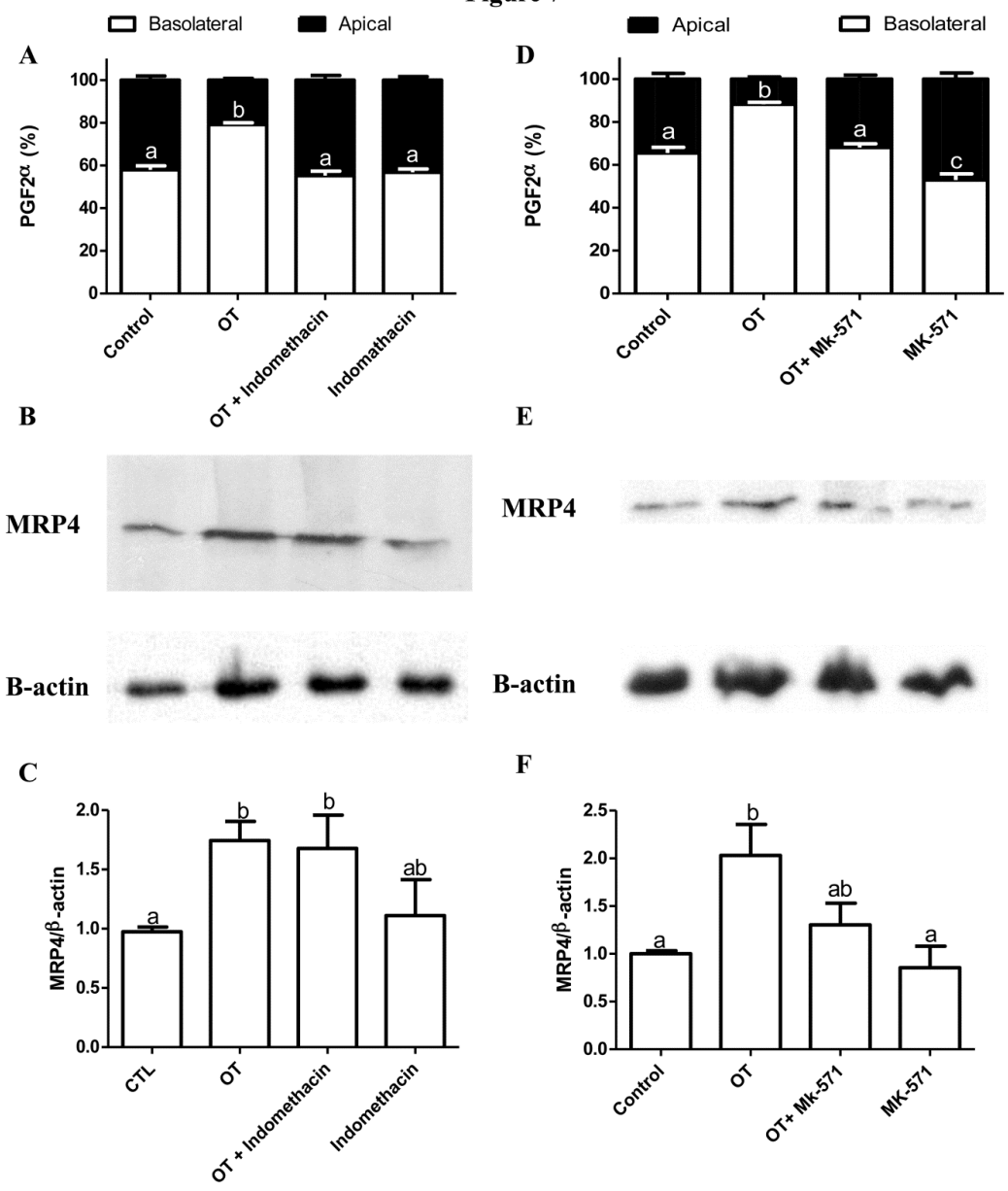


Figure 14 - Article 2: Figure 7 Influence of Indomethacin and MK-571 on MRP4 and directional secretion of PGF2α in polarized bEEL cells.

bEEL cells were cultured on collagen coated inserts as described in Fig 4 and treated with OT (500nM) and Indomethacin (25 µM) or MK-571 (12.5 µM) for 6 h. PGF2α accumulation was measured in the basolateral and apical compartments of the transwell plate. The relative distribution on each side is represented as a % of total PGF2α (A, D). The effect on MRP4 protein was studied by Western analysis (B, E) and protein levels relative to β-actin are shown in (C, F). Results represent the mean ± SEM of three independent experiments run in triplicate.

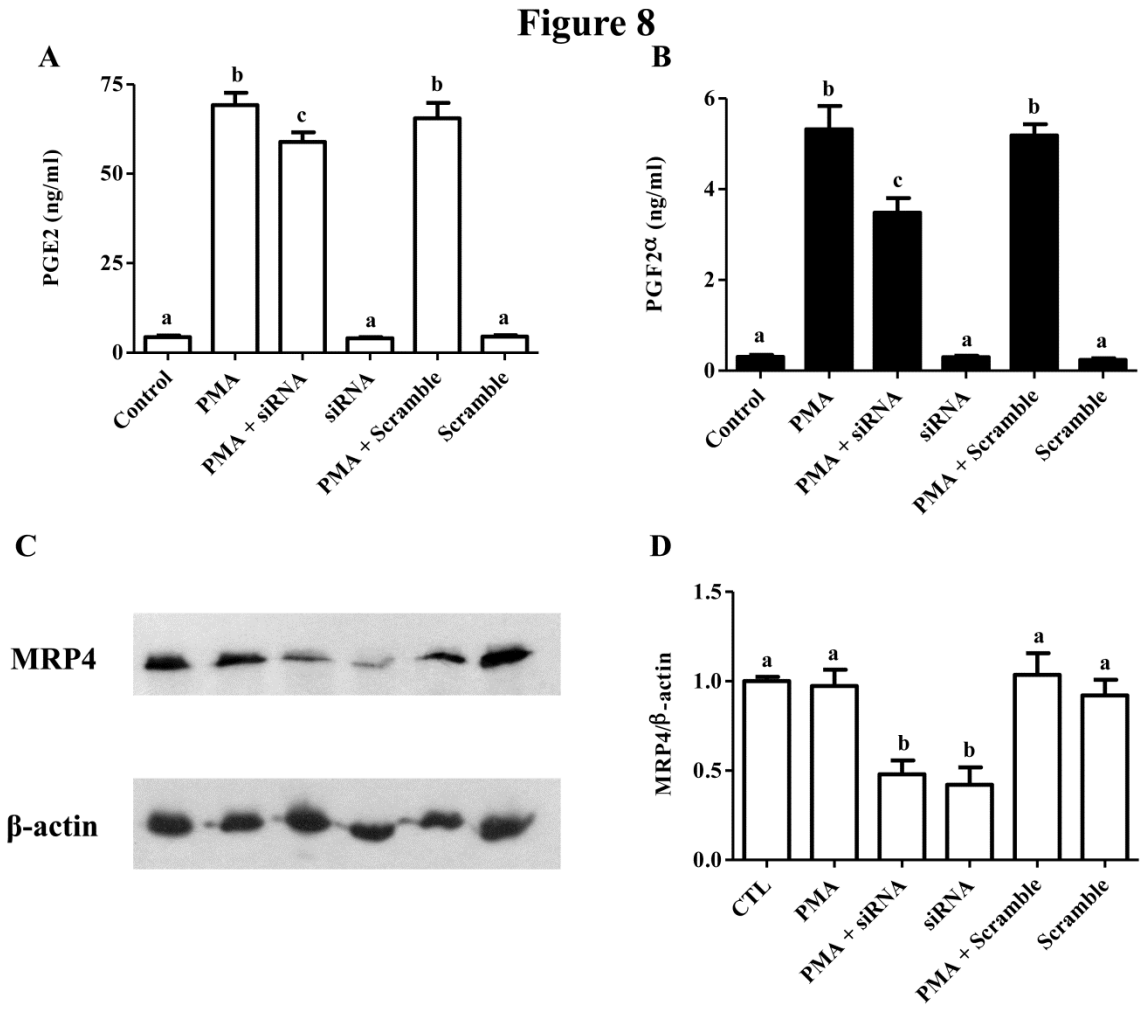


Figure 15 - Article 2: Figure 8 Extracellular accumulation of PGs in Stromal CSC cells following knockdown of MRP4 expression by siRNA.

Stromal CSC cells were transiently transfected to downregulate MRP4 with siRNA and cells were treated with PMA (10 nM) for 6 h to stimulate PG production. A,B) PGE2 and PGF2α accumulation over a 6 h culture period. C) Western blot analysis of MRP4 protein D) Densitometric analysis of MRP4 protein relative to b-actin. Results are representative or the mean ± SEM of four experiments run in triplicate.

Chapitre 4: Evaluation of the prostaglandin F synthase activity of human and bovine aldo-keto reductases: AKR1A1s complement AKR1B1s as potent PGF synthases.

Nicolas Lacroix Pépin, Pierre Chapdelaine, Michel A Fortier

Ce chapitre est présenté sous forme d'article et a été publié dans la revue :
Prostaglandins Other Lipid Mediat. 2013 Jun 6.

Centre de Recherche du CHU de Québec (CHUL), Département d'Obstétrique et Gynécologie, Axe de reproduction, santé de la mère et de l'enfant, Université Laval, Ste-Foy, Québec, Canada GIV 4G2

RÉSUMÉ

AKR1B1 de la voie des polyols a été identifiée comme synthase pour la prostaglandin F2α (PGFS). En utilisant une méthode génomique nous avons identifié dans l'endomètre 5 AKR bovines et 3 humaines avec une activité PGFS potentielle et généré les enzymes recombinantes correspondantes. L'activité PGFS des protéines recombinantes a été évaluée en utilisant un nouvel essai basé sur la génération in situ du précurseur de la biosynthèse des PG, le PGH2. PGF2α a été mesurée par ELISA et l'activité relative des différentes enzymes a été comparée. Nous avons identifié AKR1A1 et confirmé AKR1B1 comme les PGFS les plus efficaces exprimant une inhibition caractéristique en présence de methylglyoxal, de ponalrestat et de glucose.

ABSTRACT

AKR1B1 of the polyol pathway was identified as a prostaglandinF2α synthase (PGFS). Using a genomic approach we have identified in the endometrium five bovine and three human AKRs with putative PGFS activity and generated the corresponding recombinant enzymes. The PGFS activity of the recombinant proteins was evaluated using a novel assay based on in situ generation of the precursor of PG biosynthesis PGH2. PGF2α was measured by ELISA and the relative potencies of the different enzymes were compared. We identified AKR1A1 and confirmed AKR1B1 as the most potent PGFS expressing characteristic inhibition patterns in presence of methylglyoxal, ponalrestat and glucose.

INTRODUCTION

Prostaglandins (PGs) represent a family of lipid mediators acting locally in the vascular, reproductive and other systems to maintain homeostasis through complementary and sometimes opposite actions (Poyser 1995; Lim, Paria et al. 1997; Thatcher, Guzeloglu et al. 2001; Jabbour and Sales 2004; Spencer, Johnson et al. 2007; Smith, Urade et al. 2011). Prostaglandin formation is a key component of PG action and the specific target of non-steroidal anti-inflammatory drugs.

PGs are synthesized in virtually all nucleated cells of the body from essential fatty acids (EFAs), primarily arachidonic acid (AA) in the western diet. The first step in PG formation is the conversion of AA from the cell membrane to PGG2 and then PGH2 by one of two prostaglandin G/H synthases (PTGS-1 or -2) better known as cyclooxygenases (COX-1or -2). After PGH2 formation, terminal synthases generate the bioactive prostaglandins (Smith, Urade et al. 2011). Specific terminal synthases required for enzymatic production of PGs were identified for PGE2 (Tanikawa, Ohmiya et al. 2002), thromboxane A2 (TxA2) (Tanabe and Ullrich 1995), prostacyclin (PGI2) (Ullrich, Castle et al. 1981), PGD2 (Christ-Hazelhof and Nugteren 1979) and more recently PGF2α (Bresson, Boucher-Kovalik et al. 2011).

While PGF2α has not been studied as extensively as other PGs, it nevertheless plays important roles in the regulation of ocular pressure, renal absorption, cardio-vascular function, adipocyte differentiation and female reproductive function (Bresson, Lacroix-Pepin et al. 2012). In the vascular system PGF2α exhibits vasoconstrictive and pro-thrombotic responses comparable to thromboxane with the additional feature that its molecule is chemically stable and remains active until catabolism by prostaglandin dehydrogenase, usually through a single passage in the lung.

Most prostaglandin F2α synthases (PGFS) identified to date are AKRs of the 1C family and their identification goes back and forth between human and bovine since the 80's. The discovery of the bovine lung PGFS (Watanabe, Yoshida et al. 1985) previously classed as AKR1C7 (NCBI-GI: 129896, EC: 1.1.1.188) (Smith, Urade et al. 2011) led to the attribution of PGFS function to its human homologue AKR1C3 (Suzuki-Yamamoto, Nishizawa et al. 1999). Because the bovine endometrium produces huge amounts of PGF2α but does not express the bovine lung PGFS, our search for an alternate enzyme led to our identification of the role of bovine AKR1B1 (previously known as AKR1B5) in the formation of PGF2α from PGH2 in the bovine endometrium (Madore, Harvey et al. 2003). We have then extended this finding to the human where we have demonstrated that in addition to AKR1C3, AKR1B1 was able to produce PGF2α in the endometrium (Bresson, Boucher-Kovalik et al. 2011). This was a major finding because the PGFS activity of AKR1B1 proved to be much higher than that of AKR1C3 (Kabututu, Manin et al. 2009). In human, increased PGF2α production in response to IL-1β is associated with upregulation of AKR1B1, cPLA2 and COX-2 proteins (Chapdelaine, Kang

et al. 2006; Bresson, Boucher-Kovalik et al. 2011). In the bovine, we did not find any contribution of AKR1Cs (Madore, Harvey et al. 2003) and stimulation or inhibition of AKR1B1 does not explain entirely the variation in PGF2α biosynthesis (Krishnaswamy, Chapdelaine et al. 2009). Since abrogation of PGF2α release in response to AKR1B1 inhibitors was not as complete in bovine as in human endometrial cells (Bresson, Lacroix-Pepin et al. 2012), we hypothesized that another PGFS enzyme could be present.

In the present study, we have investigated if AKRs sharing at least 60% similarity with bovine AKR1B1 protein sequence were expressed in the bovine and human endometrium and if among those some exhibited PGFS activity. In order to estimate PGFS activity, we have adapted existing methods to implement a procedure whereby the PGH2 substrate was generated in situ in a cell free system. For clarity purposes, since many AKR have the same name between species while not meaning to be necessarily orthologues, "b" for bovine or "h" for human has been used throughout the text to specify the source and alleviate confusion.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following suppliers: Superscript III reverse transcriptase, one kB DNA ladder, dithiothreitol, 5x first strand buffer and TRIzol from Invitrogen Life Technologies Inc. (Burlington, ON, Canada). Random primer-pd(N)6, deoxy-NTPs, RNA Guard, rTaq DNA polymerase, PCR 10x buffer from GE healthcare Canada (Baie d'Urfé, QC, Canada). Plasmid pDrive (TA cloning kit), DNA purification kits and QuantiTect SYBR Green PCR Kit for quantitative real time PCR were from Qiagen (Mississauga, ON, Canada) using LightCycler® System (Roche Diagnostics, Laval, QC, Canada). All oligonucleotide primers were chemically synthesized using ABT 394 synthase (Perkin-Elmer, Foster City, CA). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). NADPH, methylglyoxal and 9,10-phenanthrenequinone, hematin was from Sigma-Aldrich (Oakville, ONT, Canada) and arachidonic acid, sc-560, recombinant human COX-2 and ovine COX-1 proteins were from Cayman Chemical (Ann Arbor, MI). Ponalrestat (Statil) was from Tocris Bioscience. (Ellisville, MO)

Preparation of endometrial tissues

Bovine uteri were collected at a local abattoir immediately after exsanguination, placed on ice and brought to the laboratory within 90 min. The cyclic stage of endometrial samples was determined as described previously based on gross morphology and examination of ovaries (Arosh, Parent et al. 2002). Endometrial

strips were cut into small pieces, snap-frozen in liquid nitrogen, and stored at -80 C until used for analysis. For human endometrial tissues, preparation was done as described previously (Bresson, Boucher-Kovalik et al. 2011; Bresson, Lacroix-Pepin et al. 2012) from women aged between 25 and 50 with normal cycle length (28-30 days) and no hormonal treatment. Informed consent for donation of anonymous endometrial samples was obtained before tissue collection. The research protocol was approved by the "Ethics Committee on Human Research of Centre Hospitalier Universitaire de Québec".

Molecular cloning of bovine potential PGFS

Following blasting of the genomic DNA with bovine AKR1B1 mRNA sequence, 20 homolog proteins possessing at least 60% amino acids similarity were tested using specific forward and reverse primers (supplementary table 1). These genes were synthesized and used for RT-PCR in bovine endometrial tissues. Consequently, the genes found to be expressed in the endometrium were cloned in a pDrive vector (Qiaquick PCR cloning kit, Qiagen) and then confirmed by sequencing. Finally, five bovine amplicons identified in the partial gene cloning protocol were selected based on expression level and amplified from endometrial RNA using primers listed in supplementary table 2 to generate the full length cDNA coding for complete proteins listed in supplementary tables 3 to 7.

Production of bovine and human His-tagged recombinant proteins

Five bovine genes identified as AKR1B1, 1A1, 1C1, 1C4 and DD3 were produced as recombinant proteins in BL21 DE3 pLysS. The human genes AKR1B1, 1C3 and 1A1 were produced in bacteria as for the bovine genes counterpart. Recombinant proteins including restriction enzyme sequences (supplementary table 2) were produced as described previously for AKR1B1 using a pET-16b vector that included a 8 X HIS at the N-terminal of the coding frame (Madore, Harvey et al. 2003). In summary, molecular cloning of the full-length enzyme was performed as described above for the partial fragments with the primers found in table 2. Sequencing of both strands with T7 and SP6 primers validated the identity of the cloned gene. Once in the pDrive (Qiagen), the correct full-length fragment was digested using restriction enzymes. *Nde* I/*Bam*H I was used for bovine AKR1A1, bovine AKR1B1, human AKR1A1 and human AKR1C3 and *Nde* I/*Bgl* II for bovine AKR1C1, bovine AKR1C4, bovine dihydrodiol dehydrogenase 3 (DD3) and human AKR1B1. All fragments were purified using Qiagen PCR purification Kit. Ligation was carried out at 13^{o}C overnight in presence of T4 DNA ligase (NEB). Sequencing both strands with T7 promoter and T7 terminator primers validated the identity and correct orientation of the cloned gene. The eight recombinant plasmids 8X HIS-AKRs in the prokaryotic expression vector pET-16b (Novagen) have been transformed in BL21 DE3 pLysS and grown overnight at 37°C on agar plates containing ampicillin (150μg/ml), chloramphenicol (34 μg/ml). The next day, three to five colonies of each recombinant proteins were seeded separately in 25 ml of LB containing ampicillin (150μg/ml)

and chloramphenicol (34 μg/ml) and cultured under agitation overnight at 37°C. Ten ml of the overnight culture was then seeded in 200 ml of LB medium containing only ampicillin (150μg/ml). When the culture reached an absorbance (at A600nm) between 0.5 -0.7, induction was done with 1mM IPTG for four hours under vigorous shaking at 37°C. The culture mixture was then centrifuged at 6000 x g 15 minutes and bacteria pellets were kept frozen at -80°C until lysis with 10ml of CelLytic B (Sigma) diluted 1/10 in lysis buffer (50mM NaH2PO4, 300 mM NaCl, 10mM imidazole pH8.0), containing benzonase (50 Units/ml). Lysozyme (1mg/ml) was added and the lysate was shaken vigorously at room temperature during 20 minutes. The lysate was then centrifuged at 16000 x g for 20 minutes and the supernatant (10ml) was passed through a Ni-NTA column (Superflow, size 25 ml, Qiagen). The column was washed with three volumes (30 ml) of washing buffer (50mM NaH2PO4, 300 mM NaCl, 20mM imidazole pH8.0) and elution was performed with 10 ml of the same buffer. The first two elution fractions (2ml) were collected and recombinant proteins were dialyzed twice against 2L of PBS at 4°C for 12 and 6 hrs. Proteins were then loaded on a SDS polyacrylamide gel (SDS-PAGE) and stained with Coomassie blue. Protein quantification was then done by the method of Bradford with average yields of 0.05 to 0.9 mg/ml.

Analysis of AKR1A1 and AKR1B1 by Western Blot

Protein extraction, quantification and western blot analysis were performed as described previously (Arosh, Parent et al. 2002). For each sample, aliquots of 5-10 μg protein were separated on 10% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes (Bio-RaD Laboratories Canada Ltd, Mississauga, Ontario). The membranes were blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 5% fat-free dry milk (BLOTTO) and 0.05% Tween 20. For the detection of bovine AKR1A1, the membrane was probed with a monoclonal mouse anti-human AKR1A1 (1:500) from Santa Cruz. The bovine protein (NCBI-GI: 618607, EC: 1.1.1.2) exhibits 93% identity with the human protein (NCBI-GI: 10327, EC 1.1.1.2). The bovine and human AKR1B1 polyclonal antibodies were produced in our laboratory using the corresponding recombinant proteins as antigens and were characterized previously (Madore, Harvey et al. 2003; Krishnaswamy, Chapdelaine et al. 2009; Bresson, Boucher-Kovalik et al. 2011; Bresson, Lacroix-Pepin et al. 2012). A monoclonal β-Actin antibody (Sigma-Aldrich Canada Ltd, Oakville, ONT, Canada) was used at 1:5000 in PBS-T containing 5% BLOTTO for one hour at room temperature and served as a loading and quantification control. Following the first antibody incubation, membranes were washed in PBS-T and secondary antibodies, a goat anti-rabbit (for AKR1B1) or anti-mouse (for AKR1A1 and β-Actin) horseradish peroxidase-conjugated IgG (Jackson laboratories, West Grove, PA, USA) were used at 1:10000 for 1 hour at room temperature in PBS-T containing 5% BLOTTO followed by several washes in PBS-T. The chemiluminescence signal was analyzed with an autoradiography film after treatment of the membrane with Renaissance reagent (NEN, Perkins Elmer,

Boston, MA, USA). The signal intensity of the immunoreactions was quantified by densitometry using a Multimage Light Cabinet equipped with AlphaImager 2000 software (Cell Biosciences, Santa Clara, California,)

Catalytic activity determination

AKR activity was measured in presence of 9,10-*phenanthrenequinone (10-300 µM)* and methylglyoxal (0.5-20 mM) by monitoring degradation of NADPH at 340nm. 9,10-*Phenanthrenequinone was dissolved in hot ethanol which final concentration was kept below 5%.* The activity was determined as we described previously (Jakobsson, Thoren et al. 1999; Madore, Harvey et al. 2003; Nagata, Kusakari et al. 2011). Briefly, 5µg of the purified recombinant enzymes were incubated at 25°C for 20-90 min in the presence of 200 µM NADPH in 50 mM sodium phosphate buffer, pH 7 in a final volume of 100 µl in 96 well plates. The reaction was monitored using a spectrophotometer (UVM 340) with intervals of 1 min between measurements with the MicroWin 2000 software.

In vitro PGF2α production by coupling AKR and COX enzymes with arachidonic acid

Modification to the catalytic activity procedure was done to prevent error coming from the instability of PGH2 (Jakobsson, Thoren et al. 1999; Madore, Harvey et al. 2003; Nagata, Kusakari et al. 2011). Briefly, 0.5 to 10 µg of the purified recombinant enzymes were incubated at 25°C for 2 min with 0 to 64 ng of recombinant human COX-2 or ovine COX-1 with 200 µM NADPH and 1 µM haematin in 50 mM sodium phosphate buffer, pH 7 for a final volume of 100 µl in 96 well plates. Various concentrations of arachidonic acid (0-100 µM) were added to start the reaction. Also, SC-560 a specific inhibitor of COX-1 (Smith, Zhang et al. 1998) was used at 1 and 100 nM to confirm dependence on COX activity for PGF2α formation. PGF2α measurement was done immediately at 4°C to limit further conversions. When needed, dilutions of the samples were done in 50 mM sodium phosphate buffer pH 7 and the standard curve was also done in the same buffer. The reaction plate was stored at -20°C and remaining manipulations were performed at 4°C to minimize enzymatic activity. For competitive inhibition studies, methylglyoxal was added to the reaction mixture at 0.1 or 10 mM for AKR1A1. Ponalrestat was used for AKR1B1 at 0.1 and 10 µM.

Specific Enzyme Immunoassay (ELISA) of PGF2α

PGF2α was assayed by enzyme immunoassays using acetylcholinesterase-linked PG tracer (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996) using the Cayman PGF2α EIA protocol. Sheep anti-$PGF_{2\alpha}$ serum (Bio-Quant, Ann Arbor, MI, USA) was used as the PGF2α antibody. The inter- and intra-assay coefficient of variations (n = 12) were 16 and 10% respectively. Briefly, 96-well plates were coated with rabbit anti-sheep IgG (Jackson ImmunoResearch Laboratories inc.) and 50µl of the

sample (or a dilution) was placed in each wells and then incubated with PGF2α acetylcholinesterase–linked tracer and sheep anti-PGF2α specific IgG. The plate was then incubated at 4°C overnight and then washed with 10 mM phosphate buffer (pH 7.4) prior to addition of Ellman's reagent (69 mM acetylthiocholine and 54 mM 5,5'-dithio-bis[nitrobenzoic acid] in 10 mM phosphate buffer (pH 7.4). Samples were incubated on a shaker in the dark for 3 hours and coloration read at 405 nm using a spectrophotometer (UVM 340) with the MicroWin 2000 software.

Statistical analysis

Data were analyzed using GraphPad Prism 5 program. One-way ANOVA with Bonferroni as post-hoc test with 95% confidence intervals was used for statistical significance. For graphics with less than 3 data sets, a t test was performed to ensure significance. Data are presented as the Mean ± SEM. Each experiment was repeated at least three times in triplicate or with at least three different tissues.

RESULTS

Identification of potential PGFS of the bovine and human endometrium

Among prostaglandins, PGF2α appears as the least studied member in spite of its demonstrated contribution to the reproductive and other body systems. Since all PGFS are AKRs of the 1C or 1B family, we have investigated whether other AKRs could exhibit PGFS activity. Our initial approach was to blast the bovine genome with AKR1B1 amino acid sequence using 60% similarity as the selection cut-off. Twenty different sequences (Supplementary table 1) were identified of which 5; AKR1B1, AKR1C1, AKR1C4, DD3 and AKR1A1 corresponded with AKR proteins. The other predicted sequences were either not expressed or were simply pseudo-genes. The documented PGFS AKR1B and C were present among the group but AKR1A1 came as a surprise. To complete our investigation, we have tested the corresponding human AKR1A1 and the established PGFS AKR1C3 and AKR1B1.

Expression and purification of his-tag AKR recombinant proteins.

Full length cDNA for the bovine AKR1A1 (bAKR1A1), AKR1B1 (bAKR1B1) , AKR1C1 (bAKR1C1), AKR1C4 (bAKR1C4) and DD3 (bDD3) were amplified using primers listed in supplementary table 2, sequenced and cloned in pET-16b to generate recombinant proteins in BL21

bacteria. Similarly, the full-length cDNAs coding for human AKRs 1A1 (hAKR1A1), 1B1 (hAKR1B1) and 1C3 (hAKR1C3) were produced and used to make recombinant proteins.

Equivalent amounts of purified bovine and human AKR recombinant proteins were loaded on SDS-PAGE 12% and stained with Coomassie Blue (Figure 1A). Purification of AKR1A1s, 1B1s and DD3 yielded clean preparations whereas bAKR1C1 and bAKR1C4 were slightly contaminated. This contamination may result from lower yield in recombinant protein content from these preparations. Since human and bovine AKR1A1 share 93% amino acid similarity, a commercial antibody raised against hAKR1A1 was confirmed to cross-react with the bovine protein by Western blot analysis (Figure 1B). A protein at the expected 36 kDa molecular weight was detected without cross-reaction with AKR1Bs or 1Cs. Polyclonal antibodies previously rose against the human (Bresson, Boucher-Kovalik et al. 2011; Bresson, Lacroix-Pepin et al. 2012) and the bovine (Madore, Harvey et al. 2003) AKR1B1 proteins were used respectively in figure 1C and 1D. In Figure 1C, the antibody raised against hAKR1B1 gives a strong signal for both human and bovine AKR1B1, a moderate signal for bAKR1C1 and bDD3, a weak signal for bAKR1C4 and nothing for bAKR1A1, hAKR1A1 and hAKR1C3. In figure 1D, the antibody raised against bAKR1B1 detected clearly both bovine and human AKR1B1 but exhibited major cross-reaction with bAKR1C1 and bDD3, and minor cross-reaction with hAKR1C3 and bAKR1C4. The hAKR1B1 appears slightly better than bAKR1B antibody but cross reaction with AKR1Cs and DD3 preclude from using them in preparations where these may be present.

Assessment of enzyme activity of purified recombinant AKR proteins

The AKR activity of purified recombinant human (Figure 2A and B) and bovine (Figure 2C and D) AKR1A1, 1B1, 1C1, 1C3, 1C4 and DD3 was assessed by continuous monitoring of NADPH degradation over a period up to 90 min in presence of wide spectrum AKR substrates methylglyoxal (1 mM, A and C) and 9,10-phenanthrenequinone (50 µM, B and D) (O'Connor, Ireland et al. 1999).

For methylglyoxal, AKR1A1 exhibited the highest NADPH utilization with 50% degradation in less than 5 min in bovine and human followed by both AKR1B1s which used respectively 20% (bovine) and 40-50% (human) NADPH in 12 min (Figure 2A-B). Bovine AKR1Cs and DD3 showed very slight degradation of NADPH but the human AKR1C3 showed 20% degradation.

For 9,10-phenanthrenequinone, hAKR1B1 and bDD3 exhibited the highest NADPH degradation rate at 40% in 12 min closely followed by AKR1A1 35-40% in both species and bAKR1B1 and hAKR1C3 (10%) (Figure 2D). Bovine AKR1C1 and AKR1C4 did not show visible degradation of NADPH within 12 min. The respective specific activity for each protein was calculated in the linear portion of NADPH conversion (30 min for bAKR1C1 and bAKR1C4) and is shown in table 1. Values are in the same range for both substrates as those published for the human by O'Connor (O'Connor, Ireland et al. 1999). The lower specific activity of AKR1Cs was expected from the same study. With methylglyoxal we measured a specific activity of 3.9 nmol/min/mg for bovine AKR1C1 compared to 16 nmol/min/mg for the human AKR1C1 in O'Connor study (O'Connor, Ireland et al. 1999). This is much lower than the 3332 nmol/min/mg or 4273 nmol/min/mg activities found for human AKR1A1 respectively in our study and that of O'Connor. Because methylglyoxal and 9,10-phenanthrenequinone might not be appropriate substrates for bAKR1C1 and bAKR1C4, which have never been characterized before, the same experiments were repeated with progesterone, testosterone, prostaglandin D2 and 3-pyrimidinecarboxaldehyde with comparable lack of activity. This raised concerns about the activity of these recombinant enzymes and they were not evaluated further.

Assessment of In vitro PGF2α biosynthesis

The ability of recombinant AKRs to synthesize PGF2α was tested with an in vitro cell free system where the PGH_2 substrate was generated in situ by addition of increasing concentrations of AA (10 to 100 µM) in presence of exogenous PGH synthase -1 (COX-1) or -2 (COX-2). This system was validated using hAKR1B1 the most potent PGFS known to date. The activity was expressed as PGF2α released (ng/ml) using our validated Elisa. First, AA in absence of COX did not influence measured levels of PGF2α, thus confirming the absence of cross reactivity with Elisa. Figure 3 illustrates the effect of variations in: the AA substrate (Figure 3A), hAKR1B1 (Figure 3B) or COX-1 (Figure 3C) where we observe increased PGF2α production with increasing concentrations. Inhibition of COX was used to confirm dependency on in situ PGH2 produced through COX activity. The COX-1 selective inhibitor SC-560 was more efficient to block PGF2α formation from COX-1 than from COX-2 (Figure 3D) in agreement with its reported IC_{50} of 9 nM for COX-1 and 6.3 µM for COX-2 (Smith, Zhang et al. 1998).

The present assay was developed because PGH2 is highly unstable with a reported half-life as short as 90-190 seconds and spontaneously transforms into PGE2 (44%), PGD2 (15%) and PGF2α (1,6%) for a ratio of 3 : 1 : 0.1 respectively (Yu, Xiao et al. 2011).

PGFS activity of recombinant bovine and human endometrial AKRs

The PGFS activity of 3 bovine and 3 human AKRs (Figure 4) was evaluated using conditions presented in figure 3. In the presence of COX-1 and 100 µM arachidonic acid (Figure 4A), human and bovine AKR1A1 and AKR1B1 released high levels of PGF2α whereas AKR1C3 and DD3 did not exhibit significant activity under conditions tested. In the presence of COX-2, similar results were obtained but AKR1A1 appeared to be more active than AKR1B1 (Figure 4B).

Competitive inhibition of AKR1A1 and AKR1B1 enzymes

PGF2α release by hAKR1A1 and hAKR1B1 was measured in presence of three competitive inhibitors. Methylglyoxal; (Figure 5A and D) shown in figure 2 to be a potent substrate for AKR1A1 (figure 2), ponalrestat; a potent inhibitor of the PGFS activity of AKR1B1 (Sochor, Kunjara et al. 1988; Bresson, Lacroix-Pepin et al. 2012) (Figure 5B and E), and glucose; a physiologically relevant substrate of AKR1B1 in the polyol pathway (Figure 5C and F). Methylglyoxal induced a dose dependent inhibition of the PGF2α synthase activity of AKR1A1 but not of AKR1B1, by contrast, ponalrestat and glucose inhibited significantly the PGFS activity of AKR1B1 but not that of AKR1A1. The present results illustrate that AKR1A1 and AKR1B1 have similar capacity to generate PGF2α from PGH2 but contrasting sensitivity to competitive substrates or pharmacological inhibitors.

Quantification of human and bovine AKR1A1 in the endometrium

Bovine AKR1A1 was evaluated by Western analysis and detected at a high level in adrenals and liver but not in the lungs. The protein level was higher in the liver (Figure 6A). The presence AKR1A1 was detectable in the bovine (Figure 6B) and the human (Figure 6C) endometrium.

DISCUSSION

Among prostaglandins, PGF2α appears as the least studied member. In the reproductive system however, endometrial PGF2α regulates recognition of pregnancy or the return to a new estrous cycle, initiation of parturition and menstruation. The bovine endometrium appears as a

particularly suitable model to identify mechanisms underlying PGF2α biosynthesis. Before our identification of AKR1B5, now bovine AKR1B1, all PGFS described were AKRs of the 1C family. These AKRs also homed several members of hydroxysteroid dehydrogenases. AKRs are recognized as moonlighting enzymes able to partner with several types of substrates. By contrast to the human AKR1B1, the bovine isoform does not exhibit a strong response to inducing factors. Rather, endometrial bAKR1B1 is constitutively expressed at a moderate level and increased PGF2α production results from induced COX-2 expression. In addition, inhibition of AKR1B1 never completely blocked PGF2α production in bovine endometrial cells. Our previous investigation of PGFs activity in the bovine endometrium revealed that established PGFs of the AKR1C family were not expressed or active in the bovine endometrium (Madore, Harvey et al. 2003). Therefore, we have investigated the expression, in the bovine endometrium, of additional AKR members with the hope to identify potential new PGFSs.

Our initial approach was to blast the bovine genome with AKR1B1 amino acid sequence using 60% similarity as the selection cut-off. Twenty different sequences (supplementary table 1) were identified of which 5; AKR1B1, AKR1C1, AKR1C4, DD3 (not corresponding to liver PGFS II), and AKR1A1 correctly matched with AKR proteins. The other predicted sequences were either not expressed or were simply pseudo-genes. With no surprise, AKR1B and C were present among the group but AKR1A1 came as a surprise. To complete our investigation, we have tested the corresponding human AKR1A1 together with the established PGFS AKR1C3 and AKR1B1 using the same recombinant protein approach.

The PGFS activity of the new bovine AKRs was evaluated using a novel in vitro assay in which the common precursor PGH2 was generated in situ, using recombinant COX-1 or -2 proteins in presence of AA. This assay was developed because PGH2 is expensive and is highly unstable, making it a difficult substrate to study PG biosynthesis. In addition, the preservation solvents are incompatible with assay conditions thus necessitating several extraction-dilution cycles reducing the yield in intact PGH2. PGH2 will spontaneously transform if not converted rapidly into any of native prostaglandins. Some authors describe PGH2 half-life as short as 90-190 seconds (Maclouf, Kindahl et al. 1980; Folco and Murphy 2006). Other studies using LC-MS/MS describes a half-life of less than 5 min in Tris-HCL pH 8 at 37°C. The spontaneous degradation products in absence of enzyme are PGE2 (44%), PGD2 (15%) and PGF2α (1,6%) for a ratio of 3 : 1 : 0.1 respectively and 2 other

products that are not PGI2, 15-keto PGF2α , 8-iso PGE2, 13,14-dihydro-15-keto PGE2, TBX_2 or 6-keto PGF2α by comparison with LC-MS/MS standards (Yu, Xiao et al. 2011).

We have used a combination of recombinant COX enzymes and AA to generate in situ the PGH2 necessary for PG production. The PGFS activity of the different human and bovine recombinant AKRs was determined as their ability to convert the endogenous PGH2 into PGF2α using a specific ELISA. Previous studies focused on other terminal synthases such as PGD2 (PGD synthase), PGE2 (PGE2 synthase), PGI2 (prostacyclin synthase) and TXA2 (thromboxane synthase) and their association with COX-1 and COX-2 (Smith, Urade et al. 2011). The production of PGF2α from exogenous PGH2 in vitro was used to show the PGFS activity of AKR1Bs originating from bovine (Madore, Harvey et al. 2003), mouse (Kabututu, Manin et al. 2009) and human (Kabututu, Manin et al. 2009; Bresson, Boucher-Kovalik et al. 2011; Nagata, Kusakari et al. 2011). In the present study, we show that human and bovine AKR1A1 can generate PGF2α as efficiently as AKR1B1 and much more than the established human PGFS AKR1C3. The present study confirms the high PGFS activity of AKR1B1 reported before for both species (Madore, Harvey et al. 2003; Kabututu, Manin et al. 2009; Bresson, Boucher-Kovalik et al. 2011; Nagata, Kusakari et al. 2011; Watanabe 2011).

The primary limitation with the present method is that K_m and V_{max} cannot be directly estimated because PGH2 concentration varies with COX specific activity itself variable with time. Nevertheless, we demonstrate in figure 3 that variation in any of the four components of the reaction yields a dose dependent increase in PGF2α levels thus confirming in situ generation of PGH2 and the ability of the system to identify PGFS activity. In fact, the same system can be adapted to characterize any terminal synthase pathway just by providing the appropriate recombinant enzyme followed by measurement of the corresponding PG.

Importantly, increasing the concentration of AA in absence of COX did not influence measured levels of PGF2α, thus confirming the absence of cross reactivity with the ELISA. Next, we confirmed that the production of PGF2α was proportional to the amount of AKR protein under investigation and found a clear increase with increasing AKR1B1 concentrations (Figure 3B). Inhibition of COX was then used to confirm that PGF2α release was dependent on in situ PGH2 produced through COX activity. The COX-1 selective inhibitor SC-560 was more efficient to block

PGF2α formation from COX-1 than from COX-2 (Figure 3D) in agreement with its reported IC_{50} of 9 nM for COX-1 and 6.3 µM for COX-2 (Smith, Zhang et al. 1998).

We then proceeded with competitive inhibition of AKR1A1 and AKR1B1. Methylglyoxal was chosen for AKR1A1 following demonstration in figures 2 of its high specific activity against this substrate. The selective inhibitor ponalrestat and the metabolic competitor D-glucose were chosen for AKR1B1 (Sochor, Kunjara et al. 1988; Bresson, Lacroix-Pepin et al. 2012). Methylglyoxal inhibited selectively AKR1A1 whereas ponalrestat and D-glucose competed effectively with AKR1B1.

The presence of AKR1A1 was assessed in bovine tissues and endometrial samples. The liver was the tissue that showed the highest levels (Figure 6A). The endometrium is an important site of PGF2α production, thus the levels of AKR1A1 were compared between bovine and human tissues.

From AKR1B1 crystal structure, essential amino acids for PGFS activity are Asp-43, Lys-77 and His110, the latter being the catalytic residue for PGFS activity (Nagata, Kusakari et al. 2011). Other residues stabilizing PGH2 through hydrophobic and hydrophilic interactions are Trp-20, Val-47, Tyr-48 Trp-79, Trp-111, Phe-122, Pro-218, Trp-219 and Leu-300. Human and bovine AKR1B1 share 84% amino acids identity and it goes up to 94% when considering positive substitution. The 12 amino acids involved in the ligation between NADPH and PGH2 are all conserved between the two species. This appears to explain their comparable PGFS activity and sensitivity to AKR inhibitors. By contrast, the human AKR1B1 and AKR1C3 are only 48% identical with only 6 of the 12 amino acids necessary for NADPH and PGH2 ligation conserved. For AKR1C3, PGH_2 9,11-endoperoxide reduction does not involve specific residues and PGH_2 would enter the catalytic site close to NADPH and receive the *pro-4R* hydrogen directly (Flanagan, Yosaatmadja et al. 2012). This might explain why AKR1C3 is a weak PGFS in terms of activity when compared to AKR1B1 (Figure 4).

The human and bovine AKR1A1 share 93% amino acids identity and it goes up to 98% with positive substitution. Comparison of sequences between AKR1A1 and AKR1B1 results in only 47-50% amino acids identity and up to 68-70% of positive when aligned for either human or bovine. Out of the 12 amino acids involved in the ligation between NADPH and PGH2 8 are conserved and one is similar (Val-47 to Ile-48) for bovine while 2 are similar for human (Val-47 to Ile-48 and Leu-300 to

Met-302). The difference observed between AKR1B1 and AKR1A1 is comparable to that found for AKR1C3. Yet, the PGFS activity of AKR1A1 is comparable to that of AKR1B1 (Figure 4).

The human AKR1B1 is probably the most studied AKR, mainly for its implication in driving glucose conversion in the polyol pathway and its association with diabetes complications (Chen and Zhang 2012). The role of AKR1B1 as the most potent PGFS identified to date was only recently highlighted (Kabututu, Manin et al. 2009; Bresson, Boucher-Kovalik et al. 2011; Nagata, Kusakari et al. 2011; Bresson, Lacroix-Pepin et al. 2012) bringing a new angle to look at human pathologies where this enzymes is involved (Bresson, Lacroix-Pepin et al. 2012).

AKR1A1 was one of the first AKR to be identified and characterized (Mano, Suzuki et al. 1961). It is a cytosolic enzyme that is ubiquitously expressed. Brain, kidney, liver, intestines and thyroid are the tissues where this AKR is the most abundant (Barski, Gabbay et al. 1999; O'Connor, Ireland et al. 1999). It is regulated by hStaf/ZFN143, C/EBP and PPARγ (Chen and Zhang 2012). Like AKR1B1, AKR1A1 expression can be suppressed by the action of atorvastatin on ARE response element and the PPARγ pathway in HUVEC cells (Ruf, Quintes et al. 2009). To our knowledge AKR1A1 expression has never been investigated in the endometrium. In rodents, the primary function attributed to AKR1A1 is to reduce D-glucoronidate to L-glucoronidate in the ascorbic acid pathway. This pathway does not exist in human and the role played by AKR1A1 remains to be determined (Barski, Tipparaju et al. 2008). The current findings propose PGFS activity as one of its function.

In the present study, we show that human and bovine AKR1A1 can generate PGF2α as efficiently as AKR1B1 and much more than the established human PGFS AKR1C3. The present study confirms the high PGFS activity of AKR1B1 reported before for both species (Madore, Harvey et al. 2003; Kabututu, Manin et al. 2009; Bresson, Boucher-Kovalik et al. 2011; Nagata, Kusakari et al. 2011; Watanabe 2011) while the limited PGFS activity of AKR1C3 questions its relevance in vivo (Kabututu, Manin et al. 2009; Flanagan, Yosaatmadja et al. 2012). We propose that human and bovine AKR1A1 have the potential to be functional PGFS. This finding has now to be validated in living cells in order to establish physiological relevance. This relevance was demonstrated for AKR1B1 (Bresson, Boucher-Kovalik et al. 2011) whereas AKR1C3, the former human PGFS and the bovine DD3 are unlikely to play this role in vivo due to their poor activity toward PGH2.

REFERENCES

Arosh, J. A., J. Parent, et al. (2002). "Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle." Biol Reprod 67(1): 161-9.

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod 54(2): 371-9.

Barski, O. A., K. H. Gabbay, et al. (1999). "Characterization of the human aldehyde reductase gene and promoter." Genomics 60(2): 188-98.

Barski, O. A., S. M. Tipparaju, et al. (2008). "The aldo-keto reductase superfamily and its role in drug metabolism and detoxification." Drug Metab Rev 40(4): 553-624.

Bresson, E., S. Boucher-Kovalik, et al. (2011). "The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium." J Clin Endocrinol Metab 96(1): 210-9.

Bresson, E., N. Lacroix-Pepin, et al. (2012). "The Prostaglandin F Synthase Activity of the Human Aldose Reductase AKR1B1 Brings New Lenses to Look at Pathologic Conditions." Front Pharmacol 3: 98.

Chapdelaine, P., J. Kang, et al. (2006). "Decidualization and maintenance of a functional prostaglandin system in human endometrial cell lines following transformation with SV40 large T antigen." Mol Hum Reprod 12(5): 309-19.

Chen, W. D. and Y. Zhang (2012). "Regulation of aldo-keto reductases in human diseases." Front Pharmacol 3: 35.

Christ-Hazelhof, E. and D. H. Nugteren (1979). "Purification and characterisation of prostaglandin endoperoxide D-isomerase, a cytoplasmic, glutathione-requiring enzyme." Biochim Biophys Acta 572(1): 43-51.

Flanagan, J. U., Y. Yosaatmadja, et al. (2012). "Crystal structures of three classes of non-steroidal anti-inflammatory drugs in complex with aldo-keto reductase 1C3." PLoS One 7(8): e43965.

Folco, G. and R. C. Murphy (2006). "Eicosanoid transcellular biosynthesis: from cell-cell interactions to in vivo tissue responses." Pharmacol Rev 58(3): 375-88.

Jabbour, H. N. and K. J. Sales (2004). "Prostaglandin receptor signalling and function in human endometrial pathology." Trends Endocrinol Metab 15(8): 398-404.

Jakobsson, P. J., S. Thoren, et al. (1999). "Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target." Proc Natl Acad Sci U S A 96(13): 7220-5.

Kabututu, Z., M. Manin, et al. (2009). "Prostaglandin F2alpha synthase activities of aldo-keto reductase 1B1, 1B3 and 1B7." J Biochem 145(2): 161-8.

Krishnaswamy, N., P. Chapdelaine, et al. (2009). "Development and characterization of a simian virus 40 immortalized bovine endometrial stromal cell line." Endocrinology 150(1): 485-91.

Lim, H., B. C. Paria, et al. (1997). "Multiple female reproductive failures in cyclooxygenase 2-deficient mice." Cell 91(2): 197-208.

Maclouf, J., H. Kindahl, et al. (1980). "Interactions of prostaglandin H2 and thromboxane A2 with human serum albumin." Eur J Biochem 109(2): 561-6.

Madore, E., N. Harvey, et al. (2003). "An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium." J Biol Chem 278(13): 11205-12.

Mano, Y., K. Suzuki, et al. (1961). "Enzymic studies on TPN L-hexonate dehydrogenase from rat liver." J Biochem 49: 618-34.

Nagata, N., Y. Kusakari, et al. (2011). "Catalytic mechanism of the primary human prostaglandin F2alpha synthase, aldo-keto reductase 1B1--prostaglandin D2 synthase activity in the absence of NADP(H)." FEBS J 278(8): 1288-98.

O'Connor, T., L. S. Ireland, et al. (1999). "Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members." Biochem J 343 Pt 2: 487-504.

Poyser, N. L. (1995). "The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation." Prostaglandins Leukot Essent Fatty Acids 53(3): 147-95.

Ruf, T. F., S. Quintes, et al. (2009). "Atorvastatin reduces the expression of aldo-keto reductases in HUVEC and PTEC. A new approach to influence the polyol pathway." Clin Invest Med 32(3): E219-28.

Smith, C. J., Y. Zhang, et al. (1998). "Pharmacological analysis of cyclooxygenase-1 in inflammation." Proc Natl Acad Sci U S A 95(22): 13313-8.

Smith, W. L., Y. Urade, et al. (2011). "Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis." Chem Rev 111(10): 5821-65.

Sochor, M., S. Kunjara, et al. (1988). "The effect of aldose reductase inhibitor Statil (ICI 128436) on the glucose over-utilization in kidney of diabetic rats." Biochem Pharmacol 37(17): 3349-56.

Spencer, T. E., G. A. Johnson, et al. (2007). "Fetal-maternal interactions during the establishment of pregnancy in ruminants." Soc Reprod Fertil Suppl 64: 379-96.

Suzuki-Yamamoto, T., M. Nishizawa, et al. (1999). "cDNA cloning, expression and characterization of human prostaglandin F synthase." FEBS Lett 462(3): 335-40.

Tanabe, T. and V. Ullrich (1995). "Prostacyclin and thromboxane synthases." J Lipid Mediat Cell Signal 12(2-3): 243-55.

Tanikawa, N., Y. Ohmiya, et al. (2002). "Identification and characterization of a novel type of membrane-associated prostaglandin E synthase." Biochem Biophys Res Commun 291(4): 884-9.

Thatcher, W. W., A. Guzeloglu, et al. (2001). "Uterine-conceptus interactions and reproductive failure in cattle." Theriogenology 56(9): 1435-50.

Ullrich, V., L. Castle, et al. (1981). "Spectral evidence for the cytochrome P450 nature of prostacyclin synthetase." Biochem Pharmacol 30(14): 2033-6.

Watanabe, K. (2011). "Recent reports about enzymes related to the synthesis of prostaglandin (PG) F(2) (PGF(2alpha) and 9alpha, 11beta-PGF(2))." J Biochem 150(6): 593-6.

Watanabe, K., R. Yoshida, et al. (1985). "Enzymatic formation of prostaglandin F2 alpha from prostaglandin H2 and D2. Purification and properties of prostaglandin F synthetase from bovine lung." J Biol Chem 260(11): 7035-41.

Yu, R., L. Xiao, et al. (2011). "Competitive enzymatic interactions determine the relative amounts of prostaglandins E2 and D2." J Pharmacol Exp Ther 339(2): 716-25.

Figures:

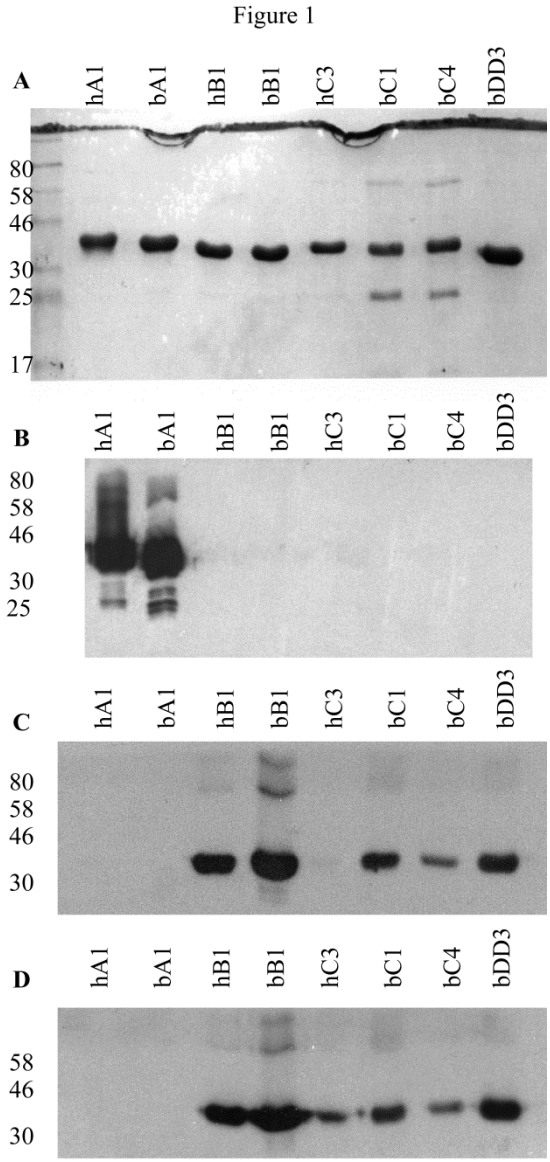


Figure 16 - Article 3: Figure 1 Isolation and purification of AKR recombinant proteins.

Recombinant proteins for human AKR1A1, 1B1 and 1C3 and bovine AKR1B1, 1C1, 1C4 and DD3 were produced and purified as described in materials and methods. Purified AKR proteins (5 µg) were loaded on 10 % SDS-PAGE and after electrophoresis, gels were stained with Coomassie Blue (A). Proteins were then transferred onto nitrocellulose membranes for Western analysis. AKR1A1 was probed with a monoclonal mouse anti-human AKR1A1 (B). In house human (C) and bovine (D) AKR1B1 polyclonal antibodies were used to probe human and bovine AKRs where we observe significant cross reactivity with AKR1Cs but not with AKR1A1.

Figure 2

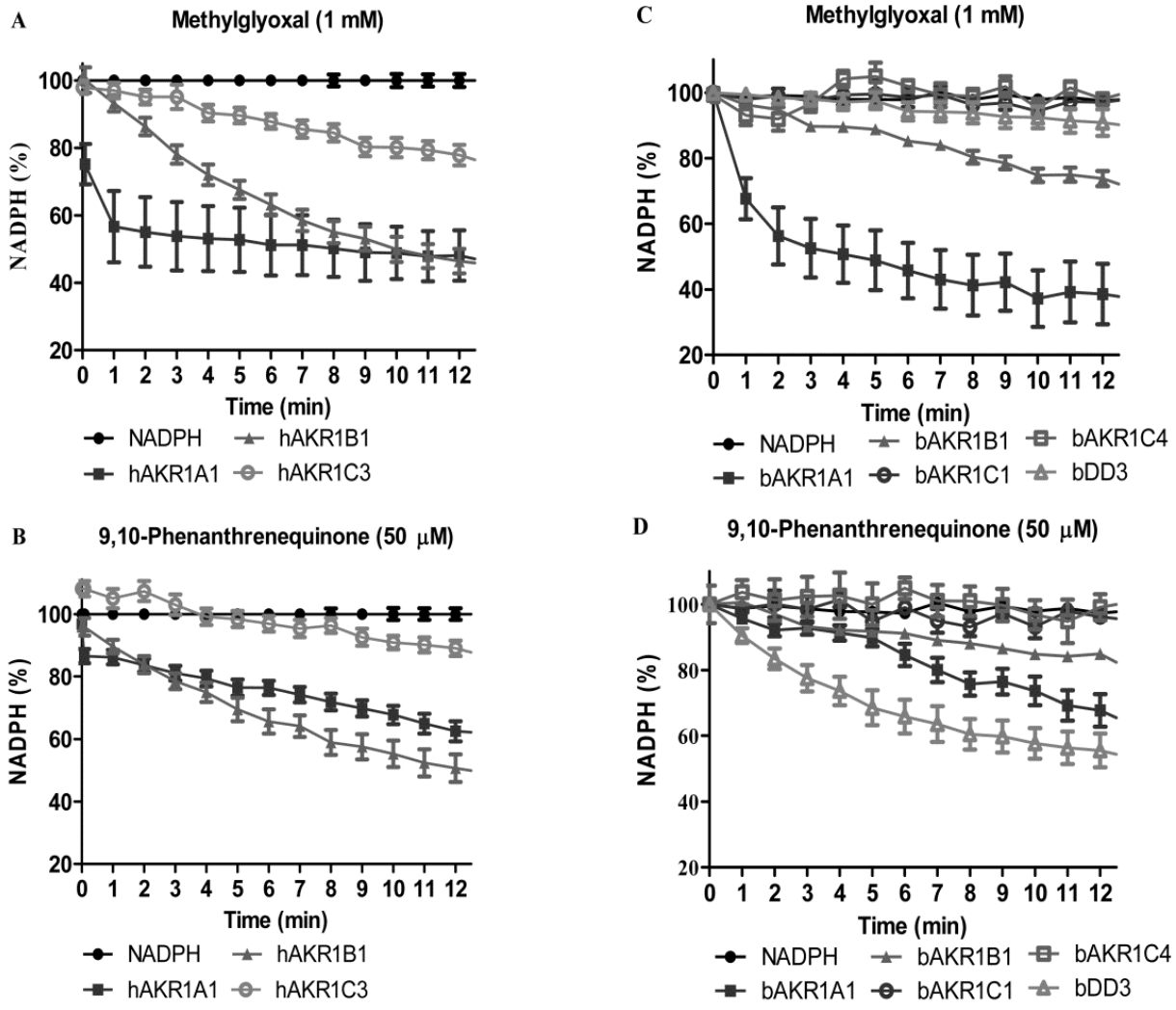


Figure 17 - Article 3: Figure 2 Assessment of activity of recombinant AKR enzymes.

The AKR activity of 5 µg of human AKR1A1, 1B1 and 1C3 (A and B) or bovine AKR1A1, 1B1, 1C1, and 1C4 as well as DD3 (C and D) was tested indirectly by measurement of NADPH catabolism. The first 12 min are shown for methylglyoxal (A, C) or 9,10-phenanthrenequinone (B, D) as substrates. Experiments were repeated three times in triplicate and the results are expressed as the mean ± SEM of the percentage of NADPH remaining in each well. Calculated specific activities are shown in table 1.

Figure 3

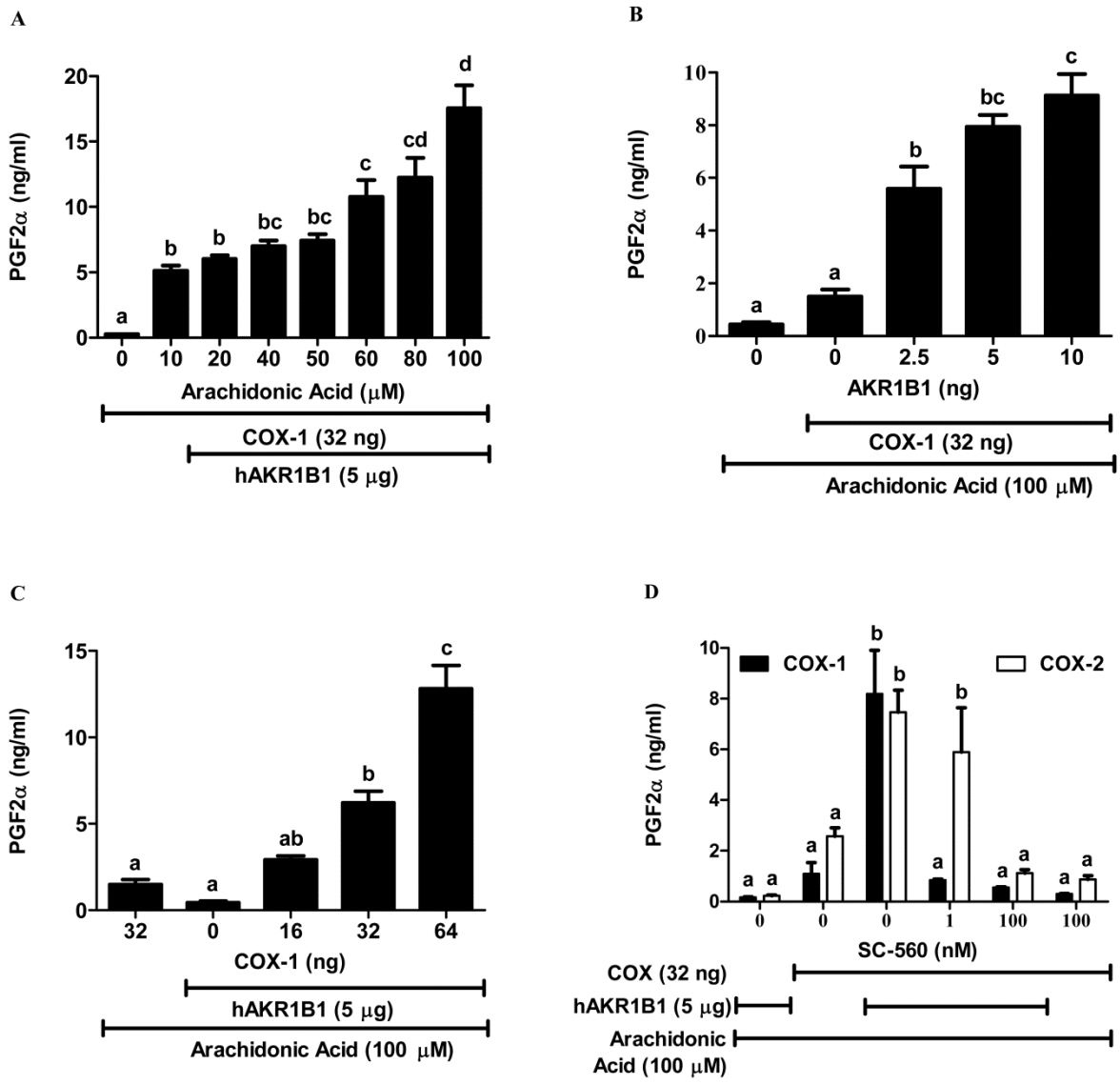


Figure 18- Article 3: Figure 3 Optimization of conditions for evaluation of PGFS activity.

The PGFS activity of hAKR1B1 was measured in vitro in a cell free system. PGH2 was generated in situ by a combination of arachidonic acid (AA) in presence of COX-1 or COX-2. The production of PGF2α was measured by ELISA. The effect of variation in the concentration of individual components of the reaction mixture in presence of fixed concentrations of other parameters is illustrated for: A) Arachidonic Acid, B) hAKR1B1 and C) recombinant ovine COX-1. In D, we illustrate the comparison of COX-1 and COX-2 in sustaining PGF2α production in presence and absence of the COX-1 selective inhibitor SC-560. Results represent the mean ± SEM of three independent experiments ran in triplicate. In D statistical analyses for COX-1 and COX-2 were done separately. Bars with different superscript are significantly different ($P<0.05$).

Figure 4

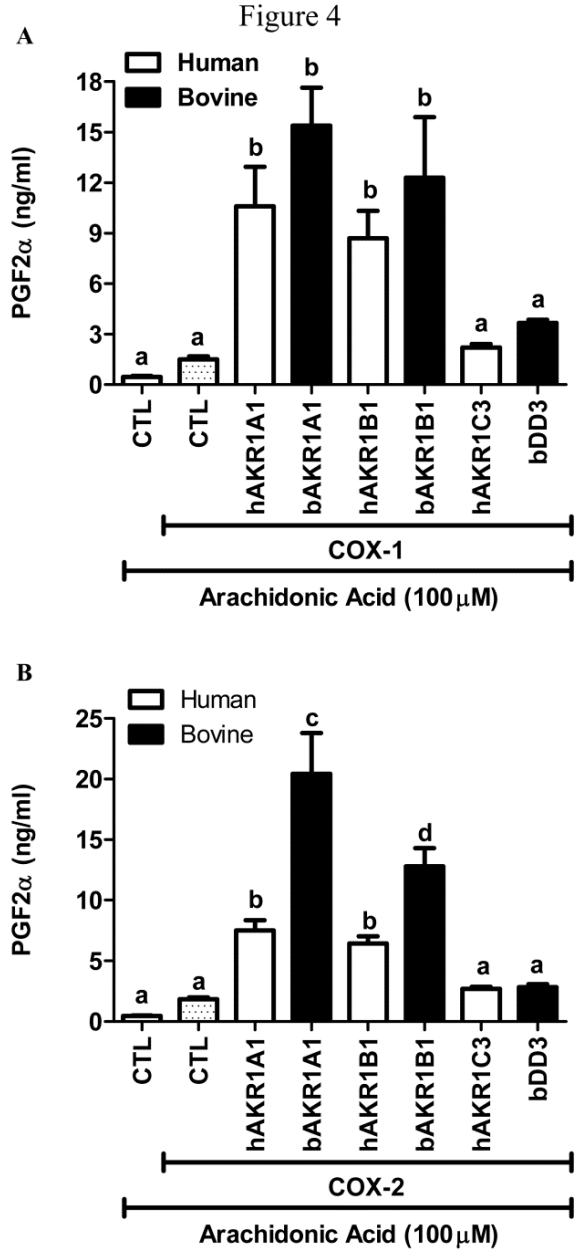


Figure 19 - Article 3: Figure 4 Evaluation of the PGFS activity of recombinant bovine AKRs.

5 µg of purified recombinant human AKR1A1, 1B1 and 1C3 and bovine AKR1A1, 1B1 and DD3 were tested using conditions determined in figure 3. The PGFS activity was determined in presence of 32 ng of COX-1 (A) or COX-2 (B) with 200µM NADPH. PGF2α release was measured by ELISA and represents the mean ± SEM of three independent experiments run in triplicate. Bars with different superscript are significantly different ($P<0.05$).

Figure 5

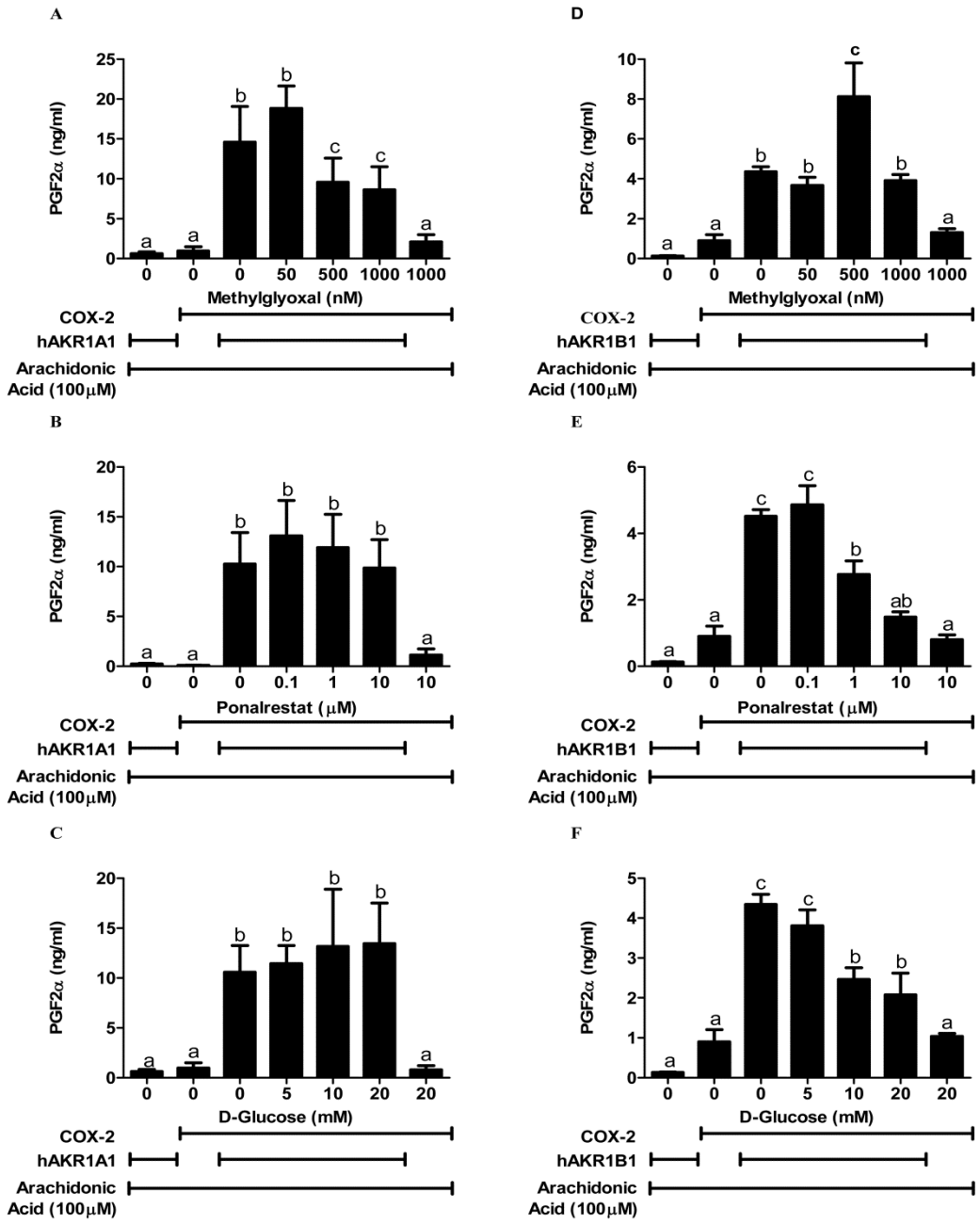


Figure 20 - Article 3: Figure 5 Competitive inhibition of the PGFS activity of AKR1A1 and AKR1B1.

The PGFS activity of 5 µg of purified recombinant human AKR1A1 (A, B and C) and AKR1B1 (D, E and F) was tested in the presence of increasing concentrations of the competitive inhibitor methylglyoxal, ARI inhibitor ponalrestat or D-glucose respectively using the same conditions as tested in figure 3. PGF2α was measured by ELISA and results represent the mean ± SEM of three independent experiments run in triplicate. Bars with different superscript are significantly different ($P<0.05$).

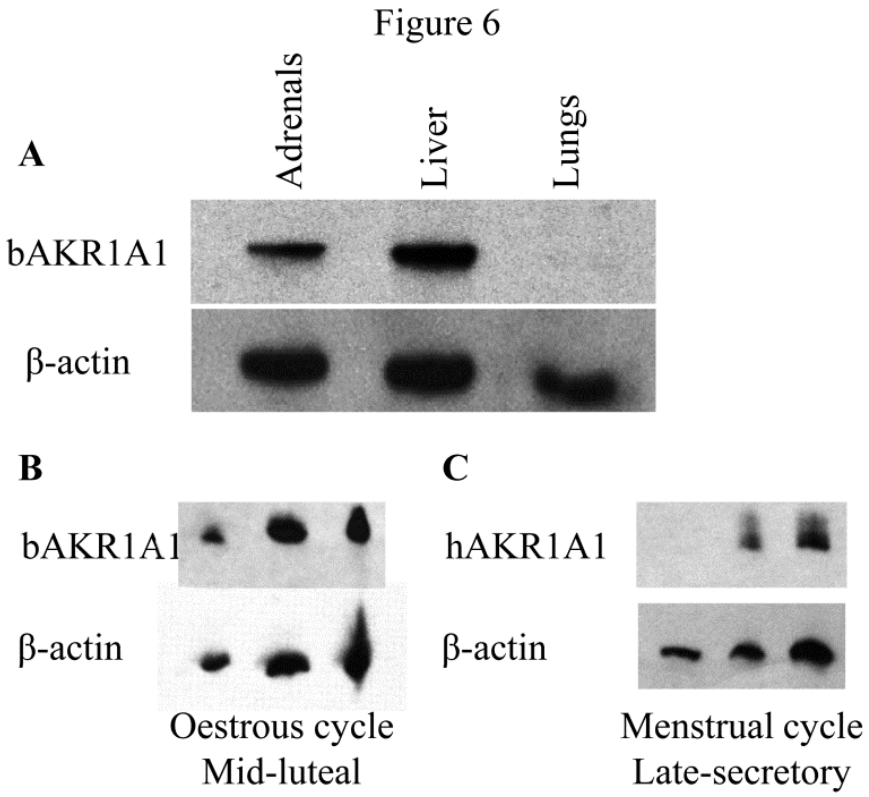


Figure 21 - Article 3: Figure 6 Analysis of AKR1A1 protein in the endometrium and other tissues.

The presence of AKR1A1 was analyzed by Western blots for bovine tissues and for bovine and human endometrial samples. 10µg of total protein extracted from the tissues were loaded on SDS-PAGE 10% and immunoblotting was done with specific antibodies against AKR1A1. Bovine adrenals, liver and lungs were tested for the presence of the 2 proteins (A). Bovine endometrial samples were taken on days 16-18 of estrous cycle (Mid-luteal phase, B) and endometrial biopsies were obtained on days 27-28 of the menstrual cycle (late secretory phase, C). Three tissues for each time period were analyzed.

Tables:

Enzyme	Specific activity (nmol/min/mg of protein)	
	Methylglyoxal (1 mM)	Phenantrenequinone (50 µM)
hAKR1A1	3332 ± 138.6	135.8 ± 5.028
bAKR1A1	1029 ± 193.3	114.3 ± 8.318
hAKR1B1	531.3 ± 49.26	332.9 ± 21.08
bAKR1B1	502.6 ± 215.8	78.13 ± 3.466
bAKR1C1	3.978 ± 1.963	26.16 ± 3.177
hAKR1C3	72.16 ± 9.783	41.35 ± 6.014
bAKR1C4	7.454 ± 3.483	6.052 ± 1.313
bDD3	81.56 ± 23.72	433.6 ± 32.83

Table 1 - Article 3: Table 1: Catalytic activities of recombinant AKRs toward dicarbonyls

Enzyme assays were performed at 25 °C in 100 mM sodium phosphate buffer, pH 7.0, using 0.2 mM NADPH and 5 µg of the protein of interest. Assays were carried out with methylglyoxal or 9,10-phenantrenequinone in triplicate and activity calculated forom the linear portion of the decay curves shown in figure 2 up to 30 min. The values shown represent means ± S.E.M. of three separate experiments.

Gene ID:	Primers
317748: AKR1B1	Foward 5'ATGGCCAACCACATCGTGCTCTAC 3' Reverse 5'AGGTATCCACGAAATCTTTCTCAC3'
616199 LOC616199: Aldose reductase-like	Foward 5'ATGGCCCTGAGTCCCAGCCCCGAG3' Reverse 5'GTACCAAGGATGAAGAGGAAGAGA3'
525193: AKR1B10	Foward 5'ATGGTCTCCTTTGTGGCGCTCAGT3' Reverse 5'TTGCTTTACTGATGATGGTATTGC3'
790476 LOC790476: Aldose reductase-like	Foward 5'ATGTCACACCCGAGGTCAGCATTG3' Reverse 5'GGACATATGGTTTGTGGTCAGAG3'
615959 AKR1E2	Foward 5'ATGGAGGCGATACCTCCTGTCCTG3' Reverse 5'TTTATTCAGAAGTCTCTCGAGCTG3'
782061 LOC782061: aldo-keto reductase family 1, member C1-like	Foward 5'ATGGATCCCAAAGGCCAGAAGGTG3' Reverse 5'GACAGAAATCCACTGAGTCAGCTA3'
782884: AKR1C3	Foward 5'ATGACAATGGATCCCAAATACCATA3' Reverse 5'GAGACAGAGATCCACTGAATCAAA3'
100139386: 20ALPHA-HSD	Foward 5'ATGGATCCCAAATACCAGAGGGTG3' Reverse 5'ATTTTTCCATTTTCATCTTTTGGA3'
617917: AKR1C3	Foward 5'GTAATGGATCCCAAAGGCCAGAGA3' Reverse 5'TCACCTATCAGTTTTCCATTTTCA3'
506594 LOC506594: prostaglandin F synthase 1-like	Foward 5'ATGTTGTGGCAGAAGAAGAAGAAA3' Reverse 5'TCAGAGGCACTGGAGTATGAATAA3'
100337056 LOC100337056: aldo-keto reductase family 1, member C3	Foward 5'ATGGATCCCAAAGGCCAGAGAGTTA3' Reverse 5'AGTTTTCCATTTTCATCTTTTGGA3'
532205 LOC532205: aldo-keto reductase family 1, member C3	Foward 5'ATGGATCCCAAAAGGCAAGTGAAG3' Reverse 5'TCAGGTATCAGTTTTCCATTTTCA3'
782922 LOC782922: prostaglandin F synthetase II-like	Foward 5'ATGGATCCCAAAAGTCAGAGG3' Reverse 5'ATCCACCGAGTCAAATATCAGTTTTC3'
100337056 LOC100337056: aldo-keto reductase family 1, member C3	Foward 5'AGAGAAGCTCTGGAAGTTACCAG3' Reverse 5'GGCTTGTTTAGGATCTTCTCCAG3'
527068 LOC527068: aldo-keto reductase family 1 member C3 -like	Foward 5'ATGGATCCCAAAAGTCAGAGA3' Reverse 5'ATTTTCATCTGTTGGGAAAAGTGT3'
538060 LOC538060: aldo-keto reductase family 1, member C1	Foward 5'ATGGATTCCAAAAGCCAGAAGGTG3' Reverse 5'ATCCACCGAGTCAAATATCAGTTTTC3'
282138: AKR1C4	Foward 5'ATGGATCCCAAAGGCCAGAGAGTG3' Reverse 5'ATTGGTTTTCCATTTTCATCTGTTG3'
100336811: LOC100336811 dihydrodiol dehydrogenase 3-like	Foward 5'CCAGGGGAGGAATTGCTTCCAAAA3' Reverse 5'GTATGTTCTCTTTGATCCGCTTCTTG3'
784927 LOC784927: prostaglandin F synthase 1-like	Foward 5'ATGGATCCCAAAAGCCAGAGAGTG3' Reverse 5'CAGTTTTCCATTTTCATCTTTGC3'
618607: AKR1A1	Foward 5'ATGGCGGCTTCCTGTATCCTCCTA3' Reverse 5'GGAGTCATAGCGTATAGTCCCATC3'

Table 2 - Article 3: Supplementary Table 1: Primers to detect presence of potential PGFS

List of used primers to detect potential PGFS in the bovine endometrium and gene identification number of the corresponding genes.

Name, NCBI Gene ID and EC number:	Primers
Human AKR1A1 NCBI-GI: 10327 EC: 1.1.1.2	pET-16b Forward 5'CCAGAATTCGTGATGGATCCACATATGGCGGCTTCCTGTGTTCTACTGCACAC 3' pET-16b Reverse 5'GTGCTGGATATCTGCAGGATCCTTAGTACGGGTCATTAAAGGGGTACAGAGG 3'
Bovine AKR1A1 NCBI-GI: 618607 EC: 1.1.1.2	pET-16b Forward 5' GCCATATGGCGGCTTCCTGTATCCTCCTAC 3' pET-16b Reverse 5' AGGATCCTCAATATGGGTCATTGAAGGGGTACAG 3'
Human AKR1B1 NCBI-GI: 231 EC: 1.1.1.21	pET-16b Forward 5'GTCCAGTGTGGTGGAATTCCACATATGGCAAGCCGTCTCCTGCTCAACAA 3' pET-16b Reverse 5'GCCACTGTGCTGGATATCTGCAGAGATCTTCAAAACTCTTCATGGAAGGGGTAATC 3'
Human AKR1C3 NCBI-GI: 8644 EC:1.1.1.213 / 1.3.1.20 / 1.1.1.188 1.1.1.63 / 1.1.1.64 / 1.1.1.112	pET-16b Forward 5'GATCCAGAATTCGTGATTTCCACATATGGATTCCAAACACCAGTGTGTAAAG 3' pET-16b Reverse 5'GTGCTGGATATCTGCAGGATCCTTAATATTCATCTGAATATGGATAATTAG 3'
Bovine AKR1C1-like NCBI-GI: 261245051 No EC number available	pET-16b Forward 5' GCCATATGGATCCCAAAGGCCAGAAAGTGA 3' pET-16b Reverse 5' GAGATCTTCAATATTCTTCAGAGAATGGAAACTCAGG 3'
Bovine AKR1C4 NCBI-GI: 282138 EC:1.1.1.188	pET-16b Forward 5' GCCATATGGATCCCAAAGGCCAGAGAGTGAAGCTTAATG 3' pET-16b Reverse 5' GAGATCTTCAATATTCTTCAACAAATGGATACTCAG 3'
Bovine Dihydrodiol dehydrogenase 3 (DD3) NCBI-GI: 261245055 EC:1.1.1.188	pET-16b Forward 5' GCCATATGGATCCCAAAGGCCAGAGAGTGAAACTTAATGAC 3' pET-16b Reverse 5'GAGATCTCTCGAGTCAATATTCTTCAGAGAATGGAAACTCAGGATGACTAACAGCAAGGTGAAGT TCGTAATATC 3'

Table 3 - Article 3: Supplementary Table 2: Cloning primers for insertion in pET-16b

List of primers used for cloning potential PGFS in the bovine endometrium and gene identification number of the corresponding genes.

Protein name	Bovine AKR1A1
NCBI current and previous names	>alcohol dehydrogenase [NADP(+)] [Bos taurus] Sequence ID: ref|NP_001069981.1| >RecName: Full=Alcohol dehydrogenase [NADP(+)]; AltName: Full=Aldehyde reductase; AltName: Full=Aldo-keto reductase family 1 member A1 Sequence ID: sp|Q3ZCJ2.1| >AK1A1_BOVIN Aldo-keto reductase family 1, member A1 (aldehyde reductase) [Bos taurus] Sequence ID: gb|AAI02167.1| >TPA: alcohol dehydrogenase [Bos taurus] Sequence ID: tpg|DAA31054.1|
cDNA sequence	ATGGCGGCTTCCTGTATCCTCCTACACACTGGGCAGAAGATGCCCCTGATTGGACTGGGCACCTG GAAGAGCGACCCTGGCCAGGTTAAAGCAGCTATTAAGTATGCCCTGAGTGTAGGCTACCGTCACA TTGACTGTGCTGCTATCTATGGCAATGAGACTGAAATCGGGGAAGCCCTGAAGGAGAATGTGGGA CCTGGCAAGTTGGTGCCTCGGGAGGAGCTCTTTGTGACTTCCAAGCTGTGGAACACAAAGCACCA CCCTGAGGATGTGGAGCCTGCCCTCCGGAAGACACTTGCTGACCTGCAGCTGGAGTATCTGGAC CTGTACCTGATGCACTGGCCTTACGCCTTTGAGCGGGGAGACAGCCCCTTCCCTAAGAATGCTGA TGGGACTATACGCTATGACTCCACCCACTACAAGGAGACCTGGAGGGCTCTGGAGGCACTCGTG GCTAAGGGGCTGGTGCGGGCTCTGGGCCTGTCCAACTTCAACAGTCGGCAGATTGACGATGTGC TCAGCGTGGCCTCTGTACGCCCAGCTGTCCTGCAGGTGGAATGCCACCCATACCTGGCTCAGAAT GAGCTGATTGCCCACTGCCAAGCACGAAACCTGGAGGTGACTGCTTATAGCCCTCTGGGCTCCTC TGATCGTGCGTGGCGTGATCCTGAGGAGCCTGTCCTGCTTAAGGAGCCAGTGGTCCTGGCACTG GCAGAAAAGCATGGTCGATCTCCAGCTCAGATCTTGCTCAGGTGGCAGGTCCAGCGGAAAGTGA GCTGCATCCCCAAGAGTGTCACGCCTTCCCGTATCCTTGAGAACATCCAGGTGTTTGACTTCACCT TTAGCCCAGAGGAGATGAAGCAGCTGGATGCCCTGAATAAAAATTTGCGATTCATTGTGCCCATGC TTACGGTGGATGGGAAGAGGGTCCCAAGAGATGCAGGGCACCCTCTGTACCCCTTCAATGACCCA TATTGACTCGAGTCTAGAGGCCANT
Protein sequence	MAASCILLHTGQKMPLIGLGTWKSDPGQVKAAIKYALSVGYRHIDCAAIYGNETEIGEALKENVGPGKLV PREELFVTSKLWNTKHHPEDVEPALRKTLADLQLEYLDLYLMHWPYAFERGDSPFPKNADGTIRYDST HYKETWRALEALVAKGLVRALGLSNFNSRQIDDVLSVASVRPAVLQVECHPYLAQNELIAHCQARNLE VTAYSPLGSSDRAWRDPEEPVLLKEPVVLALAEKHGRSPAQILLRWQVQRKVSCIPKSVTPSRILENIQ VFDFTFSPEEMKQLDALNKNLRFIVPMLTVDGKRVPRDAGHPLYPFNDPY-
M.W.	36.62 KDa

Table 4 - Article 3: Supplementary figure 3: Cloned bovine AKR1A1 protein

List of bovine cDNA and protein sequences as well as NCBI current and previous names of AKR1A1 used in this study.

Protein name	Bovine AKR1B1
NCBI current and previous names	>aldose reductase [Bos taurus] Sequence ID: ref|NP_001012537.1| >aldo-keto reductase family 1, member B1 [Bos taurus] Sequence ID: gb|AAX09075.1| >Aldo-keto reductase family 1, member B1 (aldose reductase) [Bos taurus] Sequence ID: gb|AAI10179.1| >TPA: aldose reductase [Bos taurus] Sequence ID: tpg|DAA30351.1|
cDNA sequence	ATGGCCAACCACATCGTGCTCTACACTGGCGCCAAGATGCCCATCTTGGGGCTGGGCACCTGGA AGTCCCCTCCAGGCAAAGTGACAGAGGCTGTGAAGGTGGCAATCGACCTTGGGTACCGTCACATT GACTGTGCCCACGTGTACCAGAATGAGAACGAGGTGGGTTTGGCCCTCCAGGCAAAGCTGCAGG AGAAAGTGGTGAAGCGTGAGGACCTCTTCATCGTCAGCAAGCTGTGGTGCACGTATCACGACAAG GACCTGGTGAAAGGTGCCTGCCAGAAGACGCTCAGCGACCTGAAGCTGGACTACCTGGACCTCT ACCTCATCCACTGGCCCACAGGCTTCAAGCCTGGGAAGGACTTCTTCCCATTGGATGAGGACGGC AACGTGATTCCCAGTGAGAAAGATTTCGTGGATACCTGGACGGCCATGGAAGAGCTGGTGGACGA AGGGCTGGTGAAAGCTATTGGAGTCTCCAACTTCAACCATCTCCAAGTGGAGAAGATCTTAAACAA ACCTGGCTTAAAATACAAGCCGGCGGTTAACCAGATCGAGTGCCACCCATACCTCACTCAGGAGA AGTTAATCCAGTACTGCAACTCCAAAGGCATTGTGGTGACTGCCTATAGTCCCCTCGGCTCTCCTG ACAGGCCCTGGGCCAAGCCGGAGGACCCTTCCATACTGGAGGACCCCAGGATCAAAGCGATTGC AGACAAGTACAATAAAACCACAGCCCAGGTGCTGATCCGATTCCCCATACAGAGGAACCTGATCG TGATCCCCAAGTCAGTGACACCTGAACGCATTGCTGAGAACTTCCAGGTCTTTGACTTTGAACTGG ACAAGGAGGATATGAACACCTTGCTGAGCTACAACAGGGACTGGAGGGCCTGTGCCTTGGTGAG CTGTGCCTCCCACAGGGATTACCCCTTCCACGAGGAGTTCTGACTATGTTTAAGGGCTCGAGTCT AGAGTCCCCNNNNNCNNG
Protein sequence	MANHIVLYTGAKMPILGLGTWKSPPGKVTEAVKVAIDLGYRHIDCAHVYQNENEVGLALQAKLQEKVVK REDLFIVSKLWCTYHDKDLVKGACQKTLSDLKLDYLDLYLIHWPTGFKPGKDFFPLDEDGNVIPSEKDF VDTWTAMEELVDEGLVKAIGVSNFNHLQVEKILNKPGLKYKPAVNQIECHPYLTQEKLIQYCNSKGIVVT AYSPLGSPDRPWAKPEDPSILEDPRIKAIADKYNKTTAQVLIRFPIQRNLIVIPKSVTPERIAENFQVFDFE LDKEDMNTLLSYNRDWRACALVSCASHRDYPFHEEF-
M.W.	36.06 KDa

Table 5 - Article 3: Supplementary figure 4: Cloned bovine AKR1B1 protein

List of bovine cDNA and protein sequences as well as NCBI current and previous names of AKR1B1 used in this study.

Protein name	Bovine AKR1C1
NCBI current and previous names	>aldo-keto reductase family 1, member C1-like [Bos taurus] Sequence ID: ref|NP_001159695.1| >AKR1C4 protein [Bos taurus] Sequence ID: gb|AAI03312.1| >TPA: aldo-keto reductase family 1, member C1-like [Bos taurus] Sequence ID: tpg|DAA23461.1|
cDNA sequence	ATGGATCCCAAAGGCCAGAAAGTGAAGCTTAATGATGGCCACTTCATTCCTGTCCTGGGATTTG GAACCTATGCACCTCAAGAGGTTGCTAAGAGGGATGCTCTGGAATTCACCCCATTTGCTATAGA GGTTGGGTTCCGCCATATTGACTGTGCTCATGCTTACCAAAATGAAGAGCAGATAGGTCAGGCC ATTCGAAGCAAGATGGCAGATGGCACTGTGAAGAGAGAAGACATATTCTGCACTTCAAAGCTTT GGTGCACATCCTTTCGACCAGAGTTGGTCCGACCAGCCTTGGAAAAGTCACTGAAAAGTCTTCA ACTGGACTATGTCGATCTCTATATTATGCATTACCCGCTGGCTCTGAAGCCAGGGGAGGAATTAT ATCCAAAAGATGAAAATGGAAAACTGATAGCTGACTCAGTGGATTTCTGTCTCACATGGGAGGCC CTGGAGAAGTGCAAGGACGCAGGGCTGGCCAAATCCATCGGGGTGTCCAACTTCAACCACAAG CAGCTGGAGAAGATCCTAAACAAGCCGGGGCTCAAGTACAAGCCTGTCTGCAACCAGGTGGAA TGTCACCCTTATCTCAACCAGAGGAAACTGTTGGATTTCTGCAAGTCACATGATATTGTTCTTGTT GCCTATAGTGCTCTGGGATCCCAAAGAGTAAAAGGATGGGTGAACCCAAACCACCCCGTTCTCT TGGAGGACCCAGTTCTCTCTGCCATTGCCCAGAAGCACAAGAAAACGGCAGCTCTGGTTGCCCT TCGCTACCAGATACAACGTGGGGTTGTGGTTCTGGCCAAGGGTAACAACAAGGAGTGGATCAAA GAGAACATGCAGGTGTTTGACTTTGAACTGACTCCAGAAGACATGAAAGCAATCGATGGCCTCA ACAGAAATATAAGATATTGTGATTTTCACCCGGGTGTTGGTCACCCTGAGTTTCCATTCTCTGAA GAATATTGACTCGAGTCTAGAGGCCCGTTAATCNNC
Protein sequence	MDPKGQKVKLNDGHFIPVLGFGTYAPQEVAKRDALEFTPFAIEVGFRHIDCAHAYQNEEQIGQAIRSK MADGTVKREDIFCTSKLWCTSFRPELVRPALEKSLKSLQLDYVDLYIMHYPLALKPGEELYPKDENGK LIADSVDFCLTWEALEKCKDAGLAKSIGVSNFNHKQLEKILNKPGLKYKPVCNQVECHPYLNQRKLLD FCKSHDIVLVAYSALGSQRVKGWVNPNHPVLLEDPVLSAIAQKHKKTAALVALRYQIQRGVVVLAKG NNKEWIKENMQVFDFELTPEDMKAIDGLNRNIRYCDFHPGVGHPEFPFSEEY-
M.W.	36.74 KDa

Table 6 - Article 3: Supplementary figure 5: Cloned bovine AKR1C1 protein

List of bovine cDNA and protein sequences as well as NCBI current and previous names of AKR1C1 used in this study.

Protein name	Bovine AKR1C4
NCBI current and previous names	>dihydrodiol dehydrogenase 3 [Bos taurus] Sequence ID: ref|NP_851370.1| >RecName: Full=Dihydrodiol dehydrogenase 3; AltName: Full=Prostaglandin F synthase Sequence ID: sp|P52898.1| >DDBX_BOVIN cytosolic dihydrodiol dehydrogenase 3 [Bos taurus] Sequence ID: dbj|BAA08493.1| >prostaglandin F synthase [Bos taurus] Sequence ID: dbj|BAA13690.1| >Aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4) [Bos taurus] Sequence ID: gb|AAI12520.1| >TPA: dihydrodiol dehydrogenase 3 [Bos taurus] Sequence ID: tpg|DAA23427.1|
cDNA sequence	ATGGATCCCAAAGGCCAGAGAGTGAAGCTTAATGATGGCCACTTCATTCCTGTCCTGGGATTTGG AACCTTTGCACCTCGAGAGGTTCCTAAGAGTGAAGCTCTGGAAGTCACCAAATTTGCTATAGAGGC TGGGTTCCGCCATATTGACAGTGCTCATTTGTACCAAAATGAAGAGCAGGTTGGCCAGGCCATTC GAAGCAAGATTGCCGATGGCACTGTAAAGAGAGAAGACATTTTCTACACTTCAAAGCTTTGGTCCA CTTCCCTTCGTCCAGAATTGGTCCGACCAGCCTTGGAAAAGTCACTGAATAATCTTCAACTGGACT ATGTCGATCTTTATATTATTCATTTTCCAGTGGCTCTGAAGCCAGGGGAGACACTTTTCCCAACAGA TGAAAATGGAAAACCAATATTTGACTCAGTGGATCTCTGTCGCACATGGGAGGCCCTGGAGAAGT GTAAGGACGCAGGGCTGACCAAGTCCATCGGGGTGTCCAACTTCAACCACAAGCAGCTGGAGAA GATCCTGAACAAGCCAGGGCTTAAGTACAAGCCCGTCTGCAACCAGGTGGAATGTCACCCTTATT TCAATCAGAGCAAACTGTTGGATTTCTGCAAGTCACATGATATTGTTCTTGTTGCCTATGGTGCTCT GGGATCCCAACGACTAAAAGAATGGGTGAACCCAAACCTCCCCTTTCTCTTGGAGGACCCAGTTC TCTCTGCCATTGCCAAAAAGCACAGGCAAACCCCAGCTCTGGTTGCCCTTCGCTACCAGATACAA CGTGGGGTTGTGGTTCTGGCCAAGAGTTACAACAAGAAGCGGATCAAAGAGAACATACAGGTGTT TGACTTTGAACTGACTCCAGAAGATATGAAAGCAATCGATGGCCTCAACAGCAATATGAGATATAA TGAGCTATTACTGGGTGTTGGTCACCCTGAGTATCCATTTGTTGAAGAATATTGACTCGAGGAATC ACGAATTCTGGATCCGATACTCGAGTCTAGAGTCCA
Protein sequence	MDPKGQRVKLNDGHFIPVLGFGTFAPREVPKSEALEVTKFAIEAGFRHIDSAHLYQNEEQVGQAIRSKI ADGTVKREDIFYTSKLWSTSLRPELVRPALEKSLNNLQLDYVDLYIIHFPVALKPGETLFPTDENGKPIFD SVDLCRTWEALEKCKDAGLTKSIGVSNFNHKQLEKILNKPGLKYKPVCNQVECHPYFNQSKLLDFCKS HDIVLVAYGALGSQRLKEWVNPNLPFLLEDPVLSAIAKKHRQTPALVALRYQIQRGVVVLAKSYNKKRIK ENIQVFDFELTPEDMKAIDGLNSNMRYNELLLGVGHPEYPFVEEY-
M.W.	36.79 KDa

Table 7 - Article 3: Supplementary figure 6: Cloned bovine AKR1C4 protein

List of bovine cDNA and protein sequences as well as NCBI current and previous names of AKR1C4 used in this study.

Protein name	Bovine Dihydrodiol dehydrogenase 3 (DD3)
NCBI current and previous names	>Dihydrodiol dehydrogenase 3 [Bos taurus] Sequence ID: ref|NP_001159697.1| >Prostaglandin F synthase-like1 protein [Bos taurus] Sequence ID: gb|AAN11328.1|
cDNA sequence	ATGGATCCCAAAGGCCAGAGAGTGAAACTTAATGACGGCCACTTCATTCCTGTCCTGGGATTTGG AACCTATGCACCTCCAGAGGTTGCTAAGAAGGAAGCTCTGGAATTCACCCCATTCGCTATAGAGGT TGGGTTCCGCCATATTGACTGTGCTCATGCCTACCAAAATGAAGAGGAGATTGGCCAGGTCATTC GAAGCAAGATTGCAGATGGCACTGTGAAGAGAGAAGACATATTCTGCACTTCAAAGCTTTGGTTGA CTTCCCTTCGACCAGAGTTGGTCCGACCAGCCTTGGAAAAGTCACTGAAAAATCTTCAACTGGACT ATGTCGATCTCTATATTATGCATTATCCAATGGCTCTGAAGCCAGGGGAGGAATTATTTCCAAAAGA TGAAAATGGAAAACTGATATTTGACTCAGTGGATTTCTGTCGCACGTGGGAGGCCCTGGAGAAGT GTAAGGATGCAGGGCTGGCCAAGTCCATCGGGGTGTCCAACTTCAACCACAAACAGCTGGAGAG GATCCTGAACAAGCCAGGGCTAAAGTATAAGCCTGTCTGCAACCAGGTGGAATGTCACCCTTATCT CAACCAGAGCAAACTGTTGGATTTCTGCAAGTCACATGAAATTGTTCTTGTTGCCTATGCTGGTCT GGGATCCCAACGAGTAAAAGAATGGGTGAACCAGAACCACCCCGTTCTCCTGGAGGACCCGGTT CTCTCTGCCATTTCCCAAAAGCACAAGAAAACGGCAGCTCTGATTGCCCTTCGCTACCAGATACAA CGTGGAGTTGTGGTTCTGGCCAAGGGCAACAACAAGAAGTGGATCAAAGAGAACATGCAGGTGTT TGACTTTGAACTGACTCCAGAAGACATGAAAGCAATCGATGGCCTCAACAGAAATATAAGATATTA CGAACTTCACCTTGCTGTTAGTCATCCTGAGTTTCCATTCTCTGAAGAATATTGACTCGAGTCTAGA GACCGTATNNNCC
Protein sequence	MDPKGQRVKLNDGHFIPVLGFGTYAPPEVAKKEALEFTPFAIEVGFRHIDCAHAYQNEEEIGQVIRSKIA DGTVKREDIFCTSKLWLTSLRPELVRPALEKSLKNLQLDYVDLYIMHYPMALKPGEELFPKDENGKLIFD SVDFCRTWEALEKCKDAGLAKSIGVSNFNHKQLERILNKPGLKYKPVCNQVECHPYLNQSKLLDFCKS HEIVLVAYAGLGSQRVKEWVNQNHPVLLEDPVLSAISQKHKKTAALIALRYQIQRGVVVLAKGNNKKWI KENMQVFDFELTPEDMKAIDGLNRNIRYYELHLAVSHPEFPFSEEY-
M.W.	37.03 KDa

Table 8 - Article 3: Supplementary figure 7: Cloned bovine DD3 protein

List of bovine cDNA and protein sequences as well as NCBI current and previous names of DD3 used in this study.

Chapitre 5: The prostaglandin F synthase activity of the human aldose reductase AKR1B1 brings new lenses to look at pathologic conditions.

Eva Bresson, Nicolas Lacroix-Pépin, Sofia Boucher-Kovalik, Pierre Chapdelaine, and Michel A. Fortier

Ce chapitre est présenté sous forme d'article et a été publié dans la revue :
Front Pharmacol. 2012;3:98

Unité de Recherche en Ontogénie et Reproduction, Centre Hospitalier Universitaire de Québec (CHUL), Centre de Recherche en Biologie de la Reproduction (CRBR), and Département d'Obstétrique et Gynécologie, Université Laval, Ste-Foy, QC, Canada G1V4G2

RÉSUMÉ

Les prostaglandines sont des régulateurs importants des fonctions reproductrices femelles auxlquelles les aldose réductases présentant une activité hydroxystéroïde déshydrogénase contribuent également. Notre travail sur la régulation de la fonction reproductive par les prostaglandines (PG), nous a conduit à la découverte que AKR1B5 et plus tard AKR1B1 étaient des PGF synthases physiologiquement pertinentes et hautement efficaces. PGE2 et PGF2α sont les principaux prostanoïdes produits dans l'endomètre humain et un bon équilibre dans leur production relative est essentiel pour une fertilité optimale et des menstruations normales. Des données récentes suggèrent que PGE2/EP2 et PGF2α/FP peuvent constituer un binôme fonctionnel avec une pertinence physiologique comparable à la dyade prostacycline - thromboxane dans le système vasculaire. Nous avons récemment rapporté qu'AKR1B1 a été exprimée et modulée en association avec la production PGF2α en réponse à l'IL-1β dans l'endomètre humain. Dans la présente étude, nous montrons que la AKR1B1 humaine (gène ID: 231) également connue sous le nom ALDR1 ou ALR2 est une PGF2α synthase fonctionnelle dans différents modèles de cellules et de tissus vivants. L'utilisation de cellules endométriales humaines, de cellules vasculaires musculaires lisses, de cardiomyocytes et de cellules endothéliales de la prostate nous démontrent que l'IL- 1β est capable de réguler COX-2 et AKR1B1 ainsi que la production de PGF2α à des concentrations normales de glucose. Nous montrons que l'activité du promoteur du gène AKR1B1 est augmentée par l'IL-1β en particulier autour de la région de réponse au stress multiple (MSRR) contenant deux éléments de réponse aux antioxydants putatifs (ARE) à côté de tonE et AP1. Nous montront également qu'AKR1B1 est capable de réguler la production de PGE2 par la PGF2α agissant sur son récepteur FP et que les inhibiteurs d'aldose réductase (ARI) comme l'alrestatin, le statil (ponalrestat) et l'EBPC présentent une inhibition distincte et caractéristique de la production PGF2α dans différents modèles cellulaires. L'activité PGF synthase d'AKR1B1 représente une nouvelle et importante cible pour réguler les réponses ischémiques et inflammatoires associées à plusieurs pathologies humaines.

ABSTRACT

Prostaglandins are important regulators of female reproductive functions to which aldose reductases exhibiting hydroxysteroid dehydrogenase activity also contribute. Our work on the regulation of reproductive function by prostaglandins (PGs), lead us to the discovery that AKR1B5 and later AKR1B1 were highly efficient and physiologically relevant PGF synthases. PGE2 and PGF2α are the main prostanoids produced in the human endometrium and proper balance in their relative production is important for normal menstruation and optimal fertility. Recent evidence suggests that PGE2/EP2 and PGF2α/FP may constitute a functional dyad with physiological relevance comparable to the prostacyclin-thromboxane dyad in the vascular system. We have recently reported that AKR1B1 was expressed and modulated in association with PGF2α production in response to IL-1β in the human endometrium. In the present study, we show that the human AKR1B1 (gene ID: 231) also known as ALDR1 or ALR2 is a functional PGF2α synthase in different models of living cells and tissues. Using human endometrial cells, prostate and vascular smooth muscle cells, cardiomyocytes and endothelial cells we demonstrate that IL-1β is able to up regulate COX-2 and AKR1B1 proteins as well as PGF2α production under normal glucose concentrations. We show that the promoter activity of AKR1B1 gene is increased by IL-1β particularly around the multiple stress response region (MSRR) containing two putative antioxidant response elements (ARE) adjacent to TonE and AP1.We also show that AKR1B1 is able to regulate PGE2 production through PGF2α acting on its FP receptor and that aldose reductase inhibitors (ARIs) like alrestatin, statil (ponalrestat) and EBPC exhibit distinct and characteristic inhibition of PGF2α production in different cell models. The PGF synthase activity of AKR1B1 represents a new and important target to regulate ischemic and inflammatory responses associated with several human pathologies

INTRODUCTION

The human aldose reductase ALR2 gene AKR1B1 is a notorious enzyme which has been associated with complications of diabetes for more than four decades (Srivastava, Ramana et al. 2005). AKR1B1 is considered as the rate limiting enzyme of the polyol pathway responsible for the conversion of glucose into sorbitol. The association of AKR1B1 with cardiovascular risks, neuropathies and nephropathies characteristic of diabetes is receiving increased acceptance (Alexiou, Pegklidou et al. 2009). However, the contribution of the polyol pathway to the same complications is less convincing (Del Corso, Cappiello et al. 2008). Indeed, while many factors including inflammation induce increased expression and activity of AKR1B1, this enzyme has very little affinity for glucose at physiological concentrations and a detoxification action involving reduction of lipid peroxides has been proposed (Srivastava, Ramana et al. 2005). While searching for the enzyme responsible for the production of PGF2α in the endometrium, we made the serendipitous and unexpected discovery that AKR1B5 (Madore, Harvey et al. 2003) and later the human aldose reductase AKR1B1 (Bresson, Boucher-Kovalik et al. 2011) were highly functional PGFS. Prostaglandins (PGs) are notorious mediators of pain and inflammation also associated with proliferation of cancer cells. While the same mediators are recognized as primary regulators of female reproductive function (ovulation, uterine receptivity, implantation and parturition) they also contribute to occurrence of endometrial carcinomas, menorrhagia, dysmenorrhea, endometriosis and premature labor. Among the different PGs, PGE2 and PGF2α are the main prostanoids produced in the human endometrium (Smith and Kelly 1988; Sales and Jabbour 2003). PGs are synthesized from arachidonic acid (AA) and converted to PGG_2 and PGH_2 by PGH synthases (PGHS), also known as cyclooxygenases (COX). There are two documented isoforms of PGHS in human, the constitutive COX-1 and the inducible COX-2 encoded by two distinct genes (Smith, Garavito et al. 1996). PGH_2 produced by COXs is the common precursor of all PGs generated by specific terminal synthases such as PGF synthase for PGF2α and PGE synthase for PGE2. The human terminal synthases responsible for the biosynthesis of PGE2 (Park, Pillinger et al. 2006), PGD2 (Zhou, Shaw et al. 2010), and PGI2 (Wu and Liou 2005) are well characterized, but only little is known for the enzyme responsible for PGF2α production. Before our identification of the PGF synthase activity of aldo-keto reductases (AKR) of the 1B family (Madore, Harvey et al. 2003; Bresson, Boucher-Kovalik et al. 2011) only one AKR of the 1C family, AKR1C3 was recognized as a functional PGF synthase in the human (Suzuki-Yamamoto, Nishizawa et al. 1999).

PGF2α is a biologically active prostanoid belonging to the eicosanoid family of bioactive lipids (Narumiya and FitzGerald 2001). Its biosynthesis occurs via different pathways involving reduction of PGH2 by a 9,11-endoperoxyde reductase (Watanabe 2002). Several PGFS have been identified in animals (Madore, Harvey et al. 2003), but until recently, AKR1C3 was the only isoform currently identified in human (Komoto, Yamada et al. 2006). In the bovine endometrium, using a cell-free system, we have demonstrated a strong

PGFS activity of purified recombinant AKR1B5 recently renamed as *bos taurus* AKR1B1 (Gene ID: 317748) as well as circumstantial association between its pattern of expression and PGF2α production. This represents a new putative function for this enzyme previously known for its 20α-HSD and glucose metabolism activities (Madore, Harvey et al. 2003). The human and bovine AKR1B1 (EC: 1.1.1.21) both belong to the AKR1B family and share 86% identity or homology. The human AKR1B1 (Gene ID: 231) also known as the aldose reductase or ALDR1 belongs to the AKR superfamily composed of 140 members divided into 15 families(Jin and Penning 2007). AKR1B1 (EC:1.1.1.21) is primarily known as the rate limiting enzyme for conversion of glucose to sorbitol in the polyol pathway, but recent studies revealed its ability to convert a wide array of substrates including aldehydes generated during lipid peroxidation and their glutathione (GSH) conjugates, phospholipids, atherogenic lipids and steroids (Srivastava, Ramana et al. 2005).

After identifying the bovine AKR1B1 as a potential PGFS (Madore, Harvey et al. 2003), we have shown circumstantial association with PGF2α production in human endometrial cell lines (Chapdelaine, Kang et al. 2006) and in decidualized stromal cells (Kang, Chapdelaine et al. 2006). Later, the PGFS activity of mouse AKR1B3 and AKR1B7 as well as the human AKR1B1 were tested and confirmed using purified recombinant proteins in a cell free system in vitro (Kabututu, Manin et al. 2009). Using a similar approach complemented with gain and loss of function in endometrial cell lines, we have demonstrated that the AKR1B1 was a functional PGFs in the human endometrium (Bresson, Boucher-Kovalik et al. 2011). In the present study, we have investigated further the regulation of PGF2α production by different stimulators and inhibitors as well as the promoter cis-elements responsible for IL-1β response in human endometrial cell lines. Finally, we have studied the association between AKR1B1 and PGF2α production in representative tissues where PGF2α was found to exert physiological action in vivo. We have evaluated the potential of different AKR1B1 inhibitors (ARI) to alter PGs production in living cells and in fresh endometrial tissues under normal and high glucose conditions.

MATERIALS AND METHODS

Reagents

RPMI 1640 culture medium without phenol, Superscript II reverse transcriptase, TRIzol, lipofectamine 2000, and pCR3.1 vectors were purchased from Invitrogen (Life technologies, ON, Canada). TAQ DNA polymerase and buffer used for polymerase chain reaction were from NEB (New England Biolabs, ON, Canada). RiboMax polymerase kit for siRNAs was purchased from Promega (Madison, WI, USA). Qiaquick gel extraction kit and TA cloning pDrive vector were from Qiagen (Mississauga, ON, Canada). All

oligonucleotide primers were chemically synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA). [-32P]dCTP radioactivity was bought from Perkin-Elmer Life Sciences (Markham, Ontario, Canada). Bright Star-Plus nylon membrane and UltraHyb solution were purchased from Ambion Inc. (Austin,TX, USA). The rabbit COXs antibodies were kindly provided by Dr S Kargman (Merck, Que, Canada), rabbit anti-Phospho-cPLA2 (Ser505) was from Santa-Cruz biotechnology (Santa Cruz, CA, USA) and AKR1C3 was from Abcam Inc. (Cambridge, MA). Goat anti-rabbit horse-radish peroxidase–conjugated IgG was bought from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The Western Lightning™ Chemiluminescence Reagent Plus was purchased from PerkinElmer (Woodbridge, ON, Canada). Arachidonic acid (AA), and AL-8810 were from Cayman Chemicals (Ann Arbor, MI, USA) and recombinant human IL1 was purchased from Research and Diagnostic Systems (Minneapolis, MN, USA). Aldose reductase inhibitors (ARIs), (ponalrestat (Statil), Alrestatin and EBPC) were from Tocris Bioscience (Ellisville, MO).

Cultured cell models

Human endometrial stromal (HIESC-2) and epithelial (HIEEC) cell lines were immortalized using SV40 large T antigen (Chapdelaine, Kang et al. 2006) and shown to exhibit most characteristics of freshly isolated endometrial cells (Kang, Akoum et al. 2004; Kang, Chapdelaine et al. 2006). Bovine endometrial epithelial cells were immortalized using a similar protocol and also presented most characteristics of freshly isolated cells (Krishnaswamy, Danyod et al. 2009; Krishnaswamy, Lacroix-Pepin et al. 2010; Lacroix-Pepin, Danyod et al. 2011). Human prostate smooth muscle cells, human artery smooth muscle cells, human umbilical vein endothelial cells (Huvec) and human cardiomyocytes cells were from ScienCell Research Laboratories (Carlsbad, CA, USA).

Northern blot

Northern blot analysis was performed as follows: total RNA(~20 g) was extracted with Trizol (Invitrogen, Life technologies, ON, Canada) from cultured endometrial cells, loaded on a 1.2% formaldehyde-agarose gel and electrophoresed at 100 V in 1X MOPS buffer. After electrophoresis, RNA was transferred overnight onto a nylon membrane in 10X saline-sodium citrate (SSC). The AKR1B1, mPGES-1, COX-1 and COX-2 cDNA probes were produced by digestion with restriction enzymes of different plasmids containing the corresponding cDNAs previously generated in our laboratory, each releasing a ~500 bp fragment. The resulting probes were labeled with [α-32P]dCTP (3000 Ci/mmol) using the Ready-To-Go DNA labeling Kit (GE Healthcare Life Science,Qc, Canada). Prehybridization (2-4 hours) and hybridization (overnigth) were done at 45°C using UltraHyb solution (Ambion inc, Invitrogen, Life technologies, ON, Canada). The blots were then washed at 65°C twice for 15 minutes in 0.5 X SSC and exposed for 24 to 48 hours at -80°C on BioMAx films PerkinElmer (Woodbridge, ON, Canada) to quantitate the hybridization signal intensity. Finally, 18S

ribosomal RNA stained with ethidium bromide was used to confirm uniform loading of RNA samples and a 32P-labeled oligonucleotide specific to 18S ribosomal RNA was used as an internal standard for Northern blot analysis.

Western blot

Western blot analysis was performed as we described (Chapdelaine, Kang et al. 2006). Briefly, total proteins (~20 g) extracted from culture cells were loaded in each lane and electrophoresis done on 10 % SDS-PAGE followed by electrotransfer onto nitrocellulose membrane (Bio-Rad laboratories, Mississauga, Ontario, Canada). The primary antibodies used for the present study were the rabbit AKR1B1 (dilution 1/1000), COX-1 or COX-2 (dilution 1/3000), cPLA2 (1/1000) and goat AKR1C3 (1/500) anti-serums. As an internal control, the -actin monoclonal antibody (1/5000, Sigma, Mississauga, Ontario, Canada) was used. The goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP), rabbit anti-goat IgG HRP or goat anti-mouse IgG HRP were used as secondary antibodies. The chemiluminescence was analyzed with autoradiography films at optimal times of exposure following treatment of the membranes with the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Canada).

Analysis of AKR1B1 promoter activity

A nested PCR strategy was developed to generate a long ~ 4.5 kb promoter to study the regulation of the AKR1B1 gene (AF032455). The promoter PCR fragments were amplified with PFU turbo DNA polymerase (Stratagene, Agilent technologies, USA) and cloned directly by fusion (Clontech, TAKARA Biocompany) in a pGL3 basic vector (Promega) containing the firefly luciferase gene. Constructs of AKR1B1 promoter of seven different lengths were generated using restriction enzymes targeting unique sequences in the long promoter. A short portion of the AKR1B1 promoter corresponding to the multiple stress response region (MSRR) -1710 to -1064 to the translation initiation ATG position was amplified by PCR, cloned in a TA cloning vector (pDrive, Qiagen) for sequencing and linked in a pGL3 vector (Promega) to the minimal SV40 promoter. From this, additional constructs were produced by deletion of the cis-elements corresponding to TONE and AP1 or ARE1 and 2. Stromal (HIESC2) and epithelial (HIEEC22) cells were co-transfected with the lipofectamine 2000 transfection agent (Invitrogen) and the different plasmid constructs possessing the firefly luciferase reporter and a plasmid containing the synthetic Renilla luciferase gene. After transfection, cells were treated for 24 hours with IL-1β (1ng/ml), and the promoter activity was expressed as the ratio of firefly luciferase to the renilla luciferase using the Dual-Luciferase Reporter (DLR™) Assay System (Promega, Madison, WI, USA) using a Luminoskan Ascent luminometer (ThermoElectron Corporation, Milford, MA).

Cell treatments

HIESC-2 (passages 15-22) and HIEEC-22 (passages 15-22) were cultured in RPMI 1640 without phenol red, containing 50 IU penicillin-streptomycin supplemented with 10% whole FBS during growth and 10% dextran-coated charcoal extracted FBS once cells have reached confluence and for transfection or treatments. Knockdown transfection of cells with AKR1B1 specific siRNA was performed with lipofectamine 2000 for 4 hours in culture medium without antibiotic. Unless specified, otherwise cells were treated for 24 hours with IL-1β (1ng/ml) or other agents at specified concentrations in RPMI 1640 medium without serum. For Western blot analysis (described above), the cells were grown in 24-well plates whereas for Northern blots analysis (described above), cells were in 6-well plates. At the end of the treatment period, the culture medium was recovered and stored at -20°C until evaluation of PGF2α or PGE2 production by EIA whereas protein and RNA were extracted directly from cell monolayer at the bottom of 24-well and 6-well plates respectively.

Endometrial explants culture

Endometrial tissue was obtained either from biopsies collected with an endometrial curette (Pipelle) and obtained from women aged between 25 to 50 years. Informed consent for donation of anonymous endometrial samples was obtained before tissue collection. The research protocol was approved by the Ethics Committee on Human Research of Centre Hospitalier Universitaire de Québec. Endometrial explants were prepared from fresh biopsies as described previously (Bresson, Boucher-Kovalik et al. 2011). Endometrial explants were stimulated or not with IL-1β in the presence or absence of the aldose reductase inhibitor ponalrestat (100 µM) under normal (5 mM) or high glucose (25 mM) for 24 h.

Measurement of Sorbitol

We have developed a LC/MS/MS procedure for quantitative measurement of sorbitol generated in Briefly, the culture medium was removed for estimation of PGE2 and PGF2α and sorbitol was extracted from explants by mixing with lysis buffer (200 µl), methanol (600 µl), chloroform (200 µl) and water (500 µl). The mixture was centrifuged for 5 minutes at room temperature and 100 µl of the upper phase was evaporated with a turbo-vap at 45oC for 30 min. The pellet was resuspended with 200µl of Pyridine 10%: anhydride acetic acid 10% in acetonitrile and brought at 70oC for 45 minutes. After drying samples were diluted in 100µl methanol/water (1:1) containing 5 mM ammonium acetate. The samples were spiked with Sorbitol-d8 (Omicron Biochemicals Inc., IN, USA) also used for establishment of a standard curve and analyzed on an Applied Biosystems API-5000 LC/MS/MS. The procedure was optimized to minimize interference with sugars and alcohols such as glucose, fructose, galactose, myoinositol and galactitol.

PGE2 and PGF2α

PGE2 and PGF2 were assayed by competitive EIA using acetylcholinesterase–linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996).

Statistical analysis

Data were analyzed by One or Two way ANOVA with Bonferroni as post-hoc test with 95% confidence intervals using GraphPad Prism 5 program. For data with less than 3 columns a two-tailed unpaired t test with 95% confidence intervals was performed using GraphPad Prism 5 program. Data are presented as the Mean ± SEM. Each experiment was repeated three times unless specified otherwise. Values were considered statistically significant for $P < 0.05$.

RESULTS

While glucose and oxidized lipids are passive stable substrates for AKR1B1, generation of PGF2α requires the generation of the unstable PGH2 precursor within the producing cell (Figure 1). Figure 2 illustrates that upon stimulation by IL-1β, the rate limiting enzymes cPLA2 releasing AA from membranes phospholipids, COX-2 generating the PGH2 precursor and AKR1B1 are all increased in both endometrial stromal (A) and epithelial (B) cells. The production of PGF2α is also increased following IL-1β stimulation, a response blocked by the COX-2 selective inhibitor NS-398 (C and D). As was found previously (Pelletier, Luu-The et al. 1999; Bresson, Boucher-Kovalik et al. 2011), AKR1C3 is present only in epithelial cells and is not induced by IL-1β suggesting minimal contribution to PGF2α production under these conditions.

The time course of PG biosynthetic enzymes activation following IL-1β stimulation was investigated at the mRNA level and associated with PGF2α release (Figure 3). Note that there is basal expression of AKR1B1 gene under non-stimulated conditions and that there is serial transcription of biosynthetic enzyme genes with AKR1B1 preceding COX-2 preceding mPGES-1. Accordingly, PGF2α release precedes that of PGE2 in both stromal and epithelial cells ($P<0.05$).

We have cloned the AKR1B1 promoter (~4.5 kb) and generated eight constructs to identify potential response elements sensitive to IL1-β stimulation (Figure 4). IL-1β stimulates the AKR1B1 promoter activity from -583 up in both endometrial stromal and epithelial cells (Figure 4A). A multiple stress response region (MSRR) containing two putative antioxidant response elements (ARE) adjacent to TonE and AP1 previously identified for the mouse AKR1B3 (Nishinaka and Yabe-Nishimura 2005) was also identified for the human AKR1B1 gene at -1710 to -1064 (Figure 4B). The human MSRR region was analyzed in a vector containing a

minimal promoter SV40 to identify putative cis-elements of the AKR1B1 gene promoter activity involved in IL-1β response (Figure 4C). The antioxidant response elements ARE1 and ARE2 appear particularly efficient to confer IL-1β response and may explain the quick activation of AKR1B1 and PGF2α production observed in figure 3.

The PGFsynthase activity of AKR1B1 and its stimulation by IL-1β and TNFα was investigated in cultured cells isolated from different human tissues (Figure 5). In addition to endometrial cells where we made our initial discovery, we have found that IL-1β and to a lesser extent TNFα stimulated AKR1B1, COX-2 and PGF2α production in prostatic smooth muscle cells (A), arterial smooth muscle cells (B), cardiomyocytes (C) and umbilical endothelial cells (HUVEC D). In the last two models, the observed increase in PGF2α production appeared to result from increased COX-2 expression coupled with already high preexisting levels of AKR1B1.

We have compared the inhibition potential of three aldose reductase inhibitors; alrestatin, EBPC (not shown) and ponalrestat on human endometrial cells and tested the latter on bovine endometrial epithelial cells (Figure 6). Alrestatin did not have any effect on PGF2α production (Figure 6A). EBPC inhibited PGF2α production with an apparent EC50 around 10 μM (not shown) whereas ponalrestat was the most potent with an EC50 of 1 μM (Figure 6B). Ponalrestat also inhibited PGF2α in human (Figure 6C) and bovine (Figure 6D) endometrial epithelial cells albeit with reduced efficiency exhibiting an EC50 of 50μM. Interestingly, ARIs not only inhibited PGF2α but also PGE2 in all cell models.

The potential non-selective inhibition of several components of PG biosynthesis by ARIs was investigated further (Figure 7). When AKR1B1 expression was knocked down by siRNA, similar observation of concurrent inhibition of PGF2α and PGE2 was observed (Figure 7A) suggesting potential interactions between the two biosynthetic pathways. The possibility that AKR1B1 could exert positive regulation of PGE2 production through PGF2α and its FP receptor was tested (Figure 7B). Interestingly, when AKR1B1 and PGF2α production are stimulated with IL-1β, PGF2α and the concomitant increase of PGE2 is reduced in presence of the FP receptor antagonist AL-8810 suggesting that PGF2α was able to stimulate its own and PGE2 production. The latter hypothesis was tested using the PGF2α analog bimatoprost which stimulated PGE2 release in a dose dependent manner (Figure 7C).

Finally, the relative affinity and potential competition between the PGFS and polyol pathway was tested under close to in vivo conditions (Figure 8). Fresh endometrial explants were treated with IL-1β in presence and absence of ponalrestat under normal and high glucose conditions. Interestingly, IL-1β previously demonstrated to increase AKR1B1 expression increased PGF2α or sorbitol production depending

on normal or high glucose conditions respectively. This demonstrates that PGF2α and sorbitol are formed competitively depending on the relative availability of AKR1B1 substrates.

DISCUSSION

Prostaglandins are important regulators of female reproductive function and contribute to gynecological disorders. Normal menstruation depend on an equilibrium between vasoconstrictors such as PGF2α (Lockwood and Schatz 1996; Sales and Jabbour 2003) and vasodilators such as PGE2 or nitric oxide (NO) (Tschugguel, Schneeberger et al. 1999). Excessive production of contracting prostaglandins create an ischemia-reperfusion response causing painful menstruation or dysmenorrhea (Okazaki, Matsuyama et al. 2005) whereas increased vasodilatation leads to abundant menstrual bleeding (Sales and Jabbour 2003). NSAIDs represent the most important and widely used drugs on the market and they are all efficient to treat menstrual disorders at some level. However these drugs act at an early step of biosynthesis common to all PGs and not only the isotype responsible for the pathological response. Because of its notorious role on inflammation and pain, the biosynthetic pathway leading to PGE2 has been studied extensively, but that of PGF2α is poorly documented. The data presented in this manuscript address the conditions regulating the newly identified PGFS activity of AKR1B1. In the human endometrium, it has been reported that production of PGF2α is higher in late secretory and menstrual periods of the menstrual cycle (Downie, Poyser et al. 1974). Accordingly, both AKR1B1 and AKR1C3 enzymes are present in the endometrium throughout the menstrual cycle. By contrast with AKR1B1 expressed in both stromal and glandular epithelial cells and modulated in association with endometrial PGF2α production, AKR1C3 expression is constant and completely absent in stromal cells as was reported previously (Pelletier, Luu-The et al. 1999) Zakharov 2010. The absence of the only currently accepted human PGFS AKR1C3 in stromal cells was surprising because we and others have shown that human endometrial stromal cells produce high levels of PGF2α (Huang, Liu et al. 1998; Chapdelaine, Kang et al. 2006; Kang, Chapdelaine et al. 2006). Because of a similar finding in the bovine endometrium and our identification of AKR1B5 as a functional PGFS in that system (Madore, Harvey et al. 2003), we hypothesized that the human AKR1B1, could also express PGFS activity in the human endometrium. Our initial studies showing the association between AKR1B1 expression and PGF2α production was confirmed unequivocally in the human endometrium using gain and loss of function approaches (Bresson, Boucher-Kovalik et al. 2011). The PGF synthase activity of AKR1B1 and other members of this family is now acknowledged by leaders in the field of prostaglandin synthases (Nagata, Kusakari et al. 2011; Smith, Urade et al. 2011; Watanabe 2011).

In the present study we highlight the necessary co-activation of genes of the PG biosynthesis pathway to provide the PGH2 precursor substrate to AKR1B1 in order to generate PGF2α. This is in clear contrast with detoxification or aldose reductase activities of AKR1B1 for which substrates are provided by extracellular sources (glucose) or as byproducts of intracellular reactions (oxidized lipids). Endometrial cells express constitutively GLUT2 and GLUT3 and do not require insulin for glucose entry, as such; the endometrium belongs to the category of tissues most adversely affected by poorly controlled glucose levels occurring during insulin resistance or diabetes. Figures 2 and 3 illustrate that genes and proteins of the biosynthetic pathway are increased in a time and dose dependent manner to explain increased PGF2α production following IL-1β stimulation.

In figure 3 and then in figures 6 and 7 we observe that PGE2 production closely follows that of PGF2α under both stimulatory and inhibitory conditions. We first considered these results as artifact and resulting from defective experimental design. However, when we observed that inhibition of FP receptor prevented the increase in PGE2 and that FP receptor stimulation alone was sufficient to stimulate PGE2 production, we proposed that there was a positive feedback loop between PGF2α release and production of PGE2. A link between FP activation and increased PG production could have been anticipated from previous observations in a human endometrial model (Jabbour 2005, Sales 2008) and from our own observations in bovine endometrial cells (Krishnaswamy, Lacroix-Pepin et al. 2010)given that FP and the oxytocin receptor (OT) share the same signal transduction systems. However, this is the first evidence of a specific link between FP activation and PGE2 production within a homogeneous cell system with potential physiological and pathological relevance. If we hypothesize that PGE2 acting on EP2 receptors and PGF2α acting through FP work as a dyad with opposite actions in the reproductive system, just like the thromboxane/prostacyclin dyad in the vascular system, then the interactions between the two systems are not only probable but also necessary.

Prostaglandins work like a micro endocrine system sensing altered conditions and reestablish locally, optimal conditions for tightly regulated events such as ovulation or recognition of pregnancy in the reproductive system or hemostasis in the vascular system. In this respect, PGF2α exhibits vasoconstrictive and prothrombotic responses comparable to thromboxane with the additional feature that the PGF2α molecule is chemically stable and remains active until catabolism by prostaglandin dehydrogenase, usually through a single passage in the lung. By contrast PGE2/EP2 trigger a vasodilator with anti thrombotic action similar to prostacyclin but again PGE2 is much more stable than PGI2. Thus it is important to tightly regulate the relative production and release of PGF2α and PGE2 and in this respect; our identification of a feedback loop between PGF2α and PGE2 within endometrial cells has important implications for the observed complications associated with over-expression of AKR1B1. We have initiated the characterization of the

AKR1B1 promoter and identified a multiple stress response region (MSRR) containing two putative antioxidant response elements (ARE) adjacent to TonE and AP1. The antioxidant response elements ARE1 and ARE2 appear to be involved in IL-1β response, which supports the local sensing functions of PGs to which AKR1B1 is now a contributing component. The MSRR portion of the AKR1B1 promoter will have to be investigated further under IL-1β stimulation to identify the trans-factors associated with the production of PGF2α. Because of its reported interaction with AREs, NRF2 also known as Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) is a likely candidate to link increased PGF2α release under inflammatory conditions (25).

Clinical and pathological implications: Prostaglandins are important regulators of female reproductive functions. In this respect, gene inactivation studies in the mouse have shown that COX-2 and EP2 null mice share similar phenotypes where ovulation and implantation are impaired (Lim, Paria et al. 1997; Kennedy, Zhang et al. 1999). The PGF2α/FP system is much less documented than that of PGE2, but the group of Jabbour in Edinburg has documented its involvement in endometrial pathologies including menstrual disorders and endometrial cancer(Milne and Jabbour 2003; Jabbour and Sales 2004; Sales, Milne et al. 2004; Jabbour, Sales et al. 2005; Jabbour, Sales et al. 2006; Abera, Sales et al. 2010; Catalano, Wilson et al. 2011) while we (Breuiller-Fouche, Leroy et al. 2010; Phillips, Al-Zamil et al. 2011) and others (Olson 2007) associate it with premature delivery. With that in mind, it is likely that AKR1B1 contribute to these pathologies and conversely conditions where AKR1B1 is affected such as diabetes and metabolic syndrome may exhibit a specific prognosis pattern in affected women. In addition, we claim that the female reproductive system constitutes a powerful model to understand the contribution of PGs to physio-pathological conditions in other systems. This is supported by figure 5 showing the relation between AKR1B1 and PG biosynthetic enzymes in response to IL-1β and their association with PGF2α production in smooth muscles, cardiomyocytes and endothelial cells. This is all the more important as AKR1B1 known to be highly responsive to diverse physiologic and pathologic conditions and its multiple substrate processing ability combine to create complex combinations well illustrated in figure 8. For instance, PGF2α has been associated with cardiac hypertrophy (Lai, Jin et al. 1996) and a new hamster AKR1B was identified and proposed to contribute to cardiomyopathy through PGF2α release (Sakamoto and Sugamoto 2011). Moreover, knowing that high glucose induce AKR1B1 expression we see that as long as glucose levels remain high, there will be little increase in PGF2α release, but when glucose goes down, the high levels of AKR1B1 will generate increased production of PGF2α. In turn, high PGF2α will trigger pro-ischemic responses (Yuhki, Kashiwagi et al. 2010) that fortunately will be compensated by the feedback loop release of PGE2 exerting opposite action thus bringing back homeostasis (Mandal, Zhang et al. 2005). Normal tension results from the action of EP2 opposing FP receptors in the vascular system, but adjacent tissues also express other EP receptors responsible for inflammation and pain. Therefore the relative silent condition resulting from PGE2 compensation of

overproduction of PGF2α by AKR1B1 will likely generate pain or hyperalgesia increasing the probability to take NSAIDs. Not that far ago, the best pain killer on the market with minimal gastrointestinal toxicity was rofecoxib (Vioxx), the most powerful medication to date eradicating pain by reducing mPGES-1 generated PGE2. If someone taking Vioxx was under the silent (increased AKR1B1) condition, then the compensatory PGE2/EP2 would not be available to counteract the ischemic response to PGF2α thus increasing the risks of heart failure. Interestingly, even after adjustment of risk factors, reexamination of Vioxx events (Baron, Sandler et al. 2008) reveal that people suffering from type two diabetes were eight times more at risk to die from Vioxx than non diabetics. By contrast people taking both Vioxx and Aspirin, thus blocking the AKR1B1 generated PGF2α were protected. Therefore, it is worth investigating if and how AKR1B1 potentially contributed to the failure of Vioxx.

In conclusion, the ability of AKR1B1 to release PGF2α brings a new angle to look at mechanisms responsible for pathophysiologic conditions in the woman reproductive and other systems especially in association with diabetes.

REFERENCES

Abera, A. B., K. J. Sales, et al. (2010). "EP2 receptor mediated cAMP release is augmented by PGF 2 alpha activation of the FP receptor via the calcium-calmodulin pathway." Cell Signal 22(1): 71-79.

Alexiou, P., K. Pegklidou, et al. (2009). "Aldose reductase enzyme and its implication to major health problems of the 21(st) century." Curr Med Chem 16(6): 734-752.

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod 54(2): 371-379.

Baron, J. A., R. S. Sandler, et al. (2008). "Cardiovascular events associated with rofecoxib: final analysis of the APPROVe trial." Lancet 372(9651): 1756-1764.

Bresson, E., S. Boucher-Kovalik, et al. (2011). "The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium." J Clin Endocrinol Metab 96(1): 210-219.

Breuiller-Fouche, M., M. J. Leroy, et al. (2010). "Differential expression of the enzymatic system controlling synthesis, metabolism, and transport of PGF2 alpha in human fetal membranes." Biol Reprod 83(1): 155-162.

Catalano, R. D., M. R. Wilson, et al. (2011). "Comprehensive expression analysis of prostanoid enzymes and receptors in the human endometrium across the menstrual cycle." Mol Hum Reprod 17(3): 182-192.

Chapdelaine, P., J. Kang, et al. (2006). "Decidualization and maintenance of a functional prostaglandin system in human endometrial cell lines following transformation with SV40 large T antigen." Mol Hum Reprod 12(5): 309-319.

Del Corso, A., M. Cappiello, et al. (2008). "From a dull enzyme to something else: facts and perspectives regarding aldose reductase." Curr Med Chem 15(15): 1452-1461.

Downie, J., N. L. Poyser, et al. (1974). "Levels of prostaglandins in human endometrium during the normal menstrual cycle." J Physiol 236(2): 465-472.

Huang, J. C., D. Y. Liu, et al. (1998). "Interleukin-1 beta induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells." J Clin Endocrinol Metab 83(2): 538-541.

Jabbour, H. N. and K. J. Sales (2004). "Prostaglandin receptor signalling and function in human endometrial pathology." Trends Endocrinol Metab 15(8): 398-404.

Jabbour, H. N., K. J. Sales, et al. (2005). "A positive feedback loop that regulates cyclooxygenase-2 expression and prostaglandin F2alpha synthesis via the F-series-prostanoid receptor and extracellular signal-regulated kinase 1/2 signaling pathway." Endocrinology 146(11): 4657-4664.

Jabbour, H. N., K. J. Sales, et al. (2006). "Prostaglandin receptors are mediators of vascular function in endometrial pathologies." Mol Cell Endocrinol 252(1-2): 191-200.

Jin, Y. and T. M. Penning (2007). "Aldo-keto reductases and bioactivation/detoxication." Annu Rev Pharmacol Toxicol 47: 263-292.

Kabututu, Z., M. Manin, et al. (2009). "Prostaglandin F2alpha synthase activities of aldo-keto reductase 1B1, 1B3 and 1B7." J Biochem 145(2): 161-168.

Kang, J., A. Akoum, et al. (2004). "Independent regulation of prostaglandins and monocyte chemoattractant protein-1 by interleukin-1beta and hCG in human endometrial cells." Hum Reprod 19(11): 2465-2473.

Kang, J., P. Chapdelaine, et al. (2006). "Functional characterization of prostaglandin transporter and terminal prostaglandin synthases during decidualization of human endometrial stromal cells." Hum Reprod 21(3): 592-599.

Kennedy, C. R., Y. Zhang, et al. (1999). "Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor." Nat Med 5(2): 217-220.

Komoto, J., T. Yamada, et al. (2006). "Prostaglandin F2alpha formation from prostaglandin H2 by prostaglandin F synthase (PGFS): crystal structure of PGFS containing bimatoprost." Biochemistry 45(7): 1987-1996.

Krishnaswamy, N., G. Danyod, et al. (2009). "Oxytocin receptor down-regulation is not necessary for reducing oxytocin-induced prostaglandin F(2alpha) accumulation by interferon-tau in a bovine endometrial epithelial cell line." Endocrinology 150(2): 897-905.

Krishnaswamy, N., N. Lacroix-Pepin, et al. (2010). "Epidermal growth factor receptor is an obligatory intermediate for oxytocin-induced cyclooxygenase 2 expression and prostaglandin F2 alpha production in bovine endometrial epithelial cells." Endocrinology 151(3): 1367-1374.

Lacroix-Pepin, N., G. Danyod, et al. (2011). "The multidrug resistance-associated protein 4 (MRP4) appears as a functional carrier of prostaglandins regulated by oxytocin in the bovine endometrium." Endocrinology 152(12): 4993-5004.

Lai, J., H. Jin, et al. (1996). "Prostaglandin F2 alpha induces cardiac myocyte hypertrophy in vitro and cardiac growth in vivo." Am J Physiol 271(6 Pt 2): H2197-2208.

Lim, H., B. C. Paria, et al. (1997). "Multiple female reproductive failures in cyclooxygenase 2-deficient mice." Cell 91(2): 197-208.

Lockwood, C. J. and F. Schatz (1996). "A biological model for the regulation of peri-implantational hemostasis and menstruation." J Soc Gynecol Investig 3(4): 159-165.

Madore, E., N. Harvey, et al. (2003). "An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium." J Biol Chem 278(13): 11205-11212.

Mandal, A. K., Z. Zhang, et al. (2005). "Yin-yang: balancing act of prostaglandins with opposing functions to regulate inflammation." J Immunol 175(10): 6271-6273.

Milne, S. A. and H. N. Jabbour (2003). "Prostaglandin (PG) F(2alpha) receptor expression and signaling in human endometrium: role of PGF(2alpha) in epithelial cell proliferation." J Clin Endocrinol Metab 88(4): 1825-1832.

Nagata, N., Y. Kusakari, et al. (2011). "Catalytic mechanism of the primary human prostaglandin F2alpha synthase, aldo-keto reductase 1B1--prostaglandin D2 synthase activity in the absence of NADP(H)." FEBS J 278(8): 1288-1298.

Narumiya, S. and G. A. FitzGerald (2001). "Genetic and pharmacological analysis of prostanoid receptor function." J Clin Invest 108(1): 25-30.

Nishinaka, T. and C. Yabe-Nishimura (2005). "Transcription factor Nrf2 regulates promoter activity of mouse aldose reductase (AKR1B3) gene." J Pharmacol Sci 97(1): 43-51.

Okazaki, M., T. Matsuyama, et al. (2005). "Induction of epithelial cell apoptosis in the uterus by a mouse uterine ischemia-reperfusion model: possible involvement of tumor necrosis factor-alpha." Biol Reprod 72(5): 1282-1288.

Park, J. Y., M. H. Pillinger, et al. (2006). "Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases." Clin Immunol 119(3): 229-240.

Pelletier, G., V. Luu-The, et al. (1999). "Immunocytochemical localization of type 5 17beta-hydroxysteroid dehydrogenase in human reproductive tissues." J Histochem Cytochem 47(6): 731-738.

Phillips, R. J., H. Al-Zamil, et al. (2011). "Genes for prostaglandin synthesis, transport and inactivation are differentially expressed in human uterine tissues, and the prostaglandin F synthase AKR1B1 is induced in myometrial cells by inflammatory cytokines." Mol Hum Reprod 17(1): 1-13.

Sakamoto, A. and Y. Sugamoto (2011). "Identification of a novel aldose reductase-like gene upregulated in the failing heart of cardiomyopathic hamster." Mol Cell Biochem 353(1-2): 275-281.

Sales, K. J. and H. N. Jabbour (2003). "Cyclooxygenase enzymes and prostaglandins in pathology of the endometrium." Reproduction 126(5): 559-567.

Sales, K. J. and H. N. Jabbour (2003). "Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology." Prostaglandins Other Lipid Mediat 71(3-4): 97-117.

Sales, K. J., S. A. Milne, et al. (2004). "Expression, localization, and signaling of prostaglandin F2 alpha receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the epidermal growth factor receptor and mitogen-activated protein kinase signaling pathways." J Clin Endocrinol Metab 89(2): 986-993.

Smith, S. K. and R. W. Kelly (1988). "The release of PGF2 alpha and PGE2 from separated cells of human endometrium and decidua." Prostaglandins Leukot Essent Fatty Acids 33(2): 91-96.

Smith, W. L., R. M. Garavito, et al. (1996). "Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2." J Biol Chem 271(52): 33157-33160.

Smith, W. L., Y. Urade, et al. (2011). "Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis." Chem Rev 111(10): 5821-5865.

Srivastava, S. K., K. V. Ramana, et al. (2005). "Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options." Endocr Rev 26(3): 380-392.

Suzuki-Yamamoto, T., M. Nishizawa, et al. (1999). "cDNA cloning, expression and characterization of human prostaglandin F synthase." FEBS Lett 462(3): 335-340.

Tschugguel, W., C. Schneeberger, et al. (1999). "Elevation of inducible nitric oxide synthase activity in human endometrium during menstruation." Biol Reprod 60(2): 297-304.

Watanabe, K. (2002). "Prostaglandin F synthase." Prostaglandins Other Lipid Mediat 68-69: 401-407.

Watanabe, K. (2011). "Recent reports about enzymes related to the synthesis of prostaglandin (PG) F(2) (PGF(2alpha) and 9alpha, 11beta-PGF(2))." J Biochem 150(6): 593-596.

Wu, K. K. and J. Y. Liou (2005). "Cellular and molecular biology of prostacyclin synthase." Biochem Biophys Res Commun 338(1): 45-52.

Yuhki, K., H. Kashiwagi, et al. "Roles of prostanoids in the pathogenesis of cardiovascular diseases." Int Angiol 29(2 Suppl): 19-27.

Zhou, Y., N. Shaw, et al. "Structure-function analysis of human l-prostaglandin D synthase bound with fatty acid molecules." Faseb J 24(12): 4668-4677.

FIGURES

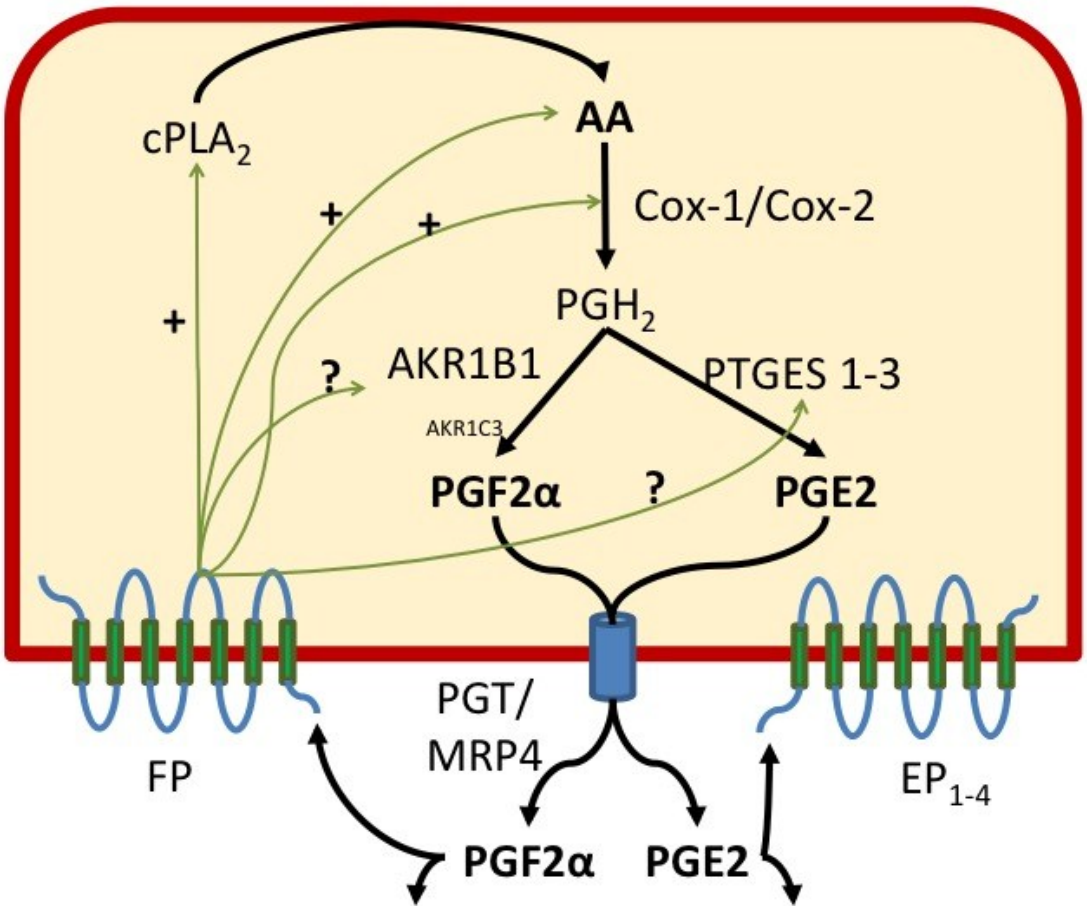


Figure 22 - Article 4: Figure 1 Prostaglandin F2α and E2 biosynthetic pathways.

In order to generate PGF2α, AKR1B1 must team up with other constitutive and inducible enzymes involved in the production of PGH2, the common precursor of all PGs. Among those, we have found that cPLA2 liberating AA from membrane phospholipids, COX-2, AKR1B1 and mPGES-1 are inducible by the cytokine IL1B. AKR1B1 apparently works with both COX-1 and COX-2 producing PGH2 to generate PGF2α whereas mPGES-1 preferentially associates with COX-2 to generate PGE2. Both epithelial and stromal cells exhibit PGF2α responsiveness and stimulation or inhibition of the FP receptor alters the production of both PGE2 and PGF2α through action at several steps of the PG biosynthesis cascade. We also present in this figure the PG transporters PGT also known as solute carrier SLCOA1 and MRP4 the multidrug resistance protein 4 or ABCC4.

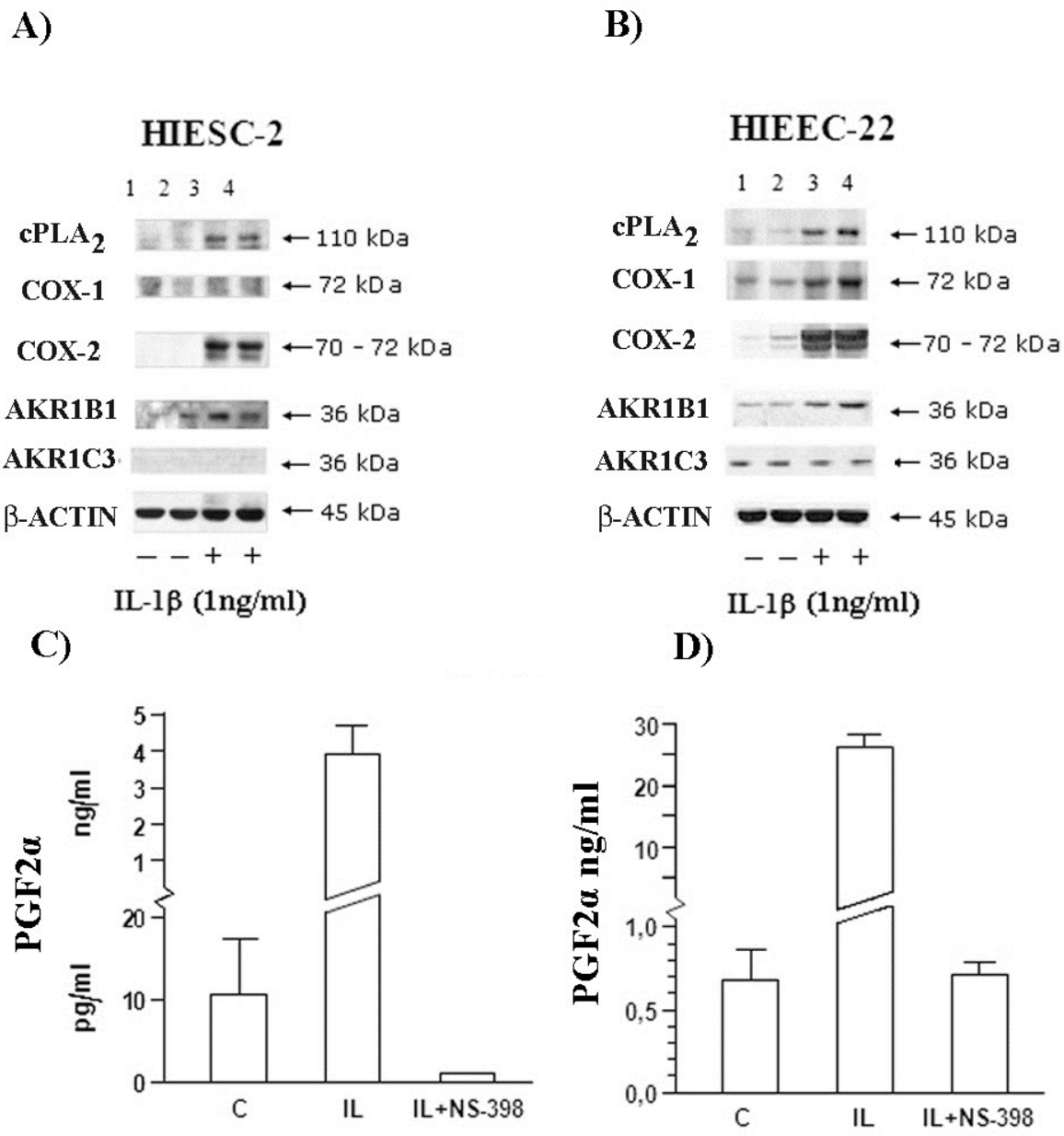


Figure 23 - Article 4: Figure 2 Influence of IL-1β on the main biosynthetic enzymes associated with PGF2α production in human endometrial stromal and epithelial cells.

Human endometrial stromal (HIESC-2) and epithelial (HIEEC-2) cells were grown to confluence and treated with IL1B for 24 h in presence and absence of NS-398 to block COX activity. PGs biosynthetic enzymes were analyzed by Western blot (HIESC-2, A; HIEEC-22 B) and associated with PGF2α production HIESC-2 (C) and HIEEC-22 (D).

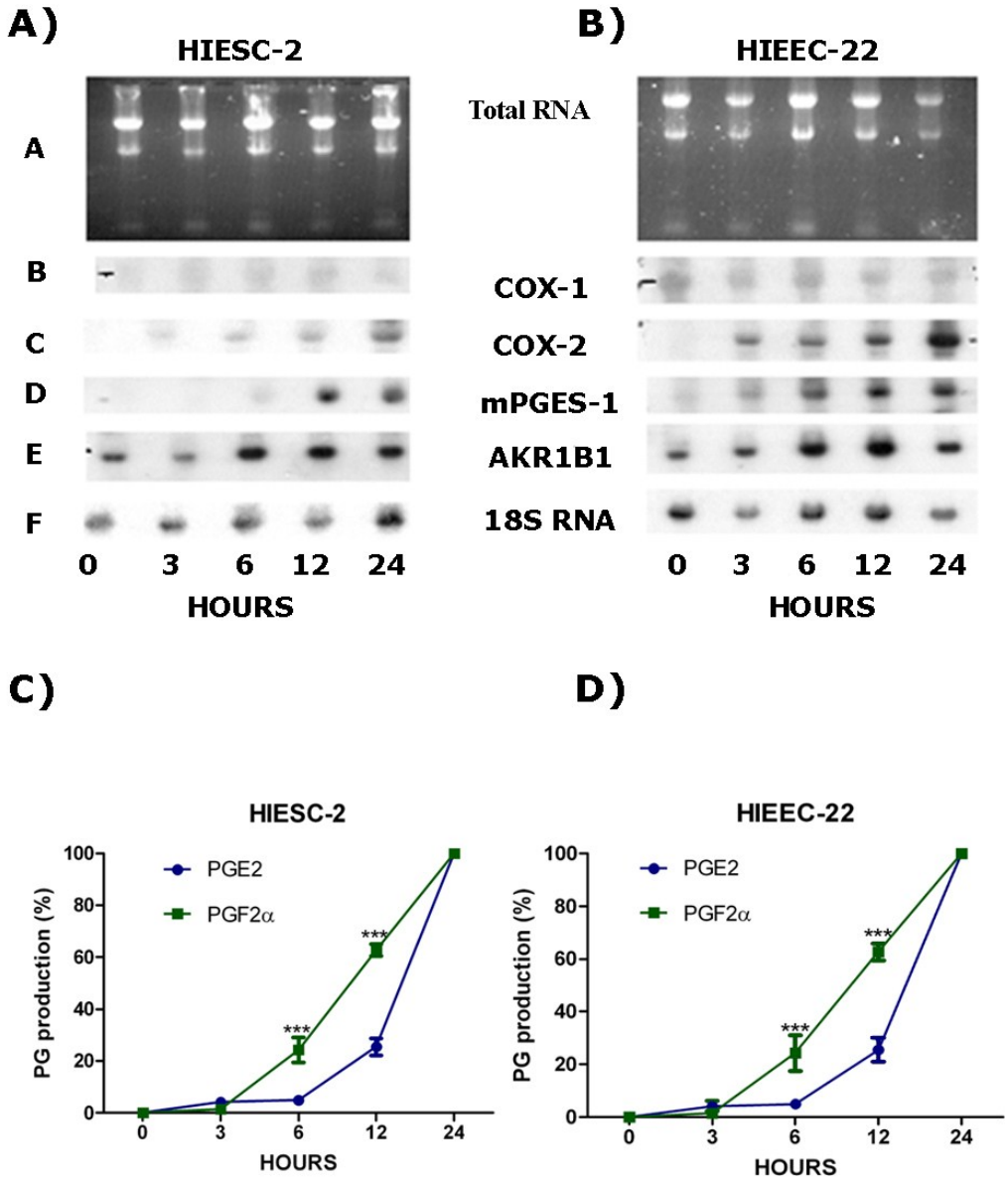


Figure 24 - Article 4: Figure 3 Time course of gene expression and PGs production in human endometrial stromal and epithelial cells following stimulation with IL-1β.

Human endometrial stromal (HIESC-2) and epithelial (HIEEC-2) cells were grown to confluence and treated with IL1B for different periods up to 24 h. Northern Blots analysis was used for quantification of AKR1B1, COX-1/-2, mPGES-1 mRNA expression. The production of PGs was evaluated as a function of time in stromal (C) and epithelial (D) endometrial cells where stimulation of PGF2α precedes that of PGE2 ($P<0.05$).

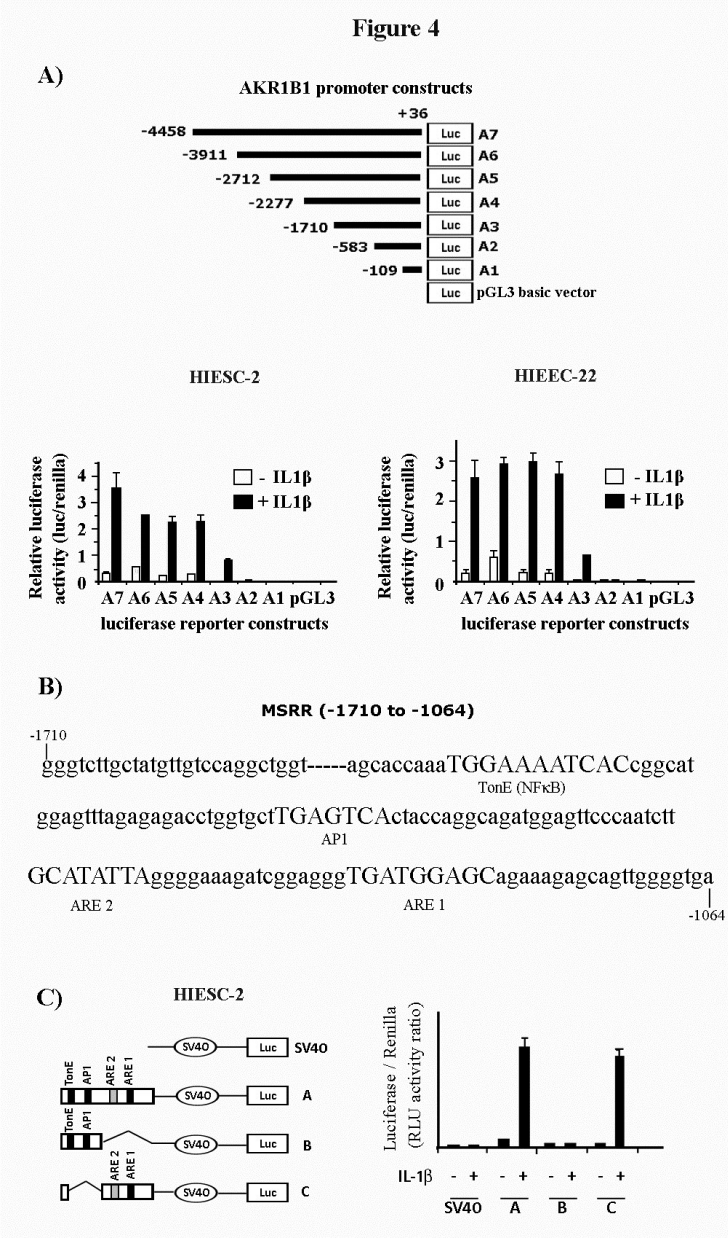


Figure 25 - Article 4: Figure 4 Analysis of AKR1B1 promoter activity involved in IL-1β response in human endometrial cells.

(A) Schematic representation of different AKR1B1 promoter constructs and corresponding activity following treatment of transfected stromal and epithelial cells treated with IL-1β. (B) Nucleotide sequence of the AKR1B1 promoter MSRR region (-1710 to -1064) showing the organization of the important cis-elements TonE (NFκB), AP1 and anti-oxidant response elements 1 and 2 (ARE1 and ARE2). (C) Relative activity of MSRR cis-elements of the AKR1B1 promoter following stimulation with IL-1β of transfected stromal endometrial cells.

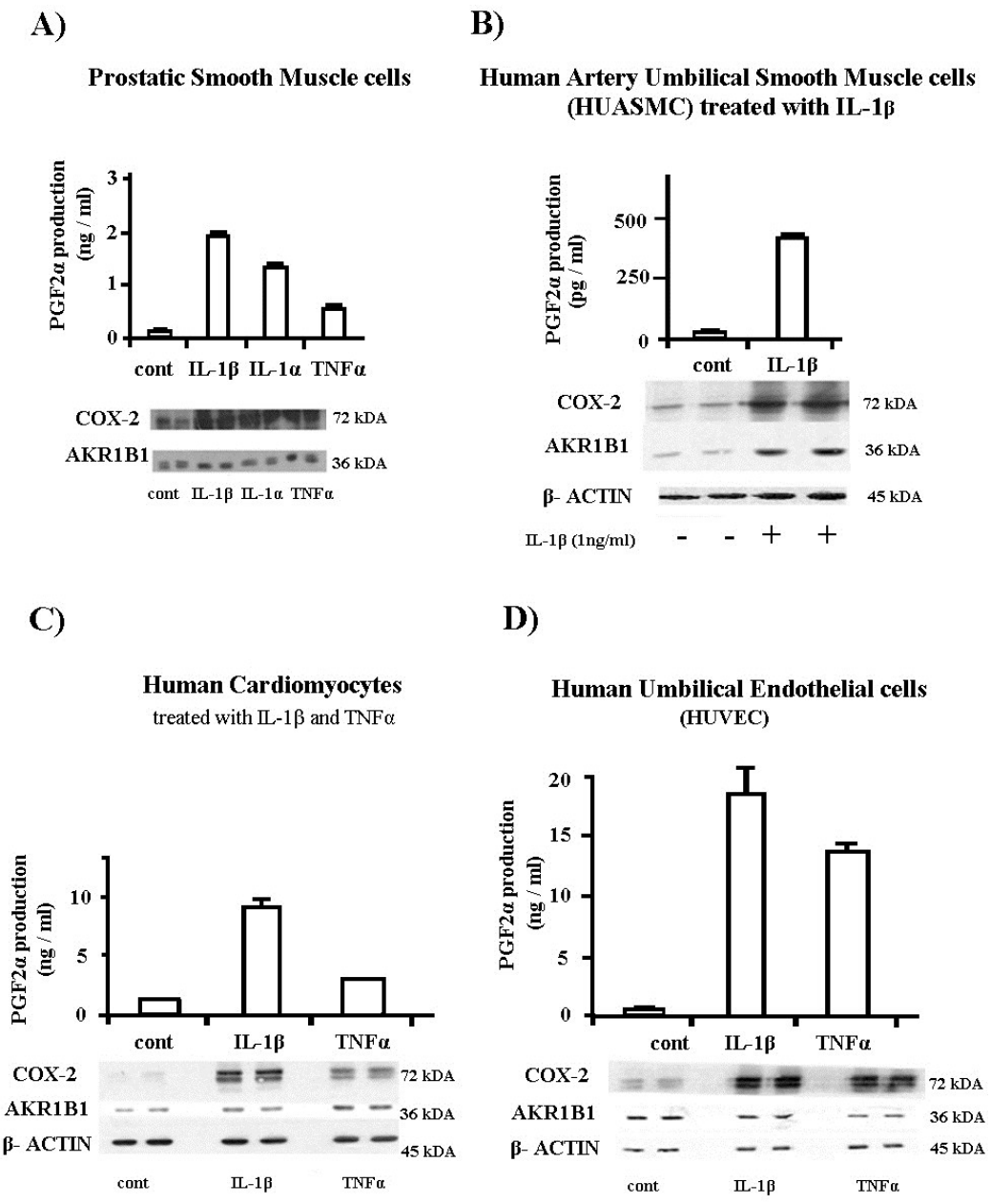


Figure 26 - Article 4: Figure 5 Association between induction of AKR1B1 and PGF2α production following cytokine stimulations in cells from different human tissues.

Cells from different human tissues were grown to confluence and treated with IL-1β, IL-1α or TNFα at 1 ng/ml for 24 h. The culture medium was recovered for assy of PGF2α by EIA and protein from attached cells recovered for Western blot analysis of COX-2 and AKR1B1. (A) prostatic smooth muscle cells, (B) human artery umbilical smooth muscle cells, (C) human cardiomyocytes (D) Human Umbilical Endothelial cells (HUVEC).

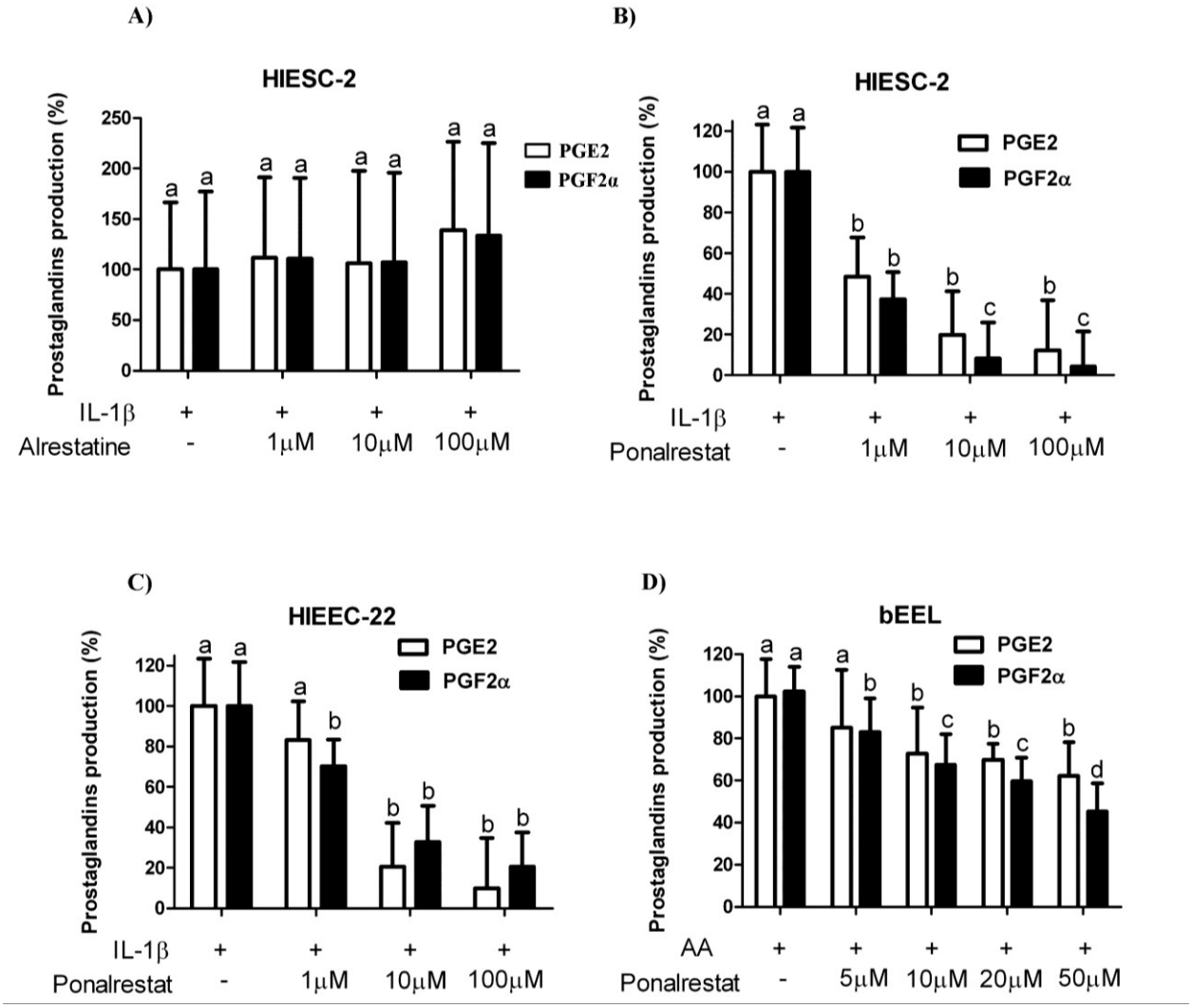


Figure 27 - Article 4: Figure 6 Influence of different aldose reductase inhibitors on PGF2α and PGE2 production in human and bovine endometrial cells.

Human endometrial stromal (HIESC-2, A-B) and human (C) or bovine epithelial (bEEL, D) cells were grown to confluence and treated with IL-1β (1 ng/ml) for 24 h in presence and absence of increasing concentrations of indicated aldose reductase inhibitors. The culture medium was recovered and PGE2 and PGF2α were assayed by EIA. A: alrestatin, B, C, D: Ponalrestat. Results are the mean ± SEM of 3 experiments run in triplicate.

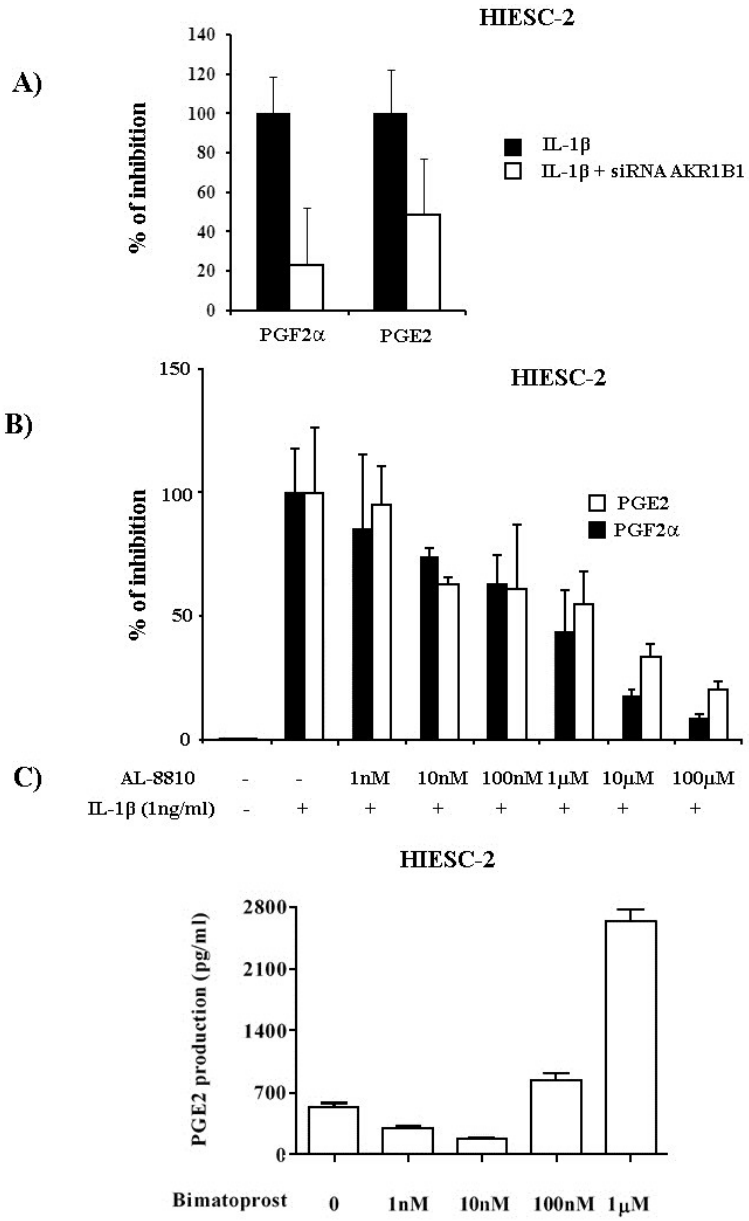


Figure 28 - Article 4: Figure 7 Influence of AKR1B1 and FP receptor inhibition or stimulation on PG production in human endometrial stromal cells.

A) Normal or AKR1B1 siRNA transfected human endometrial stromal (HIESC-2) cells were grown to confluence and treated with IL-1β for 24 h and PGE2 and PGF2α were measured in the culture medium B: HIESC-2 cells were grown to confluence and treated with IL-1β for 24 h in presence and absence of increasing concentrations of the FP receptor antagonist AL-8810 and PGE2 and PGF2α were measured in the culture medium. C: HIESC-2 cells were grown to confluence and treated for 24 h with increasing doses of the PGF2α analog and FP receptor agonist bimatoprost and PGE2 was measured in the culture medium.

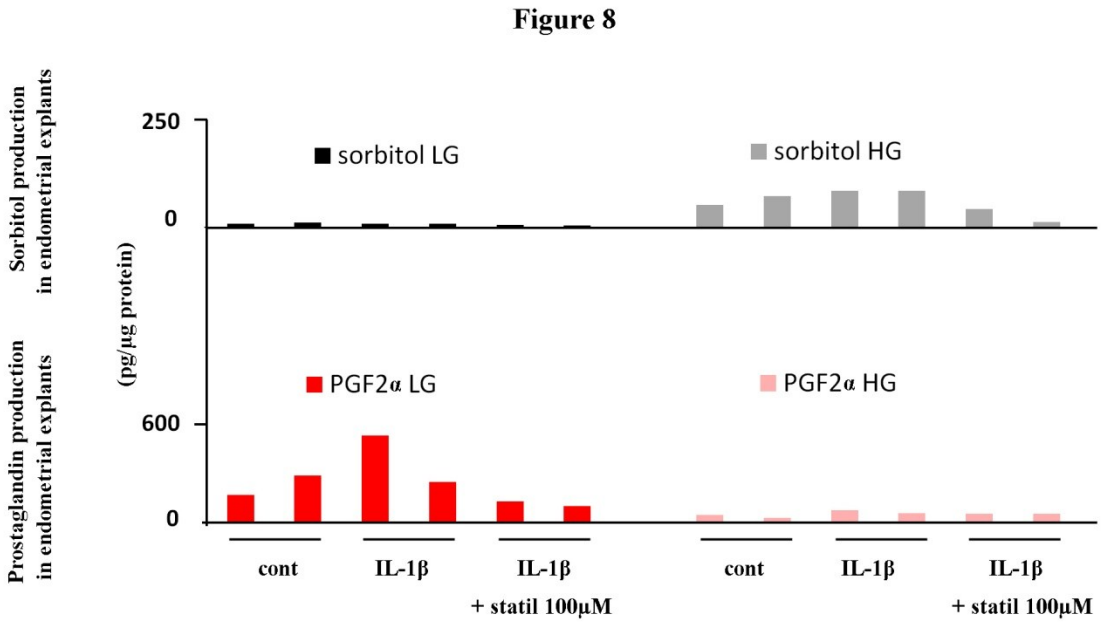


Figure 29 - Article 4: Figure 8 Influence of glucose concentration on the relative production of sorbitol and PGF2α in fresh explants from human endometrium.

Endometrial explants were prepared from fresh endometrial biopsies, cultured for 24 h and stimulated with IL-1β (1ng/ml) in presence or absence of Statil (Ponalrestat, 100µM) under normal (LG) 5mg/ml or High (HG) 25mg/ml glucose concentrations. PGF2α production was measured by EIA. Results from two different experiments are illustrated.

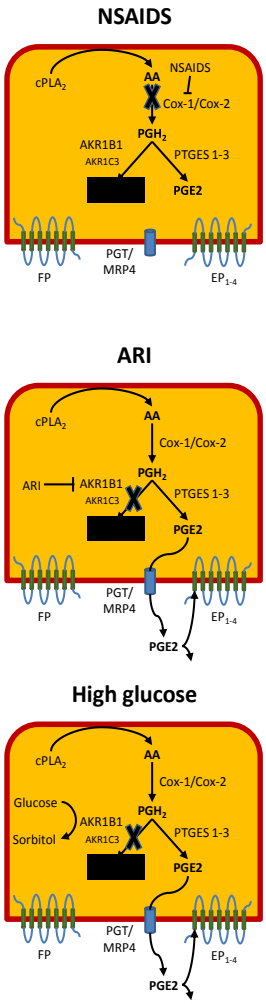


Figure 9 - Article 4: Figure 9 Regulation of PGs of the relative PGE2 and PGF2α release in endometrial cells.

Following activation of endometrial cells with the cytokine IL-1β, several enzymes leading to the production of PGF2α and PGE2 exhibit increased expression and/or activity. Treatment of cells with NSAIDs will inhibit the generation of PGH2 at the level of COX-1 and COX-2, thus blocking the release of both PGF2α and PGE2 without an alteration of the relative production of the two PGs. Inhibition of AKR1B1 activity with ARIs will inhibit preferentially PGF2α release thus creating an imbalance in favour of PGE2. However, decreased stimulation of the FP receptor should down-regulate the production of PGE2. In presence of high glucose concentration, the stimulation of AKR1B1 may be exacerbated, but glucose should inhibit competitively PGF2α release in which case reduced FP stimulation should also reduce PGE2 production. However, when glucose concentration falls back to normal, high AKR1B1 levels would favour PGF2α release and ischemic responses until PGE2 is able to compensate through EP2 and bring back a silent condition where the balance between PGF2α and PGE2 is re-established

Chapitre 6: Generation of human endometrial knockout cell lines with the CRISPR-Cas9 system confirms the prostaglandin F2α synthase activity of AKR1B1.

Nicolas Lacroix Pépin[1], Pierre Chapdelaine[1, 2], Yoima Rodriguez[1], Jacques-P. Tremblay[2],

Michel A Fortier[1]

Ce chapitre est présenté sous forme d'article et publié dans la revue :
Molecular Human reproduction. 2014

[1]Département d'Obstétrique et Gynécologie Faculté de Médecine Université Laval et Axe de reproduction, santé de la mère et de l'enfant du Centre de Recherche du CHU de Québec (CHUL), and [2]Département de Médecine Moléculaire. Faculté de Médecine Université Laval et Axe Neuroscience du Centre de Recherche du CHU de Québec (CHUL), Québec, QC, Canada GIV 4G2.

RÉSUMÉ

Les prostaglandines sont d'importants régulateurs de la function reproductive chez la femme. Les principales prostaglandines produites dans l'endomètre sont la PGE2 et la PGF2α. Relativement peu est connu sur les voies de biosynthèse menant à la formation du PGF2α. Nous avons décrit le role d'AKR1B1 dans l'accroissement de la production du PGF2α par les cellules endométriales après stimulation par l'interleukine-1β (IL-1β). Cependant, d'autres PGF synthases sont exprimées au même moment dans les cellules endométriales. Une preuve definitive du rôle d'AKR1B1 requièrerait l'interruption du gene. Malheureusement, ce dernier n'a pas d'équivalent direct chez la souris. Récemment, une technologie efficace d'édition du genome utilisant le système de la DNase Cas9 guidée par ARN et les courtes répétitions palindromiques interespacées par grappes régulières (CRISPR) a été développée. Nous avons adapté cette approche pour interrompre l'expression du gène *AKR1B1* dans notre lignée de cellules endométriales humaines. Un clone (16-2) d'origine stromale généré par le système CRISPR/Cas9 exhibe une perte complète de la protéine d'AKR1B1 et de l'expression de son messager, tandis que d'autres clones ont présenté des éditions partielles. Le présent rapport se concentre sur la caractérisation du clone 16-2 exhibant une délétion de 68 et 2 nucleotides respectivement sur chaque allèle. Les cellules de ce clone ont perdu leur habilité à produire du PGF2α mais ont aussi maintenu leur phénotype original de cellules stomales (HIESC-2) incluant la capacité de décidualiser en présence de progestérone (MPA) et de 8-bromo-AMPc. Les cellules ont aussi maintenu leur habilité à produire du PGE2 en réponse à l'IL-1β. En résumé, nous avons démontré que le nouveau système d'édition du génome CRISPR-Cas9 peut être utilisée sur des lignées cellulaires humaines pour générer des modèles de lignées stables ayant un gène délété. Nos résultats suggèrent que l'édition du génome de lignées cellulaires peut être utilisée et complémenter les modèles murins afin de valider la fonction d'un gène dans des tissus et cellules différenciés. Nos resultats confirment aussi qu'AKR1B1 est impliquée dans la synthèse de la PGF2α.

ABSTRACT

Prostaglandins are important regulators of female reproductive function. The primary PGs produced in the endometrium are PGE2 and PGF2α. Relatively little is known about the biosynthetic pathways leading to the formation of PGF2α. We have described the role of AKR1B1 in increased PGF2α production by human endometrial cells following stimulation with Interleukin-1β (IL-1β). However, alternate PGF synthases are expressed concurrently in endometrial cells. A definite proof of the role of AKR1B1 would require gene knockout; unfortunately, this gene has no direct equivalent in the mouse. Recently, an efficient genome editing technology using RNA-guided DNase Cas9 and the clustered regularly interspaced short palindromic repeats (CRISPR) system has been developed. We have adapted this approach to knockout AKR1B1 gene expression in human endometrial cell lines. One clone (16-2) of stromal origin generated by the CRISPR/Cas9 system exhibited a complete loss of AKR1B1 protein and mRNA expression, whereas other clones presented with partial edition. The present report focuses on the characterization of clone 16-2 exhibiting deletion of 68 and 2 nucleotides respectively on each of the alleles. Cells from this clone lost their ability to produce PGF2α but maintained their original stromal cell (HIESC-2) phenotype including the capacity to decidualize in presence of progesterone (MPA) and 8-bromo-cAMP. Knockout cells also maintained their ability to increase PGE2 production in response to IL-1β. In summary, we demonstrate that the new genome editing CRISPR-Cas9 system can be used in human cells to generate stable knockout cell line models. Our results suggest that genome editing of human cell lines can be used to complement mouse KO models to validate the function of genes in differentiated tissues and cells. Our results also confirm that AKR1B1 is involved in the synthesis of PGF2α.

INTRODUCTION

Prostaglandins (PGs) are synthesized by virtually all nucleated cells of the body and represent a family of lipid mediators acting locally to maintain homeostasis through complementary and sometimes opposite actions (Poyser 1995; Lim, Paria et al. 1997; Thatcher, Guzeloglu et al. 2001; Jabbour and Sales 2004; Spencer, Johnson et al. 2007; Smith, Urade et al. 2011). PGs are particularly important for normal female reproductive function and also contribute to pathological conditions in that system (Jabbour and Sales 2004). Prostaglandin F2α (PGF2α) contribute to parturition and initiation of menstruation. Painful menstruation is attributed to an ischemic response to abnormally high levels of PGF2α. The first step in PG formation is the conversion of arachidonic acid (AA) liberated from membrane phospholipids into prostaglandin G2 (PGG2) and then prostaglandin H2 (PGH2) by one of two prostaglandin G/H synthases (PTGS-1 or -2) better known as cyclooxygenases (COX-1 or -2). After PGH2 formation, terminal synthases generate the bioactive prostaglandins (Smith, Urade et al. 2011) (Fig. 1). Specific terminal synthases required for enzymatic production of PGs were identified for PGE2 (Tanikawa, Ohmiya et al. 2002), thromboxane A2 (TxA2) (Tanabe and Ullrich 1995), prostacyclin (PGI2) (Ullrich, Castle et al. 1981), prostaglandin D2 (PGD2) (Christ-Hazelhof and Nugteren 1979) and more recently for PGF2α (Bresson, Boucher-Kovalik et al. 2011). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprophen and naproxen aim at inhibiting PG biosynthesis at the level of PGHSs (Fig. 1).

While PGF2α has not been studied as extensively as other PGs, it nevertheless plays important roles in the regulation of ocular pressure, renal absorption, adipocyte differentiation as well as cardio-vascular and female reproductive functions (Bresson, Lacroix-Pepin et al. 2012). The immortalization of normal human endometrial cell lines (Chapdelaine, Kang et al. 2006) in our laboratory allowed us to study PG biosynthesis at the cellular and molecular levels and to highlight a new function of AKR1B1 as a potent PGF2α synthase (Bresson, Lacroix-Pepin et al. 2012). Indeed, we have observed that increased production of PGF2α in response to IL-1β was associated with up-regulation of AKR1B1 and COX-2 proteins (Bresson, Lacroix-Pepin et al. 2012). Most prostaglandin F2α synthases (PGFS) identified to date are Aldo-Keto Reductases (AKRs). We have demonstrated that in addition to AKR1C3, AKR1B1 was able to produce PGF2α in the endometrium (Bresson, Lacroix-Pepin et al. 2012) and AKR1A1 may also play the same function (Lacroix Pepin, Chapdelaine et al. 2013). Since several proteins with putative PGF synthase (PGFS) activity are expressed concurrently in the same cells at the same time, identification of the primary PGF synthase involved under specific conditions would require complete and selective inhibition of individual synthases. Pharmacologic inhibitors do not exhibit the required selective action and siRNAs do not lead to complete inhibition of expression (Bresson, Lacroix-Pepin et al. 2012). The mouse does not constitute an appropriate model for AKR1B1 because its activities are covered by two parent, but not identical enzymes; AKR1B3 and AKR1B7

(Kabututu, Manin et al. 2009; Pastel, Pointud et al. 2012). In spite of that, null mutations for each of AKR1B3 and AKR1B7 were generated and lead to interesting observations regarding the physiological role of AKR1Bs (Lo, Cheung et al. 2007; Kabututu, Manin et al. 2009). Unfortunately only little consideration was given to PGFS activity for AKR1B7 and none for AKR1B3. Indeed loss of AKR1B7 was associated with a 50% decrease in PGF2α levels in adipose tissue and cells while the remaining 50% could be attributed to AKR1B3 (Volat, Pointud et al. 2012). Aldose-reductase-like enzymes are highly related often co-expressed isozymes, making functional analysis of one isoform or the other a challenging task.

In order to provide a definite proof of AKR1B1 contribution to uterine prostaglandin production, genome editing of an endometrial cell line appeared as an elegant solution. Indeed it would provide a functional model comparable to a conditional endometrial knockout in the human. The recently described Clustered Regularly Interspaced Short Palindromic Repeats combined with CAS9 (CRISPRs-Cas9) system appeared as a very potent approach to reach this goal.

Recent development in genome editing brought new technologies such as Zinc Finger nucleases (ZFNases), transcription activator-like effector nucleases (TALENs) and more recently the CRISPR/Cas9 system for the generation of null mutation of targeted genes in somatic cell lines (Carroll 2012). In the present report, we have used the CRISPR/Cas9 system (Hwang, Fu et al. 2013; Mali, Yang et al. 2013) to generate null AKR1B1 human endometrial cell lines previously developed in our laboratory (Chapdelaine, Kang et al. 2006). We present also an elaborate characterization of one resulting cell line lacking the expression of AKR1B1 protein.

MATERIALS AND METHODS

Materials

Reagents were purchased from the following suppliers: RPMI without phenol red, superscript III reverse transcriptase, one kB DNA ladder, dithiothreitol, 5x first strand buffer and TRIzol, were from Life Technologies Inc. (Burlington, ON, Canada). Random primer-pd(N)6, deoxy-NTPs, RNA Guard, rTaq DNA polymerase, and PCR 10x buffer from GE healthcare Canada (Baie d'Urfé, QC, Canada). Plasmid pDrive (TA cloning kit) and DNA purification kits were from Qiagen (Mississauga, ON, Canada). All oligonucleotides were chemically synthesized and purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). Arachidonic acid (AA) was from Cayman Chemical (Ann Arbor, MI). Interleukin-1β (IL-1β) was

purchased from Research and diagnostic systems (Minneapolis, MN, USA). Design and construction of single guide RNA (sgRNA) targeting the coding sequence of AKR1B1 exon 1

The single guide RNA (sgRNA) production was based on the protocol described by Mali (Mali, Yang et al. 2013). Since NGG is the protospacer- adjacent motif (PAM) required in the target site of the sgRNA we first selected four sgRNA target sites (Fig.2C) by seeking sequences corresponding to the 5'N19NGG genomic site around exon 1 of the AKR1B1 gene (ID: 231). The gBlock® template purchased from IDT (Integrated DNA Technologies Inc. IA, US) was a 455 pb fragment containing the U6 promoter, n19 target site, sgRNA scaffold and a termination signal necessary for sgRNA expression. Our methodology uses pCR3.1 vector (Invitrogen) in which we have inserted a gBlock and was prepared as described by Mali (Mali, Yang et al. 2013).This gBlock was amplified by PCR making a PCR fragment of ~455 pb with the following primers aagcaagaagcggccgcgcgcTGTACAAAAAAGCAGGCTTTAAAG sens (BssHII) aagcaagaacccgggcccTAATGCCAACTTTGTACAAGAAAGC rev (Apa1) After amplification, the gBlock PCR product was digested with BssHII and APA1 and cloned in the pCR3.1 vector digested with the same restriction enzymes. The resulting recombinant vector containing the gBlock could then serve as template to introduce different PCR fragments (containing variable targets), which will be digested by NdeI/XbaI and then directly cloned in the recombinant vector pCR3.1. This new process allowed quick generation of new gRNA by simple replacement of a 173nt portion of the original gBlock containing alternate 19 nt targets as described below.

Generation of custom gRNA targeting AKR1B1

The gBlock inside of the recombinant pCR3.1 contains typical restriction enzyme sites where we were able to introduce different targets coming from DNA fragments obtained by PCR amplification. We only needed to synthesize one oligonucleotide containing the 19 nucleotides (N) corresponding to the different targets. For each one, we designed a single oligonucleotide (59 nts) containing 19 selected nucleotides (N, figure 2) presenting the following form: 5'GTGGAAAGGACGAAACACCGNNNNNNNNNNNNNNNNNNNGTTTTAGA GCTAGAAATAGC-3' (primer T) complementary to the following primers: ACTTGAAAGTATTTCGATTTCTTGGC TTTATATATCTTGGTGGAAAGGACGAAACACCG (Fw const) and CTTTTTCAAGTTGATAACGGACTAGCCTTATTTT AACTTGCTATTTCTAGCTCTAAAAC (Rev const). The "N"s represents the 19nts genomic target without PAM (NGG).

The specific target sgRNAs were produced by double PCR amplification with Phusion DNA polymerase (New England Biolabs) in Phusion HF buffer as follows: The three oligonucleotides were mixed together in the following proportions for the first short PCR (50 µL): 20 ng (Fw const), 20 ng (Rev const) and 5 ng (T) and a short PCR program was applied: 98°C 1 min (1 cycle), 98°C 5 sec, 55°C 10 sec, 72°C 10 sec (5 cycles), 98°C 5 sec, 60°C 10 sec, 72°C 10 sec (15 cycles), and stand at 4°C. A second PCR amplification

was done from an aliquot (2 μL) of the first short PCR with the following primers bearing restriction enzyme sites at their ends: GTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTC (53 nts) (Fw const Nde1) and GAAAGCTGGGTCTAGAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGG (59 nts) (Rev const Xba I). The second PCR reaction (100 μL) proceeded as follows: 200 ng of (Fw const, Nde1), 200 ng of (Rev const, XbaI), 2 μL aliquot of the first short PCR round, all in the HF buffer with Phusion enzyme (New England Biolabs) and the program used was: 98°C 1min (1X cycle), 98°C 5 sec, 55°C 10 sec, 72°C 10 sec (5 cycles), 98°C 5 sec, 60°C 10 sec, 72°C 10 sec (30 cycles), 72°C 2 min and stand at 4°C. At the end of the second PCR, purification of the PCR product (173 pb) was done with QIAquick PCR purification kit (Qiagen) followed by digestion with NdeI/XbaI done in NEB2 buffer for 1 hour at 37°C followed by purification with QIAquick PCR purification kit. After purification, the digested PCR product was ligated with Quick DNA ligase (New England Biolabs) with a pCR3.1 vector (Invitrogen) containing a gBlock® module (gRNA) (see Fig. 2C) digested also with NdeI/XbaI. The inserted gBlock® in pCR3.1 (see Fig. 2C) and double digestion with NdeI/XbaI allowed to insert the desired target by modification of the T primer by simple substitution of the 19Ns corresponding to the genomic target (see Fig. 2B,C).

Co-transfection of endometrial stromal cells with sgRNA and Cas9 plasmids

Human endometrial stromal cells (HIESC-2) (Chapdelaine, Kang et al. 2006) were cultured in 6-well plates and maintained in RPMI 1640 supplemented with 10% Fetal bovine serum (FBS) and penicillin-streptomycin 1X (complete medium) in a 37°C humidified incubator with 5% CO2. When the cells reached 80% confluence the complete RPMI medium of each well (3 mL) was replaced with 2 mL of fresh RPMI medium without antibiotic. Co-transfection of the cells was done as follows: for each well, 4 μg of plasmid expressing the different sgRNAs (identified as sgRNA target #14, 15, 16 and 17), (see Fig. 2B), and 2 μg of the Cas9 plasmid (Addgene, Cambridge, MA, cat no 48668) diluted in the Opti-MEM medium were mixed with 10 μL of Lipofectamine diluted in Opti-MEM medium. The complexes were added to each well and incubated for 18 hours at 37°C before medium replacement with complete RPMI. Three days after transfection, cells were harvested and genomic DNA was analyzed for the presence of mutation using the Surveyor® enzyme as described below. At the same time, since both plasmids carry the neomycin resistance gene, transfected cells were deemed overrepresented among resistant clones. A sample of the cells were cultured in presence of neomycin (400 μg/ml) for 2 days followed by two additional days in complete medium without neomycin after which cells were trypsinized, diluted and plated in 6-well plates. After 10 days, individual colonies were isolated with O-rings, harvested, seeded in 24-well plates and grown for 2 weeks until confluence when they were transferred to T25 flasks. When the latter reached confluence, cells were trypsinized and an aliquot was kept for protein analysis by Western blot. The remaining cells were frozen or expanded in T75 Flasks for additional analyses.

Genomic DNA extraction from cultured endometrial cells

Genomic DNA extraction was performed as follows: cells from individual wells were trypsinized (300 μL 1X trypsin EDTA) diluted with complete culture medium (1 mL), and recovered by centrifugation at 3000 RPM. The cell pellet was washed three times with 1 mL PBS 1X. A 25 μL lysis solution composed of EDTA 0.5M/ 10% Sarkosyl (11:1) was added to the cell pellet, supplemented with 2 μL proteinase K (10 mg/ml) and the mixture was heated at 50°C for 5 minutes. 150 μL TE 1X (Tris-HCL 10 mM pH8.0, EDTA 1 mM) and 2 μL RNase A (1 μg/μL) were then added to the lysed cells and heated at 50°C for 10 minutes. DNA was isolated and purified by serial extraction with phenol/chloroform/isoamyl alcohol (25:24:1) followed by chloroform-isoamylalcool (24:1) and after, 2 μL NaCl 5 M was added to the resulting aqueous phase, and genomic DNA was precipitated with ice cold ethanol (2 volumes) recovered by centrifugation and dried rapidly by lyophylisation. The resulting genomic DNA was dissolved in sterile water and stored at -20°C until quantification was done.

Surveyor nuclease assay for confirmation of genome modification

Genomic DNA (100 to 200 ng) of endometrial cells was extracted 72 hrs after transfection as described above. The genomic regions flanking the sgRNA target sites for exon 1 of AKR1B1 gene (Figure 2A) were PCR amplified with a forward primer (pos) 5'-CACCGGTCTGTGAAGCCCACTTTC-3' and a reverse primer (pos) 5'-GCATCAAACTGGGGTTGGACAAC-3' using Phusion® DNA Polymerase (New England Biolabs) in the presence of 3% DMSO: initial denaturation at 98°C (10 sec) for 1 cycle, denaturation at 98°C (10 sec), annealing at 58°C (20 sec) and extension at 72°C (30 sec) for 35X cycles, final extension 72°C (5 min). At the end of the PCR amplication, the products were purified using QiaQuick Spin Column (QIAGEN). A total of 400-600 ng (14 μL) of the purified PCR products were mixed with 4 μL of 5X Phusion HF Buffer (NEB) and subjected to reannealing to generate heteroduplexes: 95°C for 5 min; 95°C to 85°C ramping at – 2°C/s; 85°C to 25°C at – 0.25°C/s; and 25°C hold for 1 min. and finally hold at 4°C. After reannealing, products were treated with Surveyor nuclease and Surveyor enhancer S (Transgenomics inc. Omaha, NE, USA). All Surveyor analysis were done on 2% agarose gels containing RedSafe™ Nucleic Acid staining solution (Froggobio inc., Toronto, ON, Canada).

Sequencing analysis of AKR1B1 gene and transcript to detect insertion or deletion (INDEL)

Genomic DNA corresponding to exon 1 of AKR1B1 of each clone was amplified by PCR using Phusion® DNA polymerase in the presence of DMSO as described above but in the final extension step TAQ DNA polymerase (NEB) was added (1 μL) for an extension of 10 minutes at 68°C. The PCR products were purified using QIAquick gel extraction kit (Qiagen) from agarose gels and then cloned in the pDrive vector (TA

cloning vector, Qiagen) using QIAGEN PCR cloning kit (Qiagen). The ligated PCR products were transformed with DH5α and the next day bacteria colonies were picked and seeded in LB medium and plasmids containing inserts were analyzed by sequencing. For mRNA analysis, total RNA of the different clones was extracted from T-75 flask with 1 mL TRIzol® Reagent (Life Technologies). The first strand of cDNA was synthesized at 42°C (50 min) from 2.5 μg of RNA using SuperScript® III Reverse Transcriptase (RT, Life Technologies) with Random Hexamers (250 ng) (Life Technologies). Usually, aliquots (2 μL) of the first-strand cDNA were amplified with the following primers corresponding to exons 1, 2 and 3 of AKR1B1 mRNA: 20 pmoles of each primer 5'-AGCCATGGCAAGCCGTCTC-3' (forward) and 5'-GCACCACAGCTTGCTGAC-3' (reverse) were used in the PCR reaction using TAQ DNA polymerase (NEB): initial denaturation at 95°C (1 min) for 1X cycle, denaturation at 95°C, annealing at 58°C (30 sec), extension at 68°C (30 sec) for 35X cycles and final extension at 68°C (10 min). The final PCR product (~220 pb) corresponding to the AKR1B1 mRNA transcript was gel purified, cloned in pDrive vector and processed for cDNA sequencing.

Detection of transcripts for alternate PGF synthases by RT-PCR

The expression of alternate enzymes potentially contributing to the generation of PGF2α was investigated by RT-PCR analysis of candidate genes. Specific primers for each candidate gene were those used during our previous collaboration with Phillips et al., 2010. Briefly: for CBR1 (Carbonyl Reductase, NM_001757), forward 5'- CCTGGACGTGCTGGTCAACA-3' and reverse 5'-ACGTTCACCACTCTCCCTTG-3' for a PCR product size of ~164 pb; for HPGD (hydroxyprostaglandin dehydrogenase 15-(NAD), NM_000860) forward 5'-CTGCACCATGCACGTGAACG-3' and reverse 5'-AAGTGTCTCTCAGTTGTTGCTG-3 for a PCR product size of 229 pb'; for AKR1C3 (NM_003739), forward 5'-CAGACAAGTGA CAGGGAATGG-3' and reverse 5'-CCTCACCTGGCTTTAGAGAC-3' for a product size of ~195 pb. As positive control, human β-actin (NM_001101) was used as forward 5'-ACGGCTGCTTCCAGCTCCTCC-3' and reverse 5'-AGCCATGCCAATCTCATCTTGT-3' primers for a PCR product of ~524 bp.

Characterization of HIESC-2/CRISPR clones

Stromal clones were grown to 40% confluence in 6-well plates, fixed with methanol at 4oC and processed as previously described (Kang, Chapdelaine et al. 2005). A monoclonal antibody coupled with Cy3 was used against vimentin (Sigma) (1/500). A mAb against cytokeratin (DAKO inc., Burlington, ON, Canada) was used at a 1/500 dilution and a secondary goat anti-mouse conjugated to Alexa Fluor® 488 (Molecular Probes inc, Eugene, OR, USA) was used. The fluorescence was visualized using a Zeiss Axiovert 100-Inverted microscope (Zeiss, Germany), and images were captured and integrated using the Northern Exposure program (Empix Imaging Inc., ON, Canada).

Induction of decidualization was performed as described for our HIESC-2 cell-line (Chapdelaine, Kang et al. 2006). Briefly, cells were grown to confluence in six-well plates containing RPMI without phenol-red (Life technologies inc., Burlington, ON, Canada) containing 2% dextran-charcoal-treated FBS (DC-FBS) and 1X penicillin-streptomycin in the presence or absence of 0.5 mM 8-bromo-cAMP (Sigma, St. Louis, MO, USA) and 10–6 M medroxyprogesterone acetate (MPA) (Pharmacia Canada, Mississauga, ON, Canada). The culture medium was changed every other day and supernatants were collected after 6 days for prolactin (PRL) assay. PRL measurement was done by ELISA using the ADVIA Centaur® immunoassay system (Bayer HealthCare LLC, Tarrytown, NY, USA). The lower detection limit was 0.8µg/l.

Western Blot Analysis

Protein extraction and quantification for western blot analysis were performed as described previously (Arosh et al., 2002). For each sample, aliquots of 5-10 µg protein were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis 10% (SDS-PAGE). Proteins were then electrotransferred onto 0.45 µm nitrocellulose membranes (Bio-Rad Laboratories, Inc., Mississauga, Ontario, Canada). The membranes were blocked overnight at 4°C in phosphate-buffered saline (PBS) 0.05% Tween 20 (PBS-T) containing 5% (wt/vol) fat-free dry milk (BLOTTO). Then, the membranes were incubated in PBS-T/5% BLOTTO with the following primary antibodies: Anti-COX-2 (Merck 243) and anti-COX-1 (Merck 241) dilution 1/3000 were kindly provided by Dr S Kargman (Merck, QUE, Canada). The anti-microsomal PGE synthase 1 (anti-mPGES1) SC-166308 (1/500), AKR1A1 was probed with a monoclonal mouse anti-human AKR1A1 (1/500), CBR1 (carbonyl reductase 1) was detected with sc-70212 antibody (1/250), IGFBP-1 with sc-6072 antibody at a 1/100 dilution, 17β-HSD3 with sc-66415 antibody at a 1/200 dilution all from (Santa-Cruz biotechnology, Dallas, TX, USA). The anti-AKR1B1 (PAS-29718, Thermo scientific) was used at a 1/2000 dilution. Both the anti- AKR1B1 C-terminal probing amino acids 287~316 of AKR1B1 (AP12363PU-N) (1/1000) and anti-AKR1C3 antibody probing amino acids 17-44 used at a 1/1000 dilution (AP50133PU-N) came from ACRIS antibody GmbH. Finally, the anti-β-Actin (1/1000) (Sigma) was used as an internal standard at a 1/5000 dilution. Following incubation with the primary antibodies, the membranes were washed three times during 10 minutes in PBS-T. The membranes were then incubated in PBS-T/5% BLOTTO with the following appropriate secondary antibody either a goat anti-rabbit or anti-mouse conjugated with horseradish peroxidase (Jackson laboratories, West Grove, PA, USA). This secondary antibody was used at 1/10000 for 1 hour at room temperature followed by 3 washes of 10 minutes in PBS-T. The chemiluminescence signal was analyzed with an autoradiography film after treatment of the membrane with Renaissance reagent (NEN, Perkin Elmer, Boston, MA, USA). The signal intensity of the immunoreactions was quantified by densitometry using a Multimage Light Cabinet equipped with AlphaImager 2000 software (Cell Biosciences, Santa Clara, California,).

PGE2 and PGF2α Immunoassay

Prostaglandins were assayed by competitive EIA using acetylcholinesterase –linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996).

Statistical analysis

Data were analyzed using GraphPad Prism 5 program. One-way ANOVA with Bonferroni as post-hoc test with 95% confidence intervals was used for statistical significance. All numerical data are presented as the mean ± SEM, and differences were considered as statistically significant at the 95% confidence level ($p < 0.05$). Each experiment was repeated at least three times in triplicate.

RESULTS

Generation of null mutation of AKR1B1 gene in HIESC-2 cells

Immortalized human endometrial stromal cells (HIESC-2, (Chapdelaine, Kang et al. 2006)) were transfected with four different sgRNA plasmids targeting various nucleotide sequences of the first exon of AKR1B1 gene (Fig. 2). After 3 days, transfected cells were selected by incubation in presence of neomycin and clones were propagated for 3 weeks (Fig. 3). Mutations of the genomic DNA were assessed by a Surveyor assay on DNA extracted from transfected cells and amplified with specific primers neighboring exon 1 of the AKR1B1 gene (Fig. 4). The Cas9 plasmid alone or in combination with a plasmid expressing GFP did not generate any fragment following Surveyor digestion. However, sgRNA plasmids corresponding to targets #14, 16 and 17 but not #15, generated fragments of predicted size indicating genomic modifications (see arrows Fig. 4A). Neomycin selection of the transfected cells increased the yield of mutated cells as seen for the gRNA #16 plasmid (Fig. 4A). Western blot analysis of AKR1B1 protein in global preparations of cells co-transfected with #14, 16 and 17 gRNA plasmids individually with Cas9 did not show significant reduction of the targeted protein (Fig. 4B). After propagation, 23 clones with stromal-like morphology were selected for optimal growth rates. Proteins were extracted from each clone obtained after 2 to 3 weeks of growth and significant reduction of AKR1B1 was observed for several clones (Fig. 4C). In particular, clones 16-1 and 16-2 presented with a complete absence of AKR1B1 protein. The absence of protein was associated with a major reduction of mutated mRNA for clone 16-2 but 16-1 showed a high level of truncated mRNA expression which coding sequence started at the second methionine of exon 1 thus maintaining intact the remaining of the transcript. This and maintenance of significant production of PGF2α lead us to consider clone 16-1 as an atypical knockout that precluded further characterization.

Characterization of clones obtained after CRISPR treatment

The genomic DNA of clones 16-1, 16-2, and 16-4 was analyzed by PCR amplification with primers neighboring exon 1 of the AKR1B1 gene targeted by sgRNA#16. Figure 5C illustrates that clone 16-2 contained two different amplification products whereas clones 16-1 and 16-4 contained only one amplification product. Sequencing of the two PCR bands from clone 16-2 for which AKR1B1 protein was undetectable showed deletion of 68 nucleotides for the lower band and two nucleotides deletion for the upper PCR band (Fig. 5A). This result demonstrates that both alleles of clone 16-2 were mutated causing a frameshift of AKR1B1 transcript. Figure 5A shows that deletions correspond to the genomic area targeted by the sgRNA#16 construct. By contrast, sequencing of the single PCR band of clone 16-4 (Fig. 5B) shows that only one allele was mutated with a single nucleotide deletion thus causing a frameshift while the other allele is intact. Clone 16-4 would thus represent haplotype mutation analogous to a (–/+) genotype for AKR1B1. As shown in figure 4C, clone 16-4 is able to express AKR1B1 protein. The mRNAs were also analyzed to confirm results at the protein level (not shown).

Further analysis on the association between AKR1B1 and PGF synthase activity was limited to wild type (AKR1B1 +/+) human endometrial stromal cells HIESC-2 (Chapdelaine, Kang et al. 2006), and clones 16-2 AKR1B1 (–/–) and 16-4 AKR1B1 (–/+). Both clones presented with cell morphology similar to parental HIESC-2 cells with immunohistofluorescence positive for vimentin and negative for cytokeratin (Fig. 6A). Similarly, Western blot analysis of alternate PGF synthases (Fig. 1) confirmed the absence of AKR1A1 and AKR1C3 (Fig. 6B) but also the presence of CBR1 protein in all cell lines. By contrast, wild type endometrial epithelial cells (HIEEC) present with significant levels of all biosynthetic enzymes as we have shown previously (Bresson, Lacroix-Pepin et al. 2012). RT-PCR analysis of RNA extracted from clones 16-2, 16-4, HIESC-2 and HIEEC revealed complete absence of hydroxyprostaglandin dehydrogenase (HPGD) transcript but presence of low levels of AKR1A1 and 1C3 transcripts in all stromal cell lines and high levels of CBR1 in all cell types (results not shown). The strong expression of the CBR1 transcript and protein (Figure 6C) suggests that in absence of AKR1B1, PGF2α could be produced from conversion of PGE2 present in the cells (Figure 1).

Decidualization response in wild type and CRISPR mutated cells.

As an additional test to confirm that stromal cell phenotype was maintained, cells were submitted to a decidualization protocol that was already tested for the parental stromal cell line (Chapdelaine, Kang et al. 2006; Kang, Chapdelaine et al. 2006). Both parental and mutated cells exhibited characteristic morphological changes following treatment with 8-bromo-cAMP and MPA (Fig. 7A) including increase in cell size and plurinucleation (results not shown). Prolactin production was measured as a functional marker and cell count

was performed to normalize the results (ng prolactin /106 cells). Average cell number per well was 93, 8 and 20 x106 respectively for wild-type, 16-2 and 16-4 cells. Decidualization induced comparable prolactin increases of 18.7, 10.5 and 20.2 folds respectively for wild-type AKR1B1 (+/+), 16-2 AKR1B1 (–/–) and 16-4 AKR1B1 (–/+) cells (Fig. 7B).

Effect of AKR1B1 deletion on PGF2α production

Following treatment of cells with IL-1β (1 ng/ml) for 24 hrs, both wild-type and clone 16-4 cells exhibited a similar response, but clone 16-2 did not respond with increased production of either PGF2α or PGE2 (Fig. 8B). At the protein level (Fig. 8A), IL-1β (1 ng/ml) induced an increase in mPGES-1 and COX-2 protein expression but in clone 16-2 the relative increase of Cox-2 protein was weaker. Side by side comparison of responses to IL-1β (1 ng/ml) arachidonic acid (AA, 10 μM) in wild type and clone 16-2 cells (Fig. 9) shows higher increase in COX-2 in presence of IL-1 but otherwise comparable levels of enzymes involved in PG production (Fig. 9 A and B). The response of cell lines to AA in terms of PGF production was comparable was comparable but interestingly, PGE2 production was restored in clone 16-2 (Fig. 9 C and D).

DISCUSSION

We demonstrate here the ability of the CRISPR/Cas9 system to modify the genome of immortalized endometrial cells and we confirm the importance of the AKR1B1 gene in the production of endometrial PGF2α.

Prostaglandin F2α appears as the least studied member among prostaglandins. The PGF2α/FP system is however clearly involved in endometrial pathologies including menstrual disorders and endometrial cancer (Milne and Jabbour 2003; Jabbour and Sales 2004; Sales, List et al. 2005; Catalano, Wilson et al. 2011) and premature delivery (Phillips, Al-Zamil et al. 2011).

Before our identification of AKR1B5 in the bovine (Madore, Harvey et al. 2003), now bovine AKR1B1, all PGFS described were AKRs of the 1C family. AKRs are recognized as moonlighting enzymes able to partner with several types of substrates. The putative PGFS activity of AKR1B5 led us to investigate its human counterpart AKR1B1 well known for its role in the polyol pathway and contribution to diabetes complications. We (Bresson, Boucher-Kovalik et al. 2011; Bresson, Lacroix-Pepin et al. 2012) and others (Kabututu, Manin et al. 2009; Nagata, Kusakari et al. 2011; Phillips, Al-Zamil et al. 2011) found that the human AKR1B1 was a functional PGF synthase in various tissues.

More recently, we evaluated the activity of another AKR, AKR1A1 as a potential alternate endometrial PGFS (Lacroix Pepin, Chapdelaine et al. 2013). With AKR1C3 already expressed, the presence of AKR1B1 and AKR1A1 as alternate PGF synthases complicates the picture as to how PGF2α production is regulated under normal or pathologic conditions in the human endometrium. Because abrogation of AKR1B1 by siRNA decreases AKR1B1 expression and PGF2α output only by 50% (Bresson, Boucher-Kovalik et al. 2011), this approach did not appear sufficiently robust to address the issue. Complete knockout of individual genes involved in PGF2α appeared as the most logical solution. This was attempted in the mouse in spite of absence of AKR1B1 in this species. In mice, two distinct genes, AKR1B3 and AKR1B7 for which null mutations were generated, cover some but not all activities of AKR1B1. AKR1B3 (-/-) mice appeared protected against cerebral ischemic injury (Lo, Cheung et al. 2007), which is interesting given the documented ischemic responses to PGF2α (Norel 2007), particularly in association with menstrual pain. Unfortunately, AKR1B3-null mice were not tested for prostaglandin production level and associated pathologies. AKR1B7 null mice exhibit excessive basal adiposity and greater sensitivity to diet-induced obesity not seen with the AKR1B3 knockout model.

The recent development of genomic edition with the CRISPR/Cas9 system now opens new avenues to explore gene function in differentiated human cells. Our initial approach was to create 4 sgRNAs that aimed at exon 1 of AKR1B1 genomic sequence (Fig. 2 B). The sequences targeted by these sgRNA were then blasted to ensure minimal off-target potential. Considering the PAM sequence necessary for Cas9 digestion, target 16 (Fig. 2A) exhibited no obvious off-target (Mali, Yang et al. 2013) thus limiting the probability that other genes such as AKR1B10 an AKR1B exhibiting 68.2% homology with AKR1B1 would be affected. AKR1B10 showed only 8 out of 19 nucleotide correspondence with target 16 and lacked the PAM sequence that is essential for Cas9 activity. While the possibility for off-targets could not be eliminated completely, we believe that the process of clone generation and selection of mutated cells based on viability, morphology and PGs response kept this probability to a minimum.

The plasmids coding for the sgRNAs and Cas9 were co-transfected because the new plasmid permitting expression of both sgRNAs and Cas9 was not yet available (Cong, Ran et al. 2013). Of the 4 sgRNAs used, 3 successfully altered AKR1B1 sequence as evaluated by Surveyor (Fig. 4A). Among those, the 2 clones showing abrogation of AKR1B1 were obtained with the sgRNA #16. Sequencing of 16-2 confirmed that no AKR1B1 protein could be produced from the isolated mRNA (results not shown). The parent clone 16-4 with normal or slightly diminished AKR1B1 production (Fig. 4C) showed normal sequence for one AKR1B1 allele and a single nucleotide deletion on the second at both DNA and mRNA levels (results not shown). All genomic mutations were located at the end of the nucleotide sequence targeted by sgRNA #16.

Clone 16-2 can be assimilated to a (-\-), and clone 16-4 to a (+/-) genotype and both cell lines grew normally and exhibited morphologies comparable to wild-type (+/+) HIESC-2 cells (Fig. 6A). As expected, none of these cells of stromal origin expressed AKR1C3 (Bresson, Lacroix-Pepin et al. 2012) and did not have significant levels of AKR1A1 either (Fig. 6B). These cells did not express mRNA for the PG catabolic enzyme HPGDH as we observed for primary stromal cell cultures (Parent, Madore et al. 2006) but mRNA for CBR1, an enzyme able to convert PGE2 into PGF2α, could be detected by PCR and western blot but was not investigated further (Fig. 6C).

When treated with medroxy-progesterone acetate and 8-bromo-cAMP, all 3 cell types presented with a change in morphology characteristic of decidualization (Fig. 7A). Cells changed from spindle-shaped to ovoid with abundant cytoplasm. This change appeared more pronounced in clone 16-2 than in clone 16-4. All 3 cell types increased prolactin secretion following decidualization, (Fig. 7B).

Clones and wild type cells exhibited similar increases in PGE2 production (Fig. 8B) and COX-2 and MPGES-1 proteins (Fig. 8A). PGF2α production was highest in wild-type cells absent in clone 16-2 and intermediate in clone 16-4 in direct association with measured AKR1B1 protein level in presence of IL-1β (Fig. 8). When cells were treated with arachidonic acid, absence of AKR1B1 did not have any impact on PGE2 production (Fig. 9C) whereas PGF2α was kept at its minimum (Fig. 9D). Under both IL-1β and arachidonic acid, MPGES-1 was increased confirming that the PGE2 pathway was stimulated by IL-1β and arachidonic acid in mutated cells (Fig. 9A and B). In clone 16-2, presence of residual PGF2α in absence of AKR1B1 might be due to accessory PGF synthase activity by enzymes such as CBR1 converting PGE2 into PGF2α (Fig. 6C)

Reduced PGE2 production associated with reduced AKR1B1 derived PGF2α production following IL-1β but not arachidonic acid stimulation of prostaglandins strengthens our hypothesis that a feedback loop between PGF2α and PGE2 may exist (Bresson, Lacroix-Pepin et al. 2012). Results in figure 9 suggest that mechanisms upstream of COX-2 are responsible for increased PGE2 production in presence of PGF2α. Since arachidonic acid release by cPLA2 is dependent on Ca++ levels themselves elevated in response to FP activation by PGF2α (Jabbour and Sales 2004) and (Krishnaswamy, Danyod et al. 2009; Krishnaswamy, Lacroix-Pepin et al. 2010), this signal transduction pathway should be considered. PGE2 acting on EP2 receptors and PGF2α acting through FP work as an opposing dyad in the reproductive system, in a way similar to TXA2 and PGI2 in the vascular system, thus allowing tight regulation of cell responses. Under the circumstances, the observed interactions between the PGE2 and PGF2α systems in endometrial cells are not only probable but necessary.

In summary, the ease and efficacy of editing genome in differentiated human cells using the CRISPR/Cas9 system should have broad implications for our ability to understand complex biological systems at the functional level. Our next goal will be to use the same approach to generate complementary and multiple knockout models where key enzymes of the PGE2 and PGF2α biosynthetic cascade will be invalidated. These models will allow us to conduct in human tissues, experiments that will validate and/or complement animal studies, especially when the genes or function involved are not identical.

REFERENCES

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod **54**(2): 371-9.

Bresson, E., S. Boucher-Kovalik, et al. (2011). "The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium." J Clin Endocrinol Metab **96**(1): 210-9.

Bresson, E., N. Lacroix-Pepin, et al. (2012). "The Prostaglandin F Synthase Activity of the Human Aldose Reductase AKR1B1 Brings New Lenses to Look at Pathologic Conditions." Front Pharmacol **3**: 98.

Carroll, D. (2012). "A CRISPR approach to gene targeting." Mol Ther **20**(9): 1658-60.

Catalano, R. D., M. R. Wilson, et al. (2011). "Comprehensive expression analysis of prostanoid enzymes and receptors in the human endometrium across the menstrual cycle." Mol Hum Reprod **17**(3): 182-92.

Chapdelaine, P., J. Kang, et al. (2006). "Decidualization and maintenance of a functional prostaglandin system in human endometrial cell lines following transformation with SV40 large T antigen." Mol Hum Reprod **12**(5): 309-19.

Christ-Hazelhof, E. and D. H. Nugteren (1979). "Purification and characterisation of prostaglandin endoperoxide D-isomerase, a cytoplasmic, glutathione-requiring enzyme." Biochim Biophys Acta **572**(1): 43-51.

Cong, L., F. A. Ran, et al. (2013). "Multiplex genome engineering using CRISPR/Cas systems." Science **339**(6121): 819-23.

Hwang, W. Y., Y. Fu, et al. (2013). "Efficient genome editing in zebrafish using a CRISPR-Cas system." Nat Biotechnol **31**(3): 227-9.

Jabbour, H. N. and K. J. Sales (2004). "Prostaglandin receptor signalling and function in human endometrial pathology." Trends Endocrinol Metab **15**(8): 398-404.

Kabututu, Z., M. Manin, et al. (2009). "Prostaglandin F2alpha synthase activities of aldo-keto reductase 1B1, 1B3 and 1B7." J Biochem **145**(2): 161-8.

Kang, J., P. Chapdelaine, et al. (2006). "Functional characterization of prostaglandin transporter and terminal prostaglandin synthases during decidualization of human endometrial stromal cells." Hum Reprod **21**(3): 592-9.

Kang, J., P. Chapdelaine, et al. (2005). "Expression of human prostaglandin transporter in the human endometrium across the menstrual cycle." J Clin Endocrinol Metab **90**(4): 2308-13.

Krishnaswamy, N., G. Danyod, et al. (2009). "Oxytocin receptor down-regulation is not necessary for reducing oxytocin-induced prostaglandin F(2alpha) accumulation by interferon-tau in a bovine endometrial epithelial cell line." Endocrinology **150**(2): 897-905.

Krishnaswamy, N., N. Lacroix-Pepin, et al. (2010). "Epidermal growth factor receptor is an obligatory intermediate for oxytocin-induced cyclooxygenase 2 expression and prostaglandin F2 alpha production in bovine endometrial epithelial cells." Endocrinology **151**(3): 1367-74.

Lacroix Pepin, N., P. Chapdelaine, et al. (2013). "Evaluation of the prostaglandin F synthase activity of human and bovine aldo-keto reductases: AKR1A1s complement AKR1B1s as potent PGF synthases." Prostaglandins Other Lipid Mediat.

Lim, H., B. C. Paria, et al. (1997). "Multiple female reproductive failures in cyclooxygenase 2-deficient mice." Cell **91**(2): 197-208.

Lo, A. C., A. K. Cheung, et al. (2007). "Deletion of aldose reductase leads to protection against cerebral ischemic injury." J Cereb Blood Flow Metab **27**(8): 1496-509.

Madore, E., N. Harvey, et al. (2003). "An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium." J Biol Chem **278**(13): 11205-12.

Mali, P., L. Yang, et al. (2013). "RNA-guided human genome engineering via Cas9." Science **339**(6121): 823-6.

Milne, S. A. and H. N. Jabbour (2003). "Prostaglandin (PG) F(2alpha) receptor expression and signaling in human endometrium: role of PGF(2alpha) in epithelial cell proliferation." J Clin Endocrinol Metab **88**(4): 1825-32.

Nagata, N., Y. Kusakari, et al. (2011). "Catalytic mechanism of the primary human prostaglandin F2alpha synthase, aldo-keto reductase 1B1--prostaglandin D2 synthase activity in the absence of NADP(H)." FEBS J **278**(8): 1288-98.

Norel, X. (2007). "Prostanoid receptors in the human vascular wall." ScientificWorldJournal **7**: 1359-74.

Parent, M., E. Madore, et al. (2006). "15-Hydroxyprostaglandin dehydrogenase in the bovine endometrium during the oestrous cycle and early pregnancy." Reproduction **131**(3): 573-82.

Pastel, E., J. C. Pointud, et al. (2012). "Aldo-Keto Reductases 1B in Endocrinology and Metabolism." Front Pharmacol **3**: 148.

Phillips, R. J., H. Al-Zamil, et al. (2011). "Genes for prostaglandin synthesis, transport and inactivation are differentially expressed in human uterine tissues, and the prostaglandin F synthase AKR1B1 is induced in myometrial cells by inflammatory cytokines." Mol Hum Reprod **17**(1): 1-13.

Poyser, N. L. (1995). "The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation." Prostaglandins Leukot Essent Fatty Acids **53**(3): 147-95.

Sales, K. J., T. List, et al. (2005). "A novel angiogenic role for prostaglandin F2alpha-FP receptor interaction in human endometrial adenocarcinomas." Cancer Res **65**(17): 7707-16.

Smith, W. L., Y. Urade, et al. (2011). "Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis." Chem Rev **111**(10): 5821-65.

Spencer, T. E., G. A. Johnson, et al. (2007). "Fetal-maternal interactions during the establishment of pregnancy in ruminants." Soc Reprod Fertil Suppl **64**: 379-96.

Tanabe, T. and V. Ullrich (1995). "Prostacyclin and thromboxane synthases." J Lipid Mediat Cell Signal **12**(2-3): 243-55.

Tanikawa, N., Y. Ohmiya, et al. (2002). "Identification and characterization of a novel type of membrane-associated prostaglandin E synthase." Biochem Biophys Res Commun **291**(4): 884-9.

Thatcher, W. W., A. Guzeloglu, et al. (2001). "Uterine-conceptus interactions and reproductive failure in cattle." Theriogenology **56**(9): 1435-50.

Ullrich, V., L. Castle, et al. (1981). "Spectral evidence for the cytochrome P450 nature of prostacyclin synthetase." Biochem Pharmacol **30**(14): 2033-6.

Volat, F. E., J. C. Pointud, et al. (2012). "Depressed levels of prostaglandin F2alpha in mice lacking Akr1b7 increase basal adiposity and predispose to diet-induced obesity." Diabetes **61**(11): 2796-806.

FIGURES

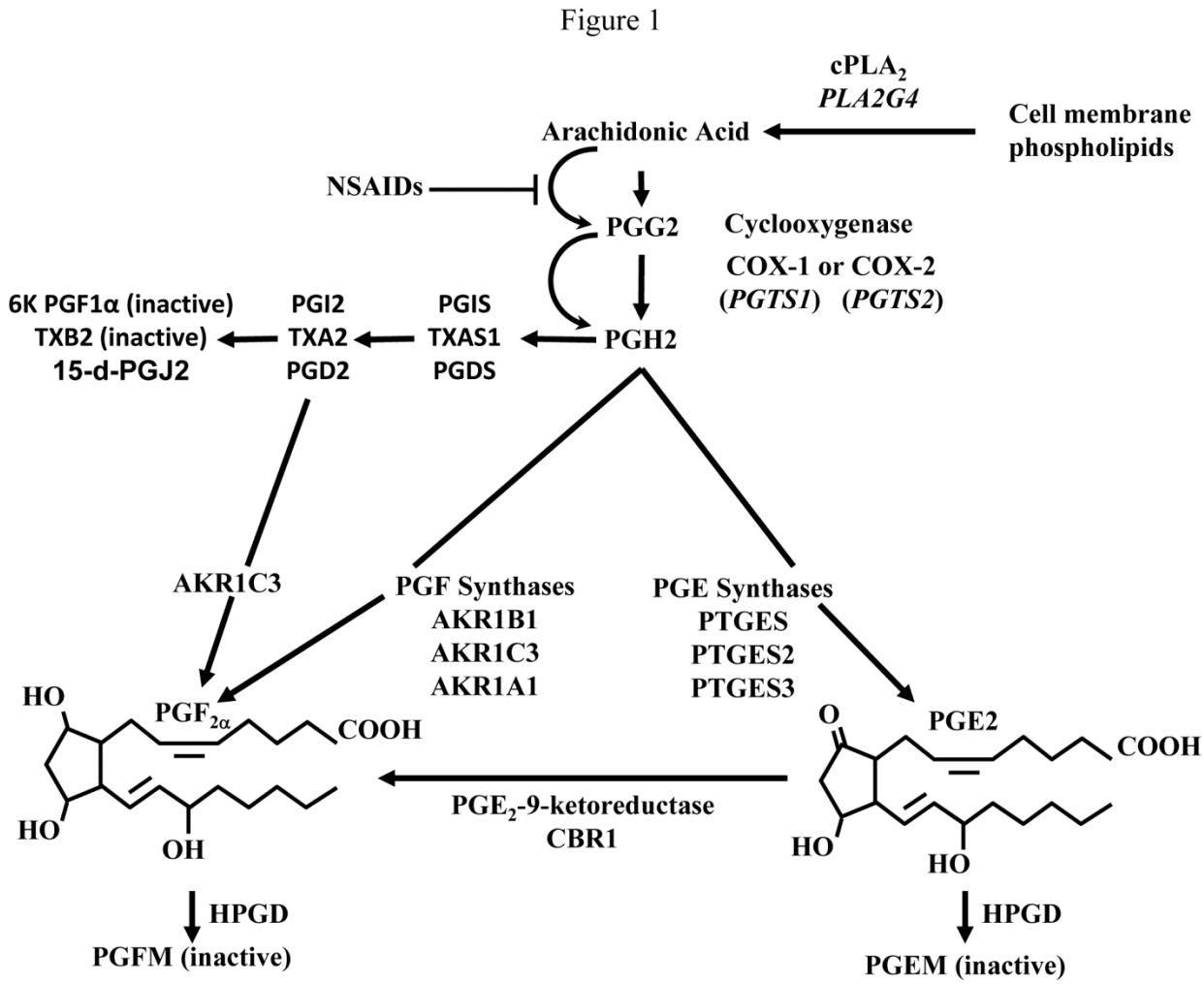


Figure 30 - Article 5: Figure 1 Prostaglandin biosynthesis pathway.

cPLA2 (PLA2G4) releases arachidonic acid (AA) from membrane phospholipids and COX enzymes (PTGS1, PTGS2) convert it to PGG2 and PGH2, the common precursor for all PGs. PGH2 is then converted into one of the active PG by specific terminal synthases such as PGE synthases (PTGES, PTGES2, PTGES3). PGF synthases (AKR1B1, AKR1C3, AKR1A1), PGD synthase (PGDS) and Thromboxane synthase 1(TXAS1). CBR1 is able to convert PGE2 into PGF2α while AKR1C3 can convert PGD2 into 9α,11β-PGF2. PGE2 and PGF2α are inactivated into PGEM and PGFM by HPGD (15-PGDH), PGD2 converts spontaneously into bioactive PGJ2 whereas unstable PGI2 and TXA2 convert into inactive 6K-PGF1α and TXBA2.

Figure 2

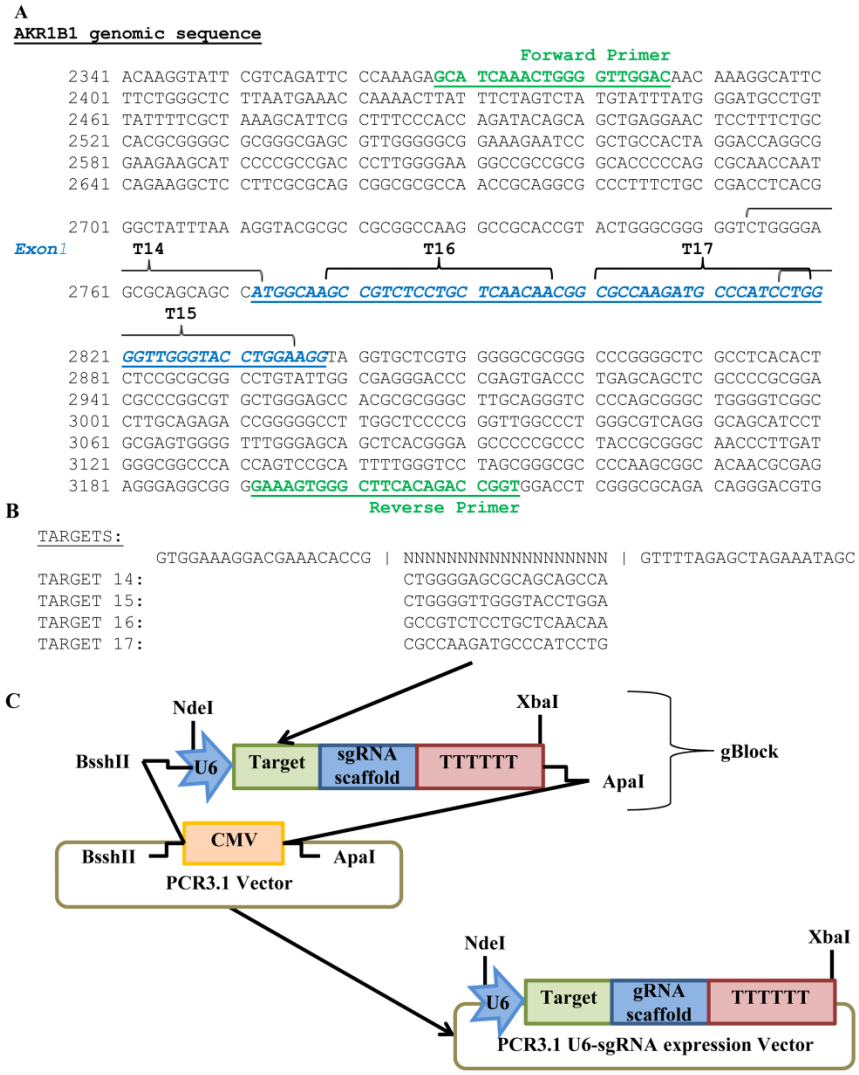


Figure 31 - Article 5: Figure 2 Human *AKR1B1* exon 1.

Part of the genomic sequence of AKR1B1 is presented in (A). Primers used to sequence are highlighted in green. The first exon of AKR1B1 is shown in bold, underlined and blue font. Oligonucleotides synthesized are represented in (B) where the 19 selected nucleotides for each target replaced the "N". Target sequences used for each sgRNAs are represented above exon 1 sequence and separately in (B). Target sequences derived from genomic sequence of AKR1B1 exon 1 (A) were added to a synthesized gBlock® from IDT (Integrated DNA technologies inc.) containing the U6 promoter + Target sequence of the sgRNA + guide RNA scaffold + termination signal. Then, the gBlock® was amplified by PCR and inserted in the pCR3.1 plasmid between the BssHII and the ApaI sites to replace the CMV promoter and create a pCR3.1 U6-gRNA plasmid (C). The sgRNA inserted in pCR3.1 U6-sgRNA plasmid contains 2 unique restriction sites (NdeI and XbaI), allowing to exchange the sequence to generate different sgRNAs by PCR amplification.

Figure 3

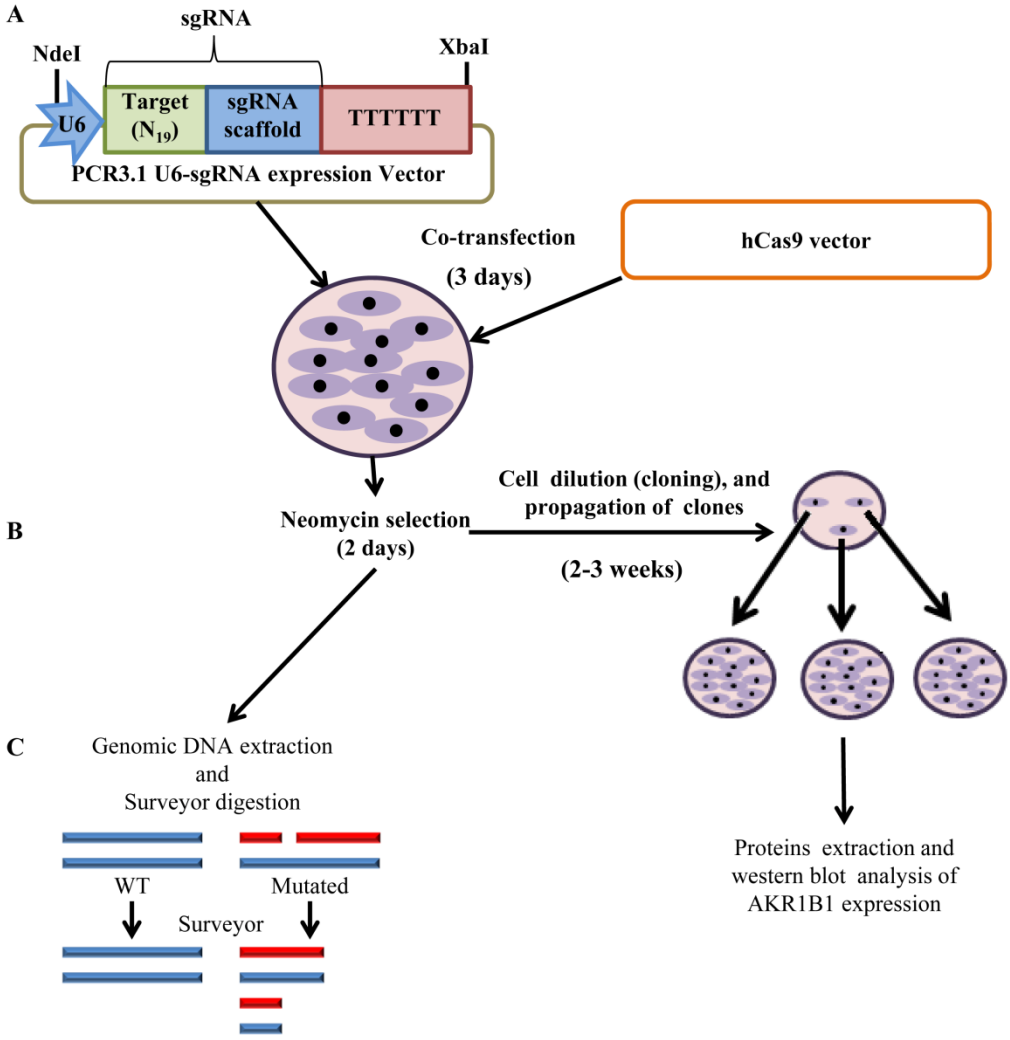


Figure 32 - Article 5: Figure 3 Use of CRISPRs/Cas9 system to silence AKR1B1 gene in human endometrial stromal cells (HIESC).

HIESC-2 cells were co-transfected with a plasmid containing Cas9 (Addgene, no 48668) and pCR3.1 U6-sgRNA plasmid using lipofectamine 2000 and maintained in culture for 3 days (A). After 2 days of neomycin selection, cells were harvested and seeded at low dilution for isolation of single clones (B). While single clone propagation was proceeding (2-3 weeks), an aliquot was used for DNA extraction and Surveyor digestion to test mutation efficiency (C). Finally, an aliquot of each clone was used to analyse AKR1B1 protein expression by Western blot. Clones that achieved successful growth were also analyzed at the protein level to probe for AKR1B1.

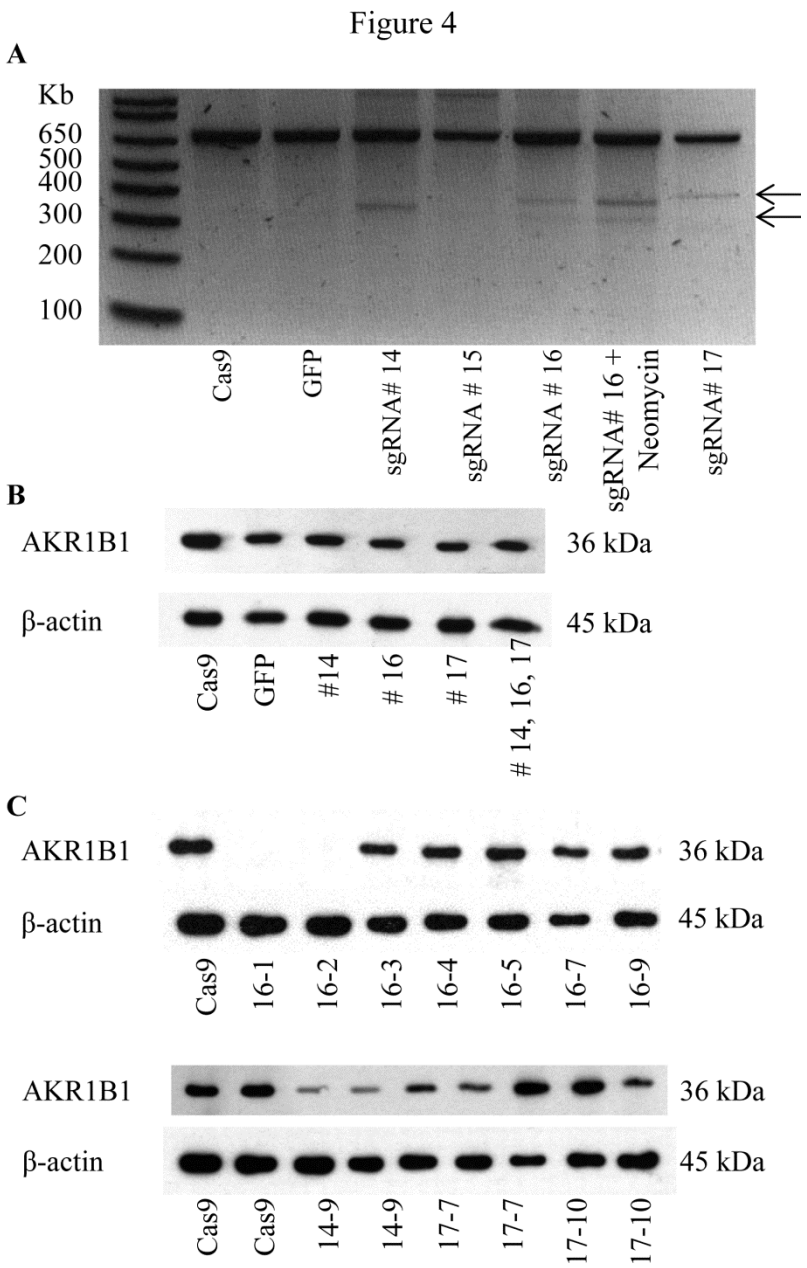


Figure 33 - Article 5: Figure 4 Surveyor and Western blot analysis of AKR1B1 expression in transfected cell clones.

HIESC cells were transfected with lipofectamine 2000 with Cas9 and one of the four pCR3.1 U6-sgRNA plasmids (figure 2) coding for a sgRNA targeting exon 1 of the human AKR1B1 gene. The DNA was extracted from the cells after 72 hours before and after neomycin selection (48 hours). Part of the AKR1B1 gene was amplified by PCR and the presence of INDELs in the target sequence was detected by digestion with the Surveyor enzyme resulting in additional bands (A). After culture, the proteins were extracted and western blot for AKR1B1 was performed (B). Individual clones were isolated for cells treated with sgRNA #16 and western blot for the presence of AKR1B1 were performed (C).

Figure 5

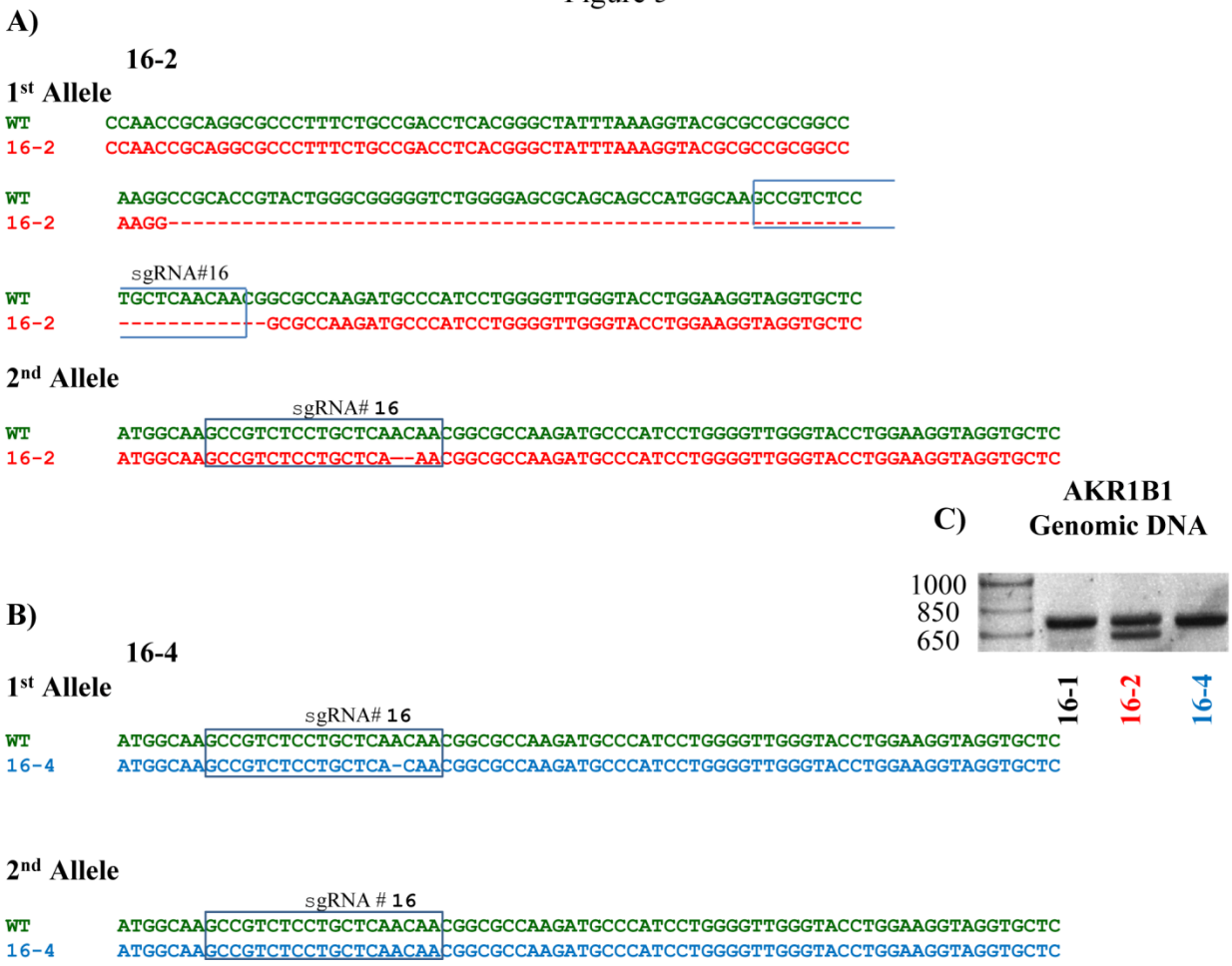


Figure 34 - Article 5: Figure 5 Sequencing of exon 1 of *AKR1B1* for clones 16-2 and 16-4.

Genomic DNA was extracted from the cells after 72 hours of culture. Part of the AKR1B1 gene was amplified by PCR and sequenced. For clone 16-2, deletions of 68 and 2 nucleotides were found for the first and second allele respectively (A). For clone 16-4 only the first allele was altered with a single nucleotide deletion (B). Representative PCR amplification products from genomic DNA of clones 16-1, 16-2 and 16-4 are shown in (C).

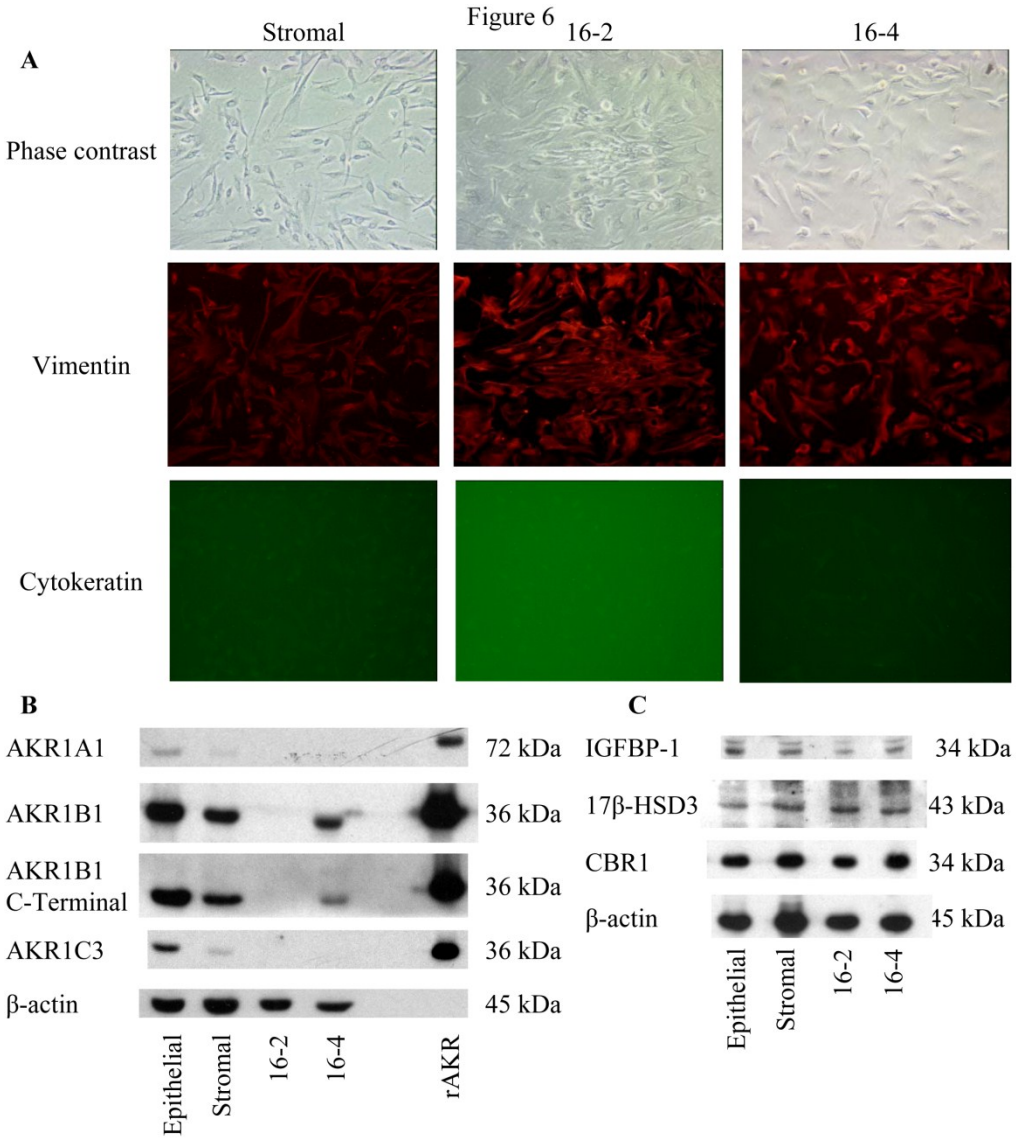


Figure 35 - Article 5: Figure 6 Phenotypic characterization of clones 16-2 (–/–) and 16-4 (+/–).

Cells of clones 16-2 and 16-4 were grown in parallel with wild type stromal cells (HIESC-2) as control for 3 days and observed under phase contrast or following immunolabeling with vimentin or cytokeratin antibodies at 100X magnification (A). Photomicrographs show that normal and mutated cells exhibit similar morphology and expression of vimentin but not cytokeratin. Western blot analysis for the presence of alternate PGF synthases was performed (B and C). AKR1A1, AKR1B1 and AKR1C3 were assessed in HIEEC-22 (epithelial), HIESC-2 (stromal), clone 16-2 and clone 16-4. Recombinant Aldo-keto Reductase proteins (rAKR) were used as internal standards (B). Western blot analysis of CBR1, IGFBP-1 and 17β-HSD3 is shown for the same cell lines (D). In all cases, β-actin was used as internal standard.

Figure 7

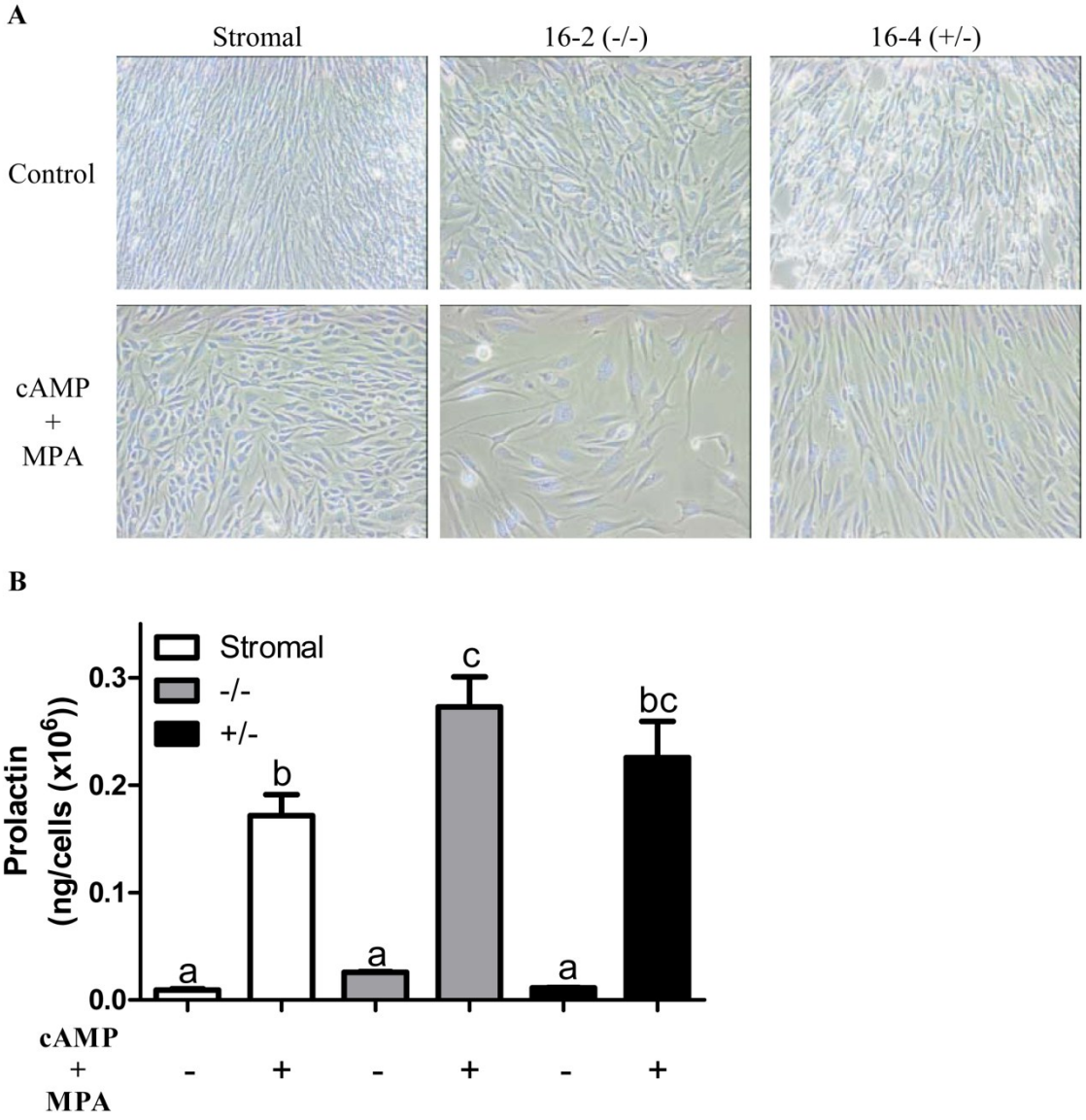
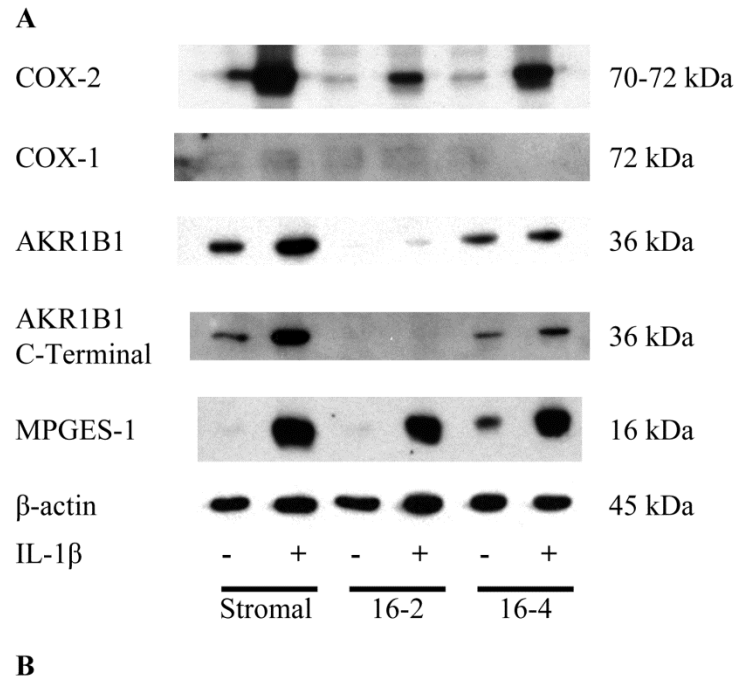


Figure 36 - Article 5: Figure 7 Decidualization of wild type HIESC-2 and clones 16-2 (–/–) and 16-4 (+/–).

Decidualization was induced by a 6-day treatment with 8-bromo-cAMP (0.5 mM) and MPA (10^{-6}M) inducing morphological changes in stromal (HIESC-2) from a spindle to an ovoid shape (magnification 100X) (A). Decidualization stimulated prolactin secretion in all cell lines (B). After decidualization cell number ranged from 10 to 130 x10^6 cells per well.

Figure 8



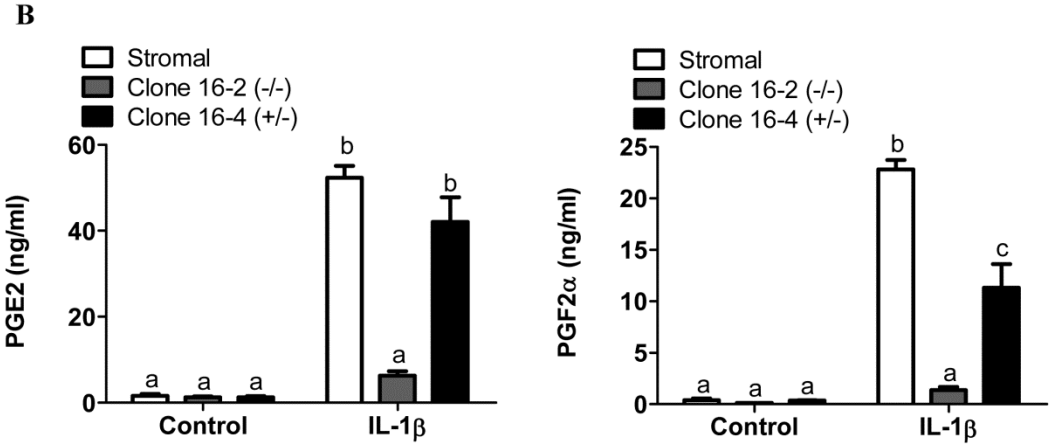


Figure 37 - Article 5: Figure 8 Correlation between prostaglandin production and expression of biosynthetic enzymes in HIESC-2 and clones 16-2 (–/–) and 16-4 (+/–).

HIESC-2 (stromal), clones 16-2 and 16-4 were grown to confluency and then treated with IL-1β (1 ng/ml). Proteins of the PGE2 and PGF2α biosynthetic cascade were then evaluated by Western blot analysis (A) The corresponding production of PGE2 and PGF2α measured by EIA (B). Results are the mean +/- SEM of 3 experiments run in triplicate.

Figure 9

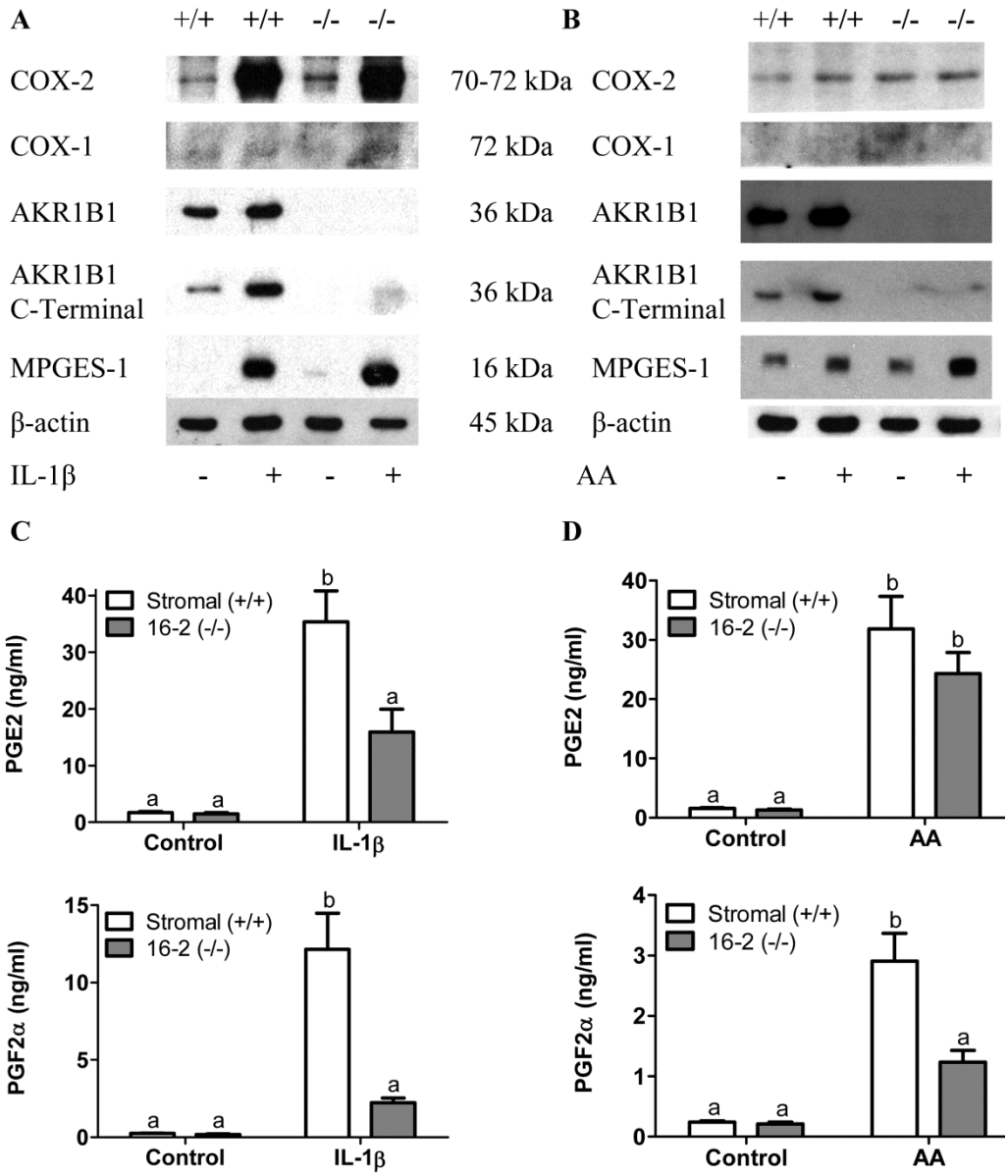


Figure 38 - Article 5: Figure 9 Correlation between PGF2α production and PGE2 synthesis.

HIESC-2 (stromal) and clone 16-2 were cultured as described earlier and then treated with IL-1β (1 ng/ml) (A and C) or Arachidonic Acid (AA) (10 μM) (B and D). Protein levels of prostaglandin biosynthetic enzymes were analyzed by Western blot (A and B), and PGE2 and PGF2α levels measured by EIA (C and D). Results are the mean +/- SEM of 3 experiments run in triplicate

Chapitre 7: A feedback loop involving the FP receptor regulates the production of PGE2 in endometrium.

Nicolas Lacroix Pépin, Yoima Rodriguez, Michel A Fortier

Ce chapitre est présenté sous forme d'article et n'a pas encoré été soumis:
Prostaglandins Other Lipid Mediat. 2014

Centre de Recherche du CHUQ, Département d'Obstétrique et Gynécologie, Unité d'Ontogénie et Reproduction, Centre de Recherche en Biologie de la Reproduction, Université Laval, Ste-Foy, Québec, Canada GIV 4G2

RÉSUMÉ

Les prostaglandines (PGs) sont impliquées dans plusieurs processus reproductifs et leur action est régulée au niveau de la biosynthèse, du catabolisme et de la transduction du signal. Nos études récentes ont montré un mécanisme de rétroaction chez l'humain entre la production de PGF2α et celle de PGE2. Dans le travail présenté ici, nous avons voulu évaluer si un mécanisme existait dans les cellules endométriales bovines. Nous avons trouvé que les deux prostaglandines sont inhibées en présence d'interféron-tau ou d'inhibiteurs d'aldo-céto réductase et que la production de PGF2α précède l'augmentation de PGE2 autant dans les cellules stromales qu'épithéliales. L'utilisation d'un inhibiteur du récepteur EP soit l'AH-6809 n'a présenté d'effet sur aucune des deux prostaglandines tandis que toutes deux étaient diminuées en présence de l'inhibiteur du récepteur FP AL-8810. La stimulation des cellules épithéliales à l'ocytocine n'a pas été affectée par ce traitement. En conclusion, nous montrons que la production de PGE2 dans les cellules endométriales épithéliales et stromales d'origine bovine semble régulée par l'effet du PGF2α sur le récepteur FP. Une boucle de rétroaction similaire à celle présente chez l'humain semble s'assurer que la production de PGE2 s'active lorsque celle de PGF2α débute et qu'elle est inhibée lorsque la quantité dePGF2α diminue.

ABSTRACT

Prostaglandins (PGs) are involved in several female reproductive processes and their action is regulated at the levels of biosynthesis, catabolism and signal transduction. Our recent studies in human identified a feedback loop regulating PGF2α and PGE2 production. In the present study we seek to evaluate if bovine endometrial cells are subjected to a similar regulation. We have found that interferon-tau and aldo-keto reductase inhibitors inhibited the production of both prostaglandins and that PGF2α production temporally preceded PGE2 increases in both stromal and epithelial cells. The use of EP inhibitor AH-6809 had no effect on either prostaglandin production in both cell types while they were both decreased in presence of FP inhibitor AL-8810. Moreover, oxytocin stimulation of epithelial cells was not affected by that treatment. In conclusion, we showed that PGE2 production was under the control of PGF2α through its FP receptor in both stromal and epithelial cells of bovine endometrium. Thus, a feedback loop similar to that found in human regulates the relative production of PGE2 and PGF2α.

INTRODUCTION

Prostaglandins (PGs) represent a family of lipid mediators acting locally in the vascular, reproductive and other systems to maintain homeostasis through complementary and sometimes opposite actions (Jabbour and Sales 2004; Smith, Urade et al. 2011). PGs are synthesized in virtually all nucleated cells of the body from essential fatty acids (EFAs). The first steps in PG formation are the release of arachidonic acid (AA) from membrane phospholipids by cPLA2 and the conversion of AA from the cell membrane to PGG2 and then PGH2 by one of the two prostaglandin G/H synthases (PTGS-1 or -2) better known as cyclooxygenases (COX-1 or -2). After PGH2 formation, terminal synthases generate the bioactive prostaglandins (Smith, Urade et al.). In the vascular system PGF2α exhibits vasoconstrictive and pro-thrombotic responses comparable to thromboxane with the additional feature that its molecule is chemically stable while PGE2 acts to increase vasodilatation. These PGs remain active until catabolism by prostaglandin dehydrogenase, usually through a single passage in the lung. In other systems, PGF2α plays important roles in the regulation of ocular pressure, renal absorption, cardio-vascular function, adipocyte differentiation and female reproductive function in human (Bresson, Lacroix-Pepin et al. 2012) and bovine (McCracken, Custer et al. 1999). PGE2 has been proposed to have multiple roles as a luteotrophic, luteostatic, or luteoprotective signal at the time of establishment of pregnancy; as an immunomodulatory mediator at fetal-maternal interface; as a mitogenic, antiapoptotic, and angiogenic factor; and either as a myometrial relaxant or stimulant (Arosh, Banu et al. 2004). Altered production of PGE2 and PGF2α has been found in human uterine fluids in association with gynecological disorders such as menorrhagia, dysmenorrhea and endometriosis (Sales and Jabbour 2003).

The apparent ambiguous action of PGE2 results from action through several receptors activating distinct signal transduction systems. EP1 is coupled to Gi and calcium channels while EP2 acts primarily through cAMP-dependent protein kinase (PKA) (cAMP/PKA/p38MAPK pathway) (Hsiao, Mak et al. 2007), whereas the EP4 acts via phosphatidylinositol 3-kinase (PI3K) as well as PKA (Regan 2003), EP3 for which there are 8 splice variants, is principally coupled with the inhibitory Gi system (Fortier, Krishnaswamy et al. 2008). In the bovine endometrium, EP4 is absent whereas EP2 and EP3 together with FP were reported at the mRNA levels with a predominance of EP2 over EP3 (Arosh, Banu et al. 2003; Arosh, Banu et al. 2004). In the human endometrium, prostanoid receptor expression is modulated throughout the cycle. EP1 maximal expression occurs during the early secretory phase and that of EP2, EP3 and EP4 during the mid-secretory phase (Catalano, Wilson et al.).

PGF2α acts through its FP receptors coupled to the heterotrimeric G-protein G_q subunit, PLCβ and Ca^{2+} release. FP is expressed in smooth muscles such as myometrium and blood vessels to induce contractility and menstrual like pain (Lundstrom and Green 1978; Abramovitz, Boie et al. 1994; Bos, Richel et

al. 2004; Jabbour and Sales 2004; Sales, List et al. 2005; Sales, Grant et al. 2008). PGF2α can also bind to the EP1 and EP3 receptors, albeit with reduced affinity (Breyer, Bagdassarian et al. 2001). PGF2α, regardless of the receptor utilized, leads to the activation of Phospholipase C-β (PLCβ), catalyzing the cleavage of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP2) into the second messengers inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers Ca^{2+} release from the endoplasmic reticulum (ER). DAG can activate PKC, but also be converted into AA by monoacylglycerol (MAG) lipase or fatty acid amidohydrolase (FAAH) and enter into the PG biosynthetic cascade (Tang, Edwards et al. 2006). The exact signaling pathways mediating PGF2α action remain somewhat obscure, but likely involve both Gq and Gi-dependent signal transduction, the relative contribution of both pathways to this signal depending on cell type and conditions (Bos, Richel et al. 2004). In Ishikawa cells stably transfected with FP receptor and treated with prostaglandin PGF2α , transphosphorylation of the epidermal growth factor receptor (EGFR) and phosphorylation of extracellular signal-regulated kinase ERK 1/2 was observed (Sales, List et al. 2005). Activation of EGFR-Ras-mitogen-activated protein kinase/ERK kinase (MEK) signaling via the FP receptor is similar to the oxytocin activation of OTR we described in bovine endometial epithelial cells (Krishnaswamy, Lacroix-Pepin et al. 2010). The FP receptor gene is expressed in epithelial cells during the mid- to late-proliferative phase of menstrual cycle (Milne and Jabbour 2003) and its expression is maximal in the proliferative phase (Catalano, Wilson et al.) but down regulated in the proliferative phase of women suffering from abundant bleeding (Smith, Jabbour et al. 2007).

We have shown that inhibition of AKR1B1 lead to reduced production of both PGE2 and PGF2α production in human and bovine endometrial cells (Bresson, Lacroix-Pepin et al. 2012) as was observed after gene silencing of AKR1B1 by siRNA (Bresson, Boucher-Kovalik et al. 2011; Bresson, Lacroix-Pepin et al. 2012). Temporal analysis of PG production in endometrial HIEEC-22 and HIESC-2 cells following stimulation with IL-1β showed that PGF2α production preceded that of PGE2 by 6 hours but that the rate of production was comparable by 24 hrs. Blocking the FP receptor with AL-8810 decreased the production of both prostaglandins following IL-1β stimulation while FP stimulation with a receptor agonist increased PGE2 production in human stromal cells. It was then hypothesized that PGE2 production was regulated by the FP receptor. Since the release of arachidonic acid by cPLA2 is dependent on Ca^{2+} (Jabbour and Sales 2004; Krishnaswamy, Danyod et al. 2009; Krishnaswamy, Lacroix-Pepin et al. 2010), it was our primary candidate as the target of FP regulation of prostaglandin production.

In the present manuscript, we sought to investigate further the potential autocrine regulation of prostaglandin production in both human and bovine endometrial cells.

MATERIALS AND METHODS

Materials

The reagents were purchased from the following suppliers: RPMI 1640 (without phenol) from Invitrogen Life Technologies Inc. (Burlington, ON, Canada). Tissue culture plates from Sarstedt (St Leonard, QC, Canada); fetal bovine serum and antibiotics from Wisent Inc. (Montréal, QC, Canada). The other chemicals used were of molecular biology grade available from Laboratoire Mat or Fisher Biotech (Québec, QC, Canada). Oxytocin and PMA were from (Sigma-Aldrich, Oakville, ONT, Canada) Arachidonic acid, Fluprostenol, NS-398, AH-6908 and AL-8810 were from Cayman (Chemicals, Ann Arbor, MI). Recombinant ovine IFN-τ was donated by Drs. F. W. Bazer and T. E. Spencer (Animal Biotechnology Laboratory, Texas A&M University, College Station, TX).

Cell culture

The bovine endometrial epithelial cell line (bEEL) and bovine caruncular stromal cells (CSC) as well as human endometrial epithelial cell line (HIEEC-22) and stromal cells (HIESC-2) were cultured as described previously (Krishnaswamy, Lacroix-Pepin et al.). Briefly, a frozen aliquot was grown in RPMI-1640 medium without phenol red (Gibco-BRL (Invitrogen), Mississauga, ON, Canada) supplemented with 10% FBS and 50 IU penicillin-streptomycin (Wisent Inc, QUE); confluent cultures were trypsinized and seeded at $4X10^4$ cells per milliliter. Confluent cells were conditioned overnight with fresh RPMI medium before treatment.

Treatment protocol

Cells were grown for 3 days in 24 well plates and culture medium was replaced with fresh serum-free RPMI 1640 which was also used as the solvent for the reagents. The cells were treated in the presence or absence of agonist and/or inhibitor for 6h for bovine cells and 24h for human cells. Supernatant was harvested and stored at -20^oC until assay of PGs.

PGE2 and PGF2α Immunoassay

Prostaglandins were assayed by competitive EIA using acetylcholinesterase–linked PG tracers (Cayman, Ann Arbor, MI, USA) as described previously (Asselin, Goff et al. 1996).

Statistical analysis

Data were analyzed using GraphPad Prism 5 program. One-way ANOVA with Bonferroni as post-hoc test with 95% confidence intervals was used for statistical significance. All numerical data are presented as the mean ± SEM, and differences were considered as statistically significant at the 95% confidence level ($p < 0.05$). Each experiment was repeated at least three times in triplicate.

RESULTS

Cell-specific stimulation in bovine endometrial cells.

As described in the materials and methods section, endometrial cells were cultured and treated with various compounds to ensure specificity of cell type in terms of response. Oxytocin was used as a stimulator of PG production specific for epithelial cells (Krishnaswamy, Danyod et al. 2009). Accordingly, both PGE2 and PGF2α were increased in response to OT in epithelial cells (Fig. 1A and B) whereas stromal cells did not respond (Fig. 1D and E). IL-1α and to a lesser extent IL-1β were shown as potent stimulators of PG production in stromal but not epithelial cells (Nishimura, Bowolaksono et al. 2004; Tanikawa, Acosta et al. 2005; Tanikawa, Kim et al. 2009). As was shown by others, bovine epithelial cells did not react to either IL-1 (Fig. 1C) but stromal cells were stimulated (Fig. 1F) with a preference for IL-1α at higher doses (Nishimura, Bowolaksono et al. 2004; Tanikawa, Acosta et al. 2005; Tanikawa, Kim et al. 2009). As previously reported, IL-1α stimulated preferentially PGE2 production. In a second experiment endometrial epithelial cells were treated with oxytocin in presence and absence of interferon-tau (Figure 2A) while stomal cells were treated with the PKC agonist PMA and interferon-tau. In all cases interferon-tau reduced the stimulation response. We have identified the primary PGFsynthase in the endometrium as AKR1B1; accordingly, epithelial and stromal cells were treated with AKR1B inhibitors (AKRi) (Madore, Harvey et al. 2003). The AKRi Ponalrestat (Statil) was found more efficient to block PG production in both epithelial (Fig. 2C) and stromal cells (Fig. 2D). This is similar to what was we reported for the human endometrial system (Bresson, Lacroix-Pepin et al. 2012).

Time course of induction of PGE2 and PGF2α in bovine cells.

The time-dependent release of PGs following stimulation with OT in epithelial cells and TNFa in stromal cells was done in bovine endometrial cells. Bovine endometrial cells were treated to asses results previously found in HIESC-2 and HIEEC-22 that PGF2α production precedes the PGE2 increase (Bresson, Lacroix-Pepin et al. 2012). Epithelial cells (bEEL) were treated with OT (500 nM) for 6 hours (Fig. 3A) and aliquots were taken at various time points over the first 120 min. No variation in PGE2 was observed during the first 60 min (Fig. 3C) while PGF2α showed an exponential increase that started after 10-20 min of incubation to become significant after 30 min (Fig. 3E). Bovine endometrial stromal cells (CSC) were treated with TNF-α (10 ng/ml) for 6 hours (Fig. 3B) and aliquots were taken over the first 120 min. No variation of PGE2 could be found during the first 120 min (Fig. 3D) while PGF2α showed an increase that started after 60 min of incubation (Fig. 3F).

Impact of PG receptor inhibitors on stimulated PGs production.

Bovine endometrial cells were treated with various compounds with or without prostaglandin receptor antagonists to test if PGF2α could modulate PGE2 as was observed in human HIESC-2 cells (Bresson, Lacroix-Pepin et al. 2012). Treatment of bovine epithelial cells (bEEL) with arachidonic acid (10 μM) increased prostaglandins as expected and EP inhibitor AH-6809 did not significantly affect either PGE2 or PGF2α production (Fig. 4A) whereas AL-8810 an FP receptor antagonist reduced both the release of both PGs (Fig. 4B). In stromal cells (CSC) treated with IL-1α (1ng/ml) AH-6809 did not influence PG production (Fig. 4C), but AL-8810 clearly reduced prostaglandin production down to control levels (Fig. 4D). In the same cells treated with TNF-α (10 ng/ml) similar responses were observed (Fig. 4E and F). By contrast, when CSC was treated with Arachidonic Acid (10 μM) both inhibitors were able to decrease the production of both PGs (Fig. 4G and H).

Evaluation of Oxytocin stimulation on FP regulation of PGE2.

The bovine oxytocin receptor signaling pathway trough PLCβ-PKC-EGFR transactivation and PI3K-Akt involved in increased PG production (Krishnaswamy, Lacroix-Pepin et al. 2010), is similar to the reported FP receptor pathway. Accordingly, the selectivity of the PG receptor antagonists used in the present study was tested in presence of oxytocin (500 nM) and PMA (10 nM). The EP receptor antagonist AH-6809 did not have any effect on PG production when bEEL cells were treated with oxytocin (Fig. 5A). Similarly, no decrease of prostaglandins could be seen with the FP receptor antagonist AL-8810 when bEEL cells were treated with oxytocin (Fig. 5B) or PMA (Fig. 5C).

DISCUSSION

Prostaglandin F2α and its signaling pathway represent a neglected aspect in the field of prostaglandin action. The PGF2α/FP system is however clearly involved in endometrial pathologies including menstrual disorders and endometrial cancer (Milne and Jabbour 2003; Jabbour and Sales 2004; Sales, List et al. 2005; Catalano, Wilson et al. 2011) as well as premature delivery (Phillips, Al-Zamil et al. 2011).

Interferon-tau is recognized as the primary embryonic signal in ruminants, but the mechanisms underlying recognition of pregnancy remain elusive. A complex cross talk involving endometrial stromal and epithelial cells and the embryo must take place during this period. Indeed, while epithelial cells are in direct contact with the embryo and IFN-tau, IFN-τ stimulated genes (ISGs) are mainly located in the stroma of the cattle endometrium (Spencer, Sandra et al. 2008; Mansouri-Attia, Aubert et al. 2009). In case of non-productive cycles, oxytocin stimulation of epithelial PGF2α initiates luteolysis. In our model, oxytocin strongly

stimulated the production of both PGE2 and PGF2α in epithelial (Fig.1A and B) but not in stromal cells (Fig. 1D and E) in direct relation with the reported presence of OT receptors. The stimulated prostaglandin production was inhibited by interferon-tau in both cell types (Fig. 2A and B). It is generally accepted that INF-τ can down regulate the expression of OTR but we have shown that this was not a strict requirement for IFN to inhibit OT stimulated PG production (Krishnaswamy, Danyod et al. 2009). In addition to OT action on epithelial cells, it was proposed that TNF-α could induce the secretion of subluteolytic levels of PGF2α by stromal cells (Skarzynski, Uenoyama et al. 1999; Okuda and Sakumoto 2006).

Increasing evidence suggests that interleukins play critical roles for recognition of pregnancy in both human and cattle (Leung, Cheng et al. 2001; Nishimura, Bowolaksono et al. 2004; Tanikawa, Acosta et al. 2005; Okuda and Sakumoto 2006). In vivo, administration of IL-1α into the uterus was able to prolong the lifespan of the corpus luteum (Majewska, Woclawek-Potocka et al. 2010) and in the bovine endometrium IL-1α was shown to stimulate prostaglandins specifically in stromal cells (Nishimura, Bowolaksono et al. 2004; Tanikawa, Lee et al. 2008; Tanikawa, Kim et al. 2009). Accordingly, we observe in the present study that IL-1α stimulates PGE2 production without altering PGF2α (Fig. 1C and F).

In the bovine endometrium, AKR1B1 appears as the main enzyme responsible for the production of PGF2α (Madore, Harvey et al. 2003); and can be inhibited by AKR1B inhibitor such as ponalrestat (statil) (Madore, Harvey et al. 2003; Lacroix Pepin, Chapdelaine et al. 2013). The potency of different AKR1B inhibitors to inhibit PGF2a biosynthesis may however differ because ponalrestat was much more efficient than EBPC (Fig. 2C and D). Since ponalrestat is a fairly selective inhibitor of AKR1B1, we would have expected that only PGF2α would be decreased, but PGE2 was also reduced. This finding is similar to what we observed in human endometrial cells (Bresson, Boucher-Kovalik et al. 2011; Bresson, Lacroix-Pepin et al. 2012) and in response to interferon-tau in bovine endometrial cells (Parent, Chapdelaine et al. 2002; Parent, Villeneuve et al. 2003; Arosh, Banu et al. 2004; Parent and Fortier 2005; Krishnaswamy, Danyod et al. 2009) (Fig. 2A and B).

When we observed in human that inhibition of FP receptor prevented the increase in PGE2 and that FP receptor stimulation alone was sufficient to stimulate PGE2 production, we proposed that there was a positive feedback loop between PGF2α release and production of PGE2 (Bresson, Lacroix-Pepin et al. 2012). A link between FP activation and increased PG production could have been anticipated from previous observations in a human endometrial model (Jabbour, Sales et al. 2005; Sales, Grant et al. 2008) and from our own observations in bovine endometrial cells (Krishnaswamy, Danyod et al. 2009). However, this is the first evidence of a specific link between FP activation and PGE2 production within a homogeneous cell system with potential physiological and pathological relevance in both human and bovine.

One of the first evidence of a feedback loop in human was the fact that increased PGF2α production was observed 6 hrs. before that of PGE2 (Bresson, Lacroix-Pepin et al. 2012). In the present study, increased PGF2α production can be detected 20-30 min after stimulation of epithelial cells with oxytocin, whereas PGE2 production does not change in the first hour (Fig. 3C and E) but accelerates to become higher at 6 hrs (Fig. 3A). In stromal cells, upon stimulation with TNF-α, PGF2α production becomes significantly increased after 60 min while PGE2 production does not change in the first 2 hrs (Fig. 3D and F) before increasing sharply to become higher at 6 hrs (Fig. 3B).

To test if the feedback loop observed in bovine could be triggered by both EP and FP receptor, we used the EP2 antagonist AH-6809 and the FP antagonist AL-8810. In both epithelial and stromal cells, AH-6809 did not affect the production of either prostaglandin in the two cell types. On the other hand, AL-8810 induced a decrease of both PGE2 and PGF2α in both cell types (Fig. 3). Since oxytocin and FP receptor share similar transduction signals, we tested the possibility that oxytocin stimulation could compensate for FP inhibition. Indeed, AH-6809 did not have any effect on PG production; neither did AL-8810 (Fig. 5A and B). PMA, a direct stimulator of PKC was used to mimic OTR or FP signal and was not affected by AL-8810 either (Fig 5C).

Taken together, these data show that PGE2 production seems to be stimulated by increased PGF2α levels and that in absence of PGF2α, PGE2 production is compromised. If we hypothesize that PGE2 acting on EP2 receptors and PGF2α acting through FP work as a dyad with opposite actions in the reproductive system, just like the thromboxane/prostacyclin dyad in the vascular system, then the interactions between the two systems are not only probable but also necessary. This feedback loop does not seem to be working for both prostaglandins but being rather a compensatory mechanism for aberrant PGF2α production. Further characterization is required to clearly demonstrate such a loop. The use of an FP receptor agonist in combination with COX inhibitors could indicate which enzymes are directly regulated by FP receptor. A knockout approach for the bovine PGFS or FP receptor could also provide additional indication of the impact of PGF2α on PGE2 production.

REFERENCES

Abramovitz, M., M. Adam, et al. (2000). "The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs." Biochim Biophys Acta 1483(2): 285-93.

Abramovitz, M., Y. Boie, et al. (1994). "Cloning and expression of a cDNA for the human prostanoid FP receptor." J Biol Chem 269(4): 2632-6.

Arosh, J. A., S. K. Banu, et al. (2003). "Molecular cloning and characterization of bovine prostaglandin E2 receptors EP2 and EP4: expression and regulation in endometrium and myometrium during the estrous cycle and early pregnancy." Endocrinology 144(7): 3076-91.

Arosh, J. A., S. K. Banu, et al. (2004). "Temporal and tissue-specific expression of prostaglandin receptors EP2, EP3, EP4, FP, and cyclooxygenases 1 and 2 in uterus and fetal membranes during bovine pregnancy." Endocrinology 145(1): 407-17.

Arosh, J. A., S. K. Banu, et al. (2004). "Effect of interferon-tau on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2." Endocrinology 145(11): 5280-93.

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod 54(2): 371-9.

Bos, C. L., D. J. Richel, et al. (2004). "Prostanoids and prostanoid receptors in signal transduction." Int J Biochem Cell Biol 36(7): 1187-205.

Bresson, E., S. Boucher-Kovalik, et al. (2011). "The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium." J Clin Endocrinol Metab 96(1): 210-9.

Bresson, E., N. Lacroix-Pepin, et al. (2012). "The Prostaglandin F Synthase Activity of the Human Aldose Reductase AKR1B1 Brings New Lenses to Look at Pathologic Conditions." Front Pharmacol 3: 98.

Breyer, R. M., C. K. Bagdassarian, et al. (2001). "Prostanoid receptors: subtypes and signaling." Annu Rev Pharmacol Toxicol 41: 661-90.

Catalano, R. D., M. R. Wilson, et al. "Comprehensive expression analysis of prostanoid enzymes and receptors in the human endometrium across the menstrual cycle." Mol Hum Reprod 17(3): 182-92.

Catalano, R. D., M. R. Wilson, et al. (2011). "Comprehensive expression analysis of prostanoid enzymes and receptors in the human endometrium across the menstrual cycle." Mol Hum Reprod 17(3): 182-92.

Clapham, D. E. and E. J. Neer (1997). "G protein beta gamma subunits." Annu Rev Pharmacol Toxicol 37: 167-203.

Fortier, M. A., K. Krishnaswamy, et al. (2008). "A postgenomic integrated view of prostaglandins in reproduction: implications for other body systems." J Physiol Pharmacol 59 Suppl 1: 65-89.

Hsiao, H. Y., O. T. Mak, et al. (2007). "TNF-alpha/IFN-gamma-induced iNOS expression increased by prostaglandin E2 in rat primary astrocytes via EP2-evoked cAMP/PKA and intracellular calcium signaling." Glia 55(2): 214-23.

Jabbour, H. N. and K. J. Sales (2004). "Prostaglandin receptor signalling and function in human endometrial pathology." Trends Endocrinol Metab 15(8): 398-404.

Krishnaswamy, N., P. Chapdelaine, et al. (2009). "Development and characterization of a simian virus 40 immortalized bovine endometrial stromal cell line." Endocrinology 150(1): 485-91.

Krishnaswamy, N., G. Danyod, et al. (2009). "Oxytocin receptor down-regulation is not necessary for reducing oxytocin-induced prostaglandin F(2alpha) accumulation by interferon-tau in a bovine endometrial epithelial cell line." Endocrinology 150(2): 897-905.

Krishnaswamy, N., N. Lacroix-Pepin, et al. (2010). "Epidermal growth factor receptor is an obligatory intermediate for oxytocin-induced cyclooxygenase 2 expression and prostaglandin F2 alpha production in bovine endometrial epithelial cells." Endocrinology 151(3): 1367-74.

Lacroix Pepin, N., P. Chapdelaine, et al. (2013). "Evaluation of the prostaglandin F synthase activity of human and bovine aldo-keto reductases: AKR1A1s complement AKR1B1s as potent PGF synthases." Prostaglandins Other Lipid Mediat.

Lee, J., S. K. Banu, et al. (2012). "Early pregnancy induced expression of prostaglandin E2 receptors EP2 and EP4 in the ovine endometrium and regulated by interferon tau through multiple cell signaling pathways." Mol Cell Endocrinol 348(1): 211-23.

Leung, S. T., Z. Cheng, et al. (2001). "The effects of lipopolysaccharide and interleukins-1alpha, -2 and -6 on oxytocin receptor expression and prostaglandin production in bovine endometrium." J Endocrinol 168(3): 497-508.

Lundstrom, V. and K. Green (1978). "Endogenous levels of prostaglandin F2alpha and its main metabolites in plasma and endometrium of normal and dysmenorrheic women." Am J Obstet Gynecol 130(6): 640-6.

Madore, E., N. Harvey, et al. (2003). "An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium." J Biol Chem 278(13): 11205-12.

Majewska, M., I. Woclawek-Potocka, et al. (2010). "Is interleukin-1alpha a luteotrophic or luteolytic agent in cattle?" Reproduction 139(3): 665-72.

Mansouri-Attia, N., J. Aubert, et al. (2009). "Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation." Physiol Genomics 39(1): 14-27.

Markworth, J. F. and D. Cameron-Smith (2011). "Prostaglandin F2α stimulates PI3K/ERK/mTOR signaling and skeletal myotube hypertrophy." Am J Physiol Cell Physiol 300(3): C671-82.

McCracken, J. A., E. E. Custer, et al. (1999). "Luteolysis: a neuroendocrine-mediated event." Physiol Rev 79(2): 263-323.

Melien, O., G. H. Thoresen, et al. (1998). "Activation of p42/p44 mitogen-activated protein kinase by angiotensin II, vasopressin, norepinephrine, and prostaglandin F2alpha in hepatocytes is sustained, and like the effect of epidermal growth factor, mediated through pertussis toxin-sensitive mechanisms." J Cell Physiol 175(3): 348-58.

Milne, S. A. and H. N. Jabbour (2003). "Prostaglandin (PG) F(2alpha) receptor expression and signaling in human endometrium: role of PGF(2alpha) in epithelial cell proliferation." J Clin Endocrinol Metab 88(4): 1825-32.

Nishimura, R., A. Bowolaksono, et al. (2004). "Possible role of interleukin-1 in the regulation of bovine corpus luteum throughout the luteal phase." Biol Reprod 71(5): 1688-93.

Ohmichi, M., K. Koike, et al. (1997). "Role of mitogen-activated protein kinase pathway in prostaglandin F2alpha-induced rat puerperal uterine contraction." Endocrinology 138(8): 3103-11.

Okuda, K. and R. Sakumoto (2006). "Regulation of uterine function by cytokines in cows: Possible actions of tumor necrosis factor-alpha, interleukin-1 alpha and interferon-tau." Animal Science Journal 77(3): 266-274.

Parent, J. and M. A. Fortier (2005). "Expression and contribution of three different isoforms of prostaglandin E synthase in the bovine endometrium." Biol Reprod 73(1): 36-44.

Parent, J., C. Villeneuve, et al. (2003). "Influence of different isoforms of recombinant trophoblastic interferons on prostaglandin production in cultured bovine endometrial cells." Biol Reprod 68(3): 1035-43.

Phillips, R. J., H. Al-Zamil, et al. (2011). "Genes for prostaglandin synthesis, transport and inactivation are differentially expressed in human uterine tissues, and the prostaglandin F synthase AKR1B1 is induced in myometrial cells by inflammatory cytokines." Mol Hum Reprod 17(1): 1-13.

Regan, J. W. (2003). "EP2 and EP4 prostanoid receptor signaling." Life Sci 74(2-3): 143-53.

Sales, K. J., V. Grant, et al. (2008). "Prostaglandin E2 and F2alpha activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element." Mol Cell Endocrinol 285(1-2): 51-61.

Sales, K. J. and H. N. Jabbour (2003). "Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology." Prostaglandins Other Lipid Mediat 71(3-4): 97-117.

Sales, K. J., T. List, et al. (2005). "A novel angiogenic role for prostaglandin F2alpha-FP receptor interaction in human endometrial adenocarcinomas." Cancer Res 65(17): 7707-16.

Skarzynski, D. J., Y. Miyamoto, et al. (2000). "Production of prostaglandin f(2alpha) by cultured bovine endometrial cells in response to tumor necrosis factor alpha: cell type specificity and intracellular mechanisms." Biol Reprod 62(5): 1116-20.

Smith, O. P., H. N. Jabbour, et al. (2007). "Cyclooxygenase enzyme expression and E series prostaglandin receptor signalling are enhanced in heavy menstruation." Hum Reprod 22(5): 1450-6.

Smith, W. L., Y. Urade, et al. (2011). "Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis." Chem Rev 111(10): 5821-65.

Smrcka, A. V. (2008). "G protein betagamma subunits: central mediators of G protein-coupled receptor signaling." Cell Mol Life Sci 65(14): 2191-214.

Spencer, T. E., O. Sandra, et al. (2008). "Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches." Reproduction 135(2): 165-79.

Tanikawa, M., T. J. Acosta, et al. (2005). "Regulation of prostaglandin synthesis by interleukin-1alpha in bovine endometrium during the estrous cycle." Prostaglandins Other Lipid Mediat 78(1-4): 279-90.

Tanikawa, M., T. S. Kim, et al. (2009). "Cell-type specificity of interleukins 1alpha and 1beta on prostaglandin and plasminogen activator production in bovine endometrial cells." Anim Reprod Sci 114(1-3): 32-42.

Tanikawa, M., H. Y. Lee, et al. (2008). "Regulation of prostaglandin biosynthesis by interleukin-1 in cultured bovine endometrial cells." J Endocrinol 199(3): 425-34.

FIGURES

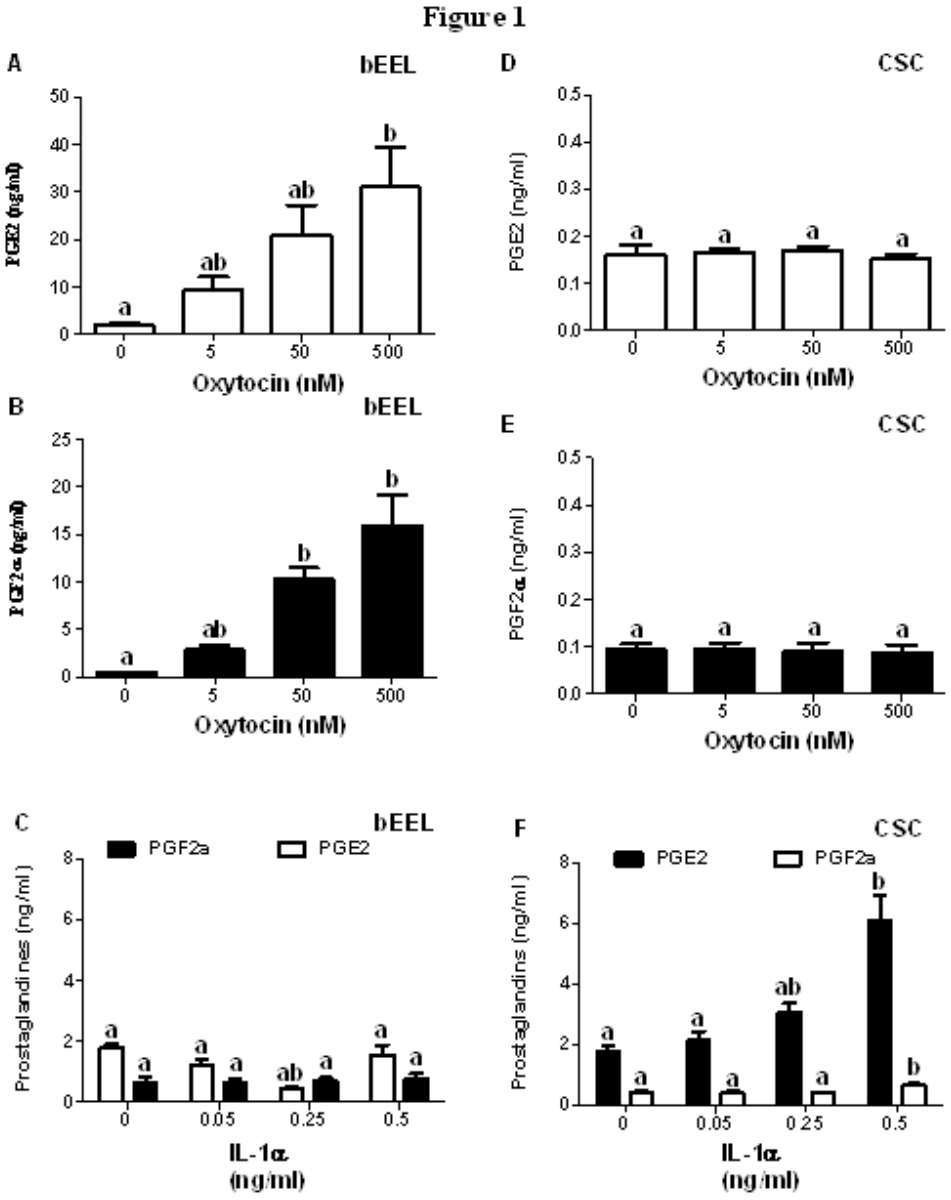


Figure 39 - Article 6: Figure 1 Cell-specific stimulation in bovine endometrial cells.

Bovine endometrial epithelial cells (bEEL) (A, B and C) and endometrial stromal celles (CSC) (D, E and F) were cultured as described in materials and methods and treated with various concentration of Oxytocin (0-500 nM) (A, B, D and E) or IL-1α (1 ng/ml) (C and F) for 6 hrs. PGE2 (A, C, D and F) and PGF2α (B, C, E and F) was measured. Results represent the mean ± SEM of three independent experiments run in triplicate. Bars with different superscripts are significantly different (P<0.05).

Figure 2

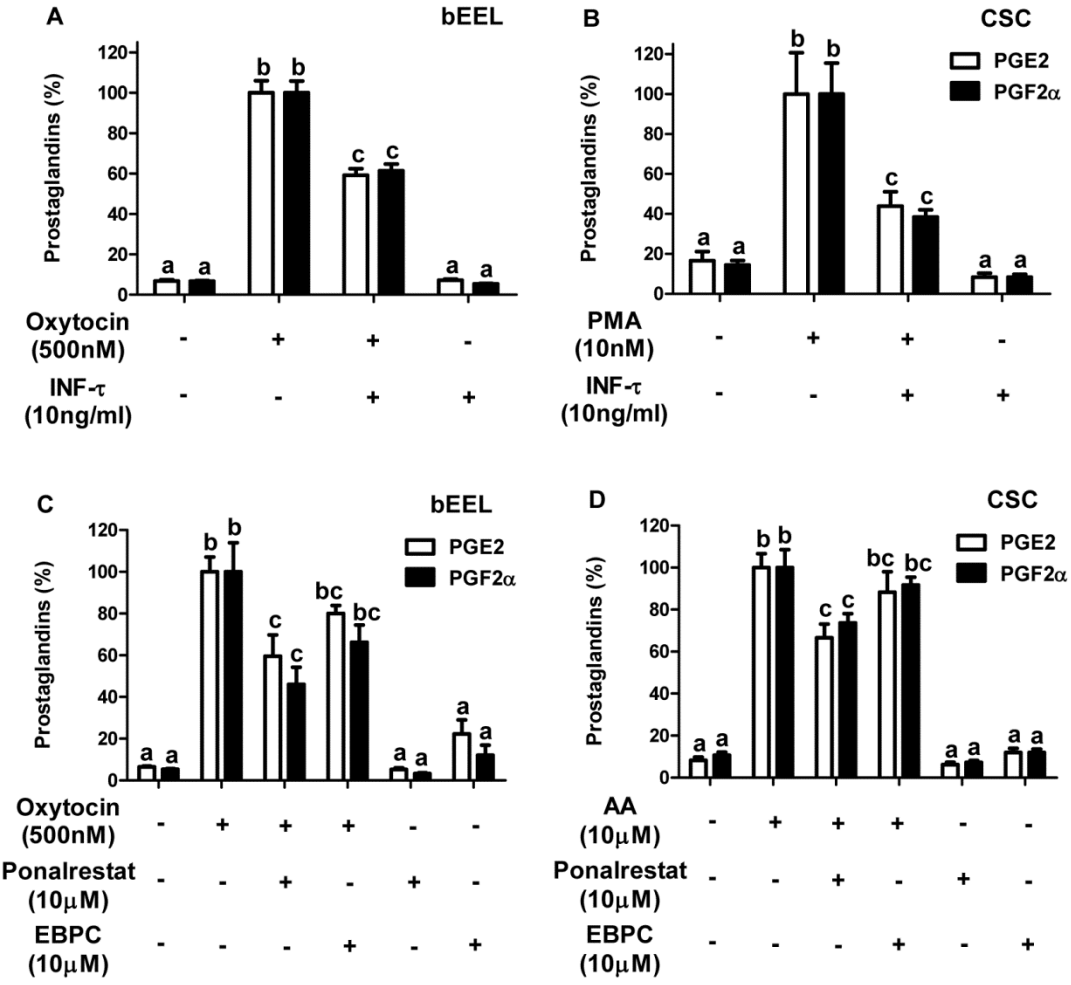


Figure 40 - Article 6: Figure 2 Inhibition of prostaglandin production.

Bovine endometrial epithelial cells (bEEL) (A and C) and endometrial stromal celles (CSC) (B and D) were cultured as described in materials and methods and treated with Oxytocin (500 nM) (A and C), PMA (1 ng/ml) (B) or Arachidonic acid (10 μM) (D) for 6 hrs in presence of interferon-tau (10ng/ml) (A and B) or AKR inhibitor Ponalrestat or EBPC (C and D). PGE2 and PGF2α were measured. Results represent the mean ± SEM of three independent experiments run in triplicate (in percentage of PGs produced compared to maximal condition (stimulation without receptor inhibitor). Bars with different superscripts are significantly different ($P<0.05$).

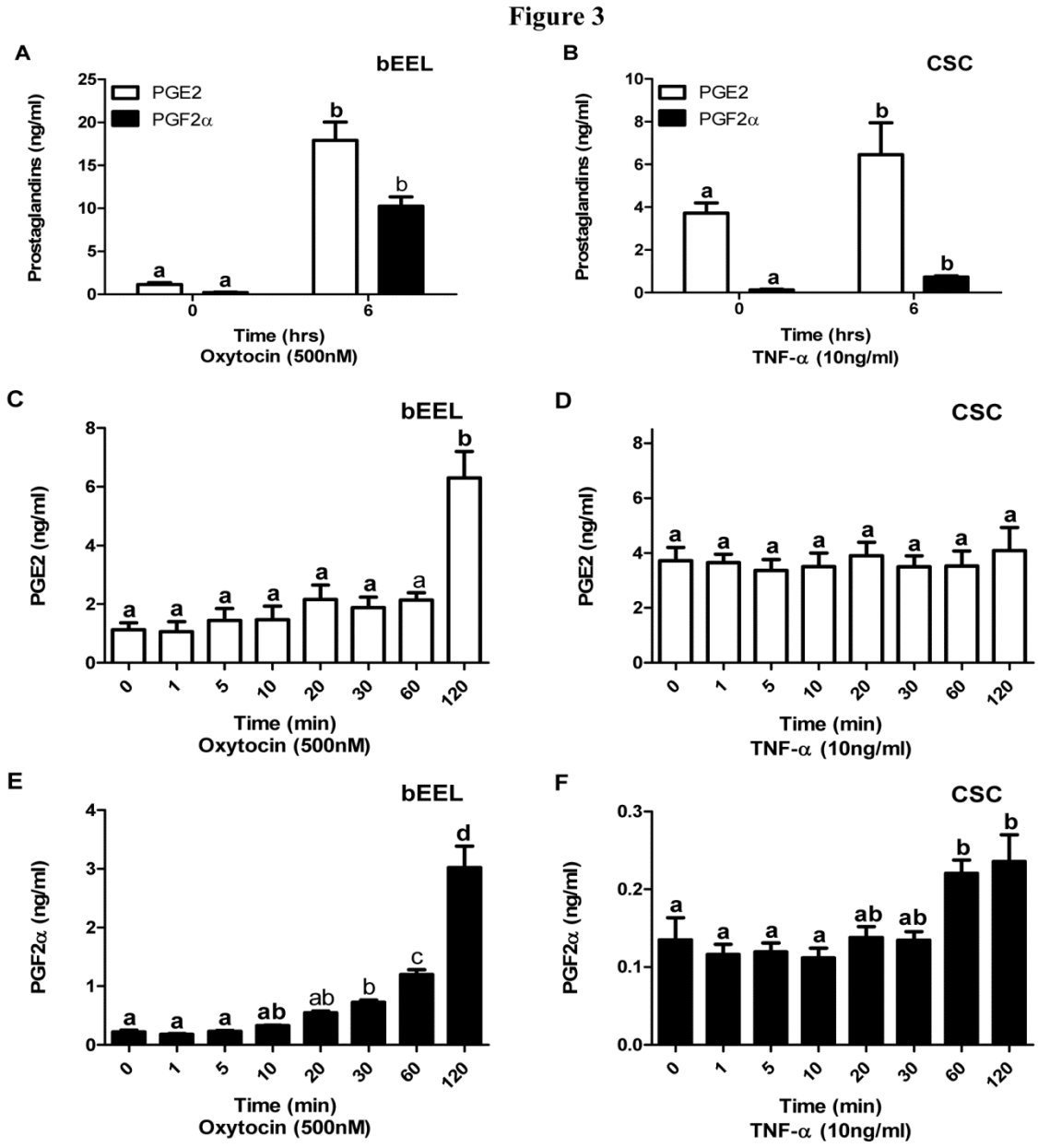


Figure 41 - Article 6: Figure 3 Time course induction of PGE2 and PGF2α in bovine cells.

Bovine endometrial epithelial cells (bEEL) and endometrial stromal cells (CSC) were cultured as described in materials and methods and treated with OT (500 nM) or TNF-α (10 ng/ml) for 6 hrs (A and B). Aliquots were taken at 1, 5, 10, 20, 30, 60, 120 min (C, D, E and F). PGE2 (A, B, C and D) and PGF2α (A, B, E and F) were measured. Results represent the mean ± SEM of three independent experiments run in triplicate. Bars with different superscripts are significantly different (P<0.05).

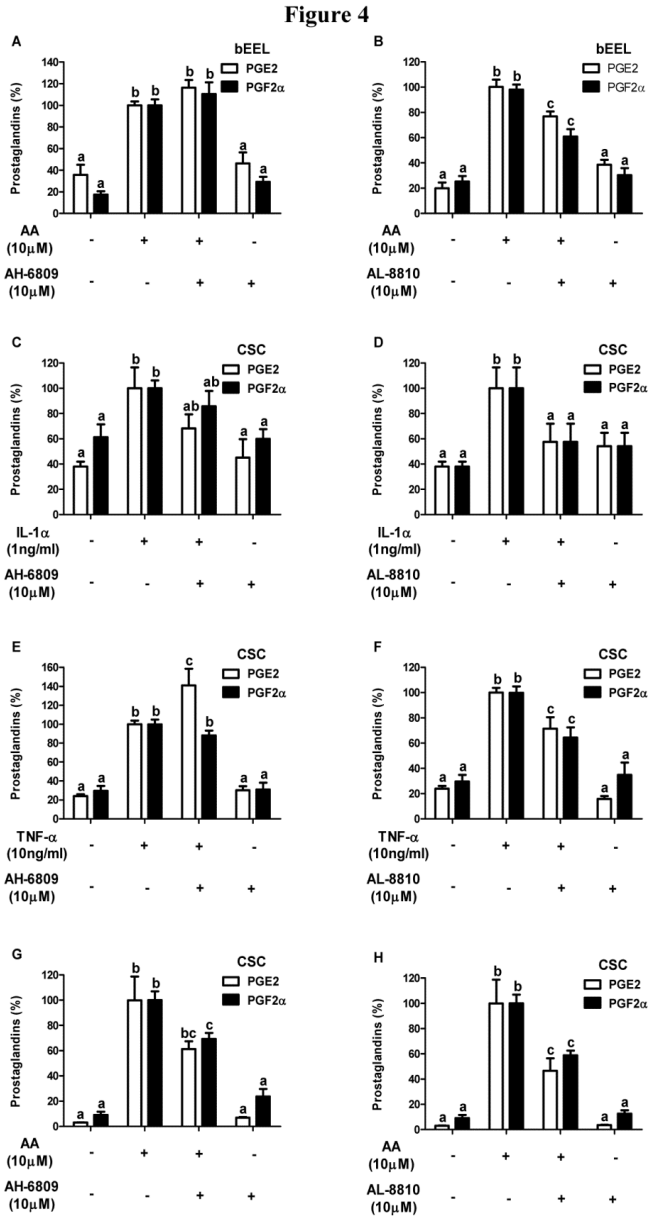


Figure 42 - Article 6: Figure 4 Impact of prostaglandins receptor inhibitor on stimulated PGs production in bovine endometrial cells.

Bovine endometrial epithelial cells (bEEL) and endometrial stromal cells (CSC) were cultured as described in materials and methods and treated with Arachidonic Acid (10 μM) (A and B), IL-1α (1ng/ml) (C and D) or TNF-α (10ng/ml) (E and F) for 6 hrs in presence of either EP receptor inhibitor AH-6809 (10 μM) (A, C and E) or FP receptor inhibitor AL-8810 (10 μM) (B, D and F). PGE2 and PGF2α were measured. Results represent the mean ± SEM of three independent experiments run in triplicate in percentage of PGs produced compared to maximal condition (stimulation without receptor inhibitor). Bars with different superscripts are significantly different ($P<0.05$).

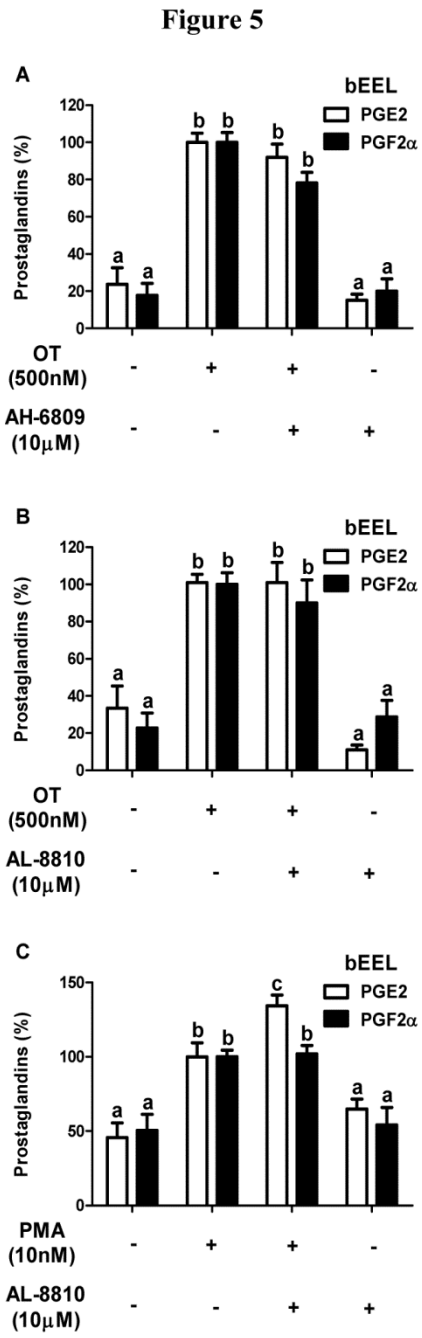


Figure 43 - Article 6: Figure 5 Impact of prostaglandins receptor inhibitor on Oxytocin and PMA stimulated PGs production in bovine endometrial epithelial cells.

Bovine endometrial epithelial cells (bEEL) were treated with either Oxytocin (500 nM) (A and B) or PMA (10 nM) (C) for 6hrs in presence of either EP receptor inhibitor AH-8809 (10 μM) (A) or FP receptor inhibitor AL-8810 (10 μM) (B and C). PGE2 and PGF2α were measured for these conditions. Results represent the mean ± SEM of three independent experiments run in triplicate in percentage of PGs produced compared to maximal condition (stimulation without inhibitor). Bars with different superscripts are significantly different ($P<0.05$).

Chapitre 8: discussion générale et conclusions

La prostaglandine F2α est beaucoup moins étudiée que la PGE2 dans les maladies humaines (Miller 2006). Pourtant, le système PGF2α/FP est impliqué dans plusieurs pathologies et il est étonnant de constater que les mécanismes entourant sa régulation et son impact ne soient pas mieux étudiés. L'augmentation de la PGF2α en fin de cycle chez le bovin et son interruption par l'interféron-tau est au centre de la cyclicité bovine et de la reconnaissance de la gestation. Ceci en fait probablement l'exemple le plus frappant de son importance physiologique. Le premier objectif de mon projet de recherche de doctoral visait par conséquent à mieux caractériser les phénomènes entourant la production de PGF2α chez le bovin. Ceci, en étudiant les mécanismes stimulant la production de PGF2α, son transport et sa synthèse afin d'évaluer comment l'IFN-τ pourrait les influencer.

Premièrement, la modulation de la réponse en prostaglandines à l'ocytocine est le principal mode d'action de l'IFN-τ lors de la reconnaissance de la gestation. Puisqu'aucune enzyme ni récepteur ne semble être diminué alors que la cellule diminue sa production de prostaglandines en réaction à l'IFN-τ, le signal en aval du récepteur OT semble être la cible directe de l'IFN-τ. La voie métabolique menant à l'augmentation de COX-2 et du PGF2α suite à la liaison de l'ocytocine à son récepteur était somme toute assez mal caractérisée chez le bovin (Roberts, Chen et al. 2008). Les résultats présentés dans le chapitre 2 ont permis de caractériser de façon plus approfondie les acteurs impliqués dans cette voie afin de pouvoir déceler des cibles potentielles.

Cette étude a montré l'impact d'EGFR-ERK1/2-Akt sur l'augmentation de COX-2 et du PGF2α. Comme l'IFN-τ peut diminuer les prostaglandines sans affecter directement la hausse de COX-2 (Krishnaswamy, Danyod et al. 2009) ceci semble indiquer que son mécanisme d'action principal ne se situe pas au niveau du blocage total de cette voie métabolique. L'impact de l'IFN-τ sur les autres enzymes de biosynthèse et les sites de production n'a cependant pas encore été élucidé. Comme COX-2 n'est pas diminué par l'IFN-τ, si l'inhibition se produit au niveau de l'expression d'une enzyme de biosynthèse, cela nous laisse donc les phospholipases ou les synthases terminales comme candidates. Les deux prostaglandines étant inhibées de façon équivalente par l'IFN-τ, il ne semble pas que ce dernier en favorise une plus qu'une autre par son action d'inhibition (Danet-Desnoyers, Wetzels et al. 1994), suggérant que son mode d'action est équivalent pour les deux. Cela pourrait donc laisser croire que c'est cPLA2 qui est affectée. Une autre possibilité est que l'IFN-τ agisse sur la compartimentation/association des enzymes de biosynthèse, les éloignant des sites de production des prostaglandines. On sait déjà que des associations entre les COX et les

mPGES existent (Murakami, Naraba et al. 2000; Tanioka, Nakatani et al. 2000; Murakami, Nakashima et al. 2003) et il serait très possible qu'une association similaire soit aussi existante pour la PGFS bovine. Le lieu exact de la production des diverses prostaglandines dans les cellules endométriales bovines est un facteur pour le moment inconnu. Trois sites principaux sont possibles. La membrane périnucléaire, des phagosomes ou encore des corps lipidiques sont des sites de production déjà démontrés dans d'autres modèles cellulaires (Bandeira-Melo, Weller et al. 2011). Il serait intéressant de traiter les cellules épithéliales ou stromales à l'IFN-τ afin de voir s'il influence le site de production des prostaglandines, par exemple, en diminuant la formation des corps lipidiques.

Deuxièmement, une autre cible potentielle de l'action de l'IFN-τ dans son action sur le signal lutéolytique du PGF2α est le passage des prostaglandines de l'utérus vers l'ovaire afin de permettre leur action sur le corps jaune (Banu, Lee et al. 2010; Lee, McCracken et al. 2010). Comme PGT est principalement un transporteur d'influx (Chan, Endo et al. 2002), il reste à déterminer si l'efflux des prostaglandines se produit via un transporteur ou par diffusion passive (Schuster 2002; Chi, Khersonsky et al. 2006; Banu, Lee et al. 2008; Banu, Lee et al. 2010). La lenteur et le manque d'efficacité de la diffusion passive (Reid, Wielinga et al. 2003) rendent ce mécanisme improbable pour un métabolite ayant une action de régulation via des récepteurs extracellulaires. Nos résultats du chapitre 3 ont permis d'élucider l'importance de MRP4 dans l'efflux de prostaglandines par les cellules épithéliales bovines. Cependant, la production polarisée des cellules épithéliales semble indépendante de l'action de l'IFN-τ, signifiant que son action ne se situe pas à cette étape mais bien lors leur formation.

Les cellules stromales doivent absorber par influx les prostaglandines des cellules épithéliales et les relâcher par efflux pour permettre un transport rapide. Néanmoins, étant donné que les cellules stromales ne sont pas polarisées, il se formera très certainement un gradient de prostaglandines du stroma jusqu'à la circulation. Dès qu'une cellule relâche des prostaglandines, elles ont la possibilité d'exercer un impact autocrine et paracrine en fonction de l'expression des récepteurs appropriés. Il serait donc très intéressant d'évaluer dans cette condition comment le récepteur FP stimulé par les fortes concentrations de PGF2α produites par les cellules épithéliales influencera les cellules stromales. Entre autres, comme l'ocytocine semble réguler la quantité de MRP4 dans les cellules épithéliales, il serait intéressant de voir l'effet du PGF2α en relation avec la quantité de MRP4 dans le stroma puisque OTR et FP ont des voies de signalisation très similaires.

Troisièmement, la formation de PGF2α chez le bovin semble provenir de l'activité PGFS de bAKR1B1 (Madore, Harvey et al. 2003). Cependant, dans les cellules épithéliales (Krishnaswamy, Chapdelaine et al. 2009) ou stomales (Krishnaswamy, Danyod et al. 2009), aucune variation des niveaux

protéiques de bAKR1B1 n'a pu être observée malgré des fluctuations importantes de PGF2α. Ceci est en opposition à la nette augmentation de hAKR1B1 en réponse à l'IL-1β dans les cellules endométriales humaines (Bresson, Boucher-Kovalik et al. 2011). L'absence de régulation de la protéine combinée à l'absence de réponse convaincante suite à l'utilisation d'inhibiteurs de hAKR1B1 nous a poussés à douter que bAKR1B1 soit la seule PGFS active dans l'endomètre bovin. Nous avons donc tenté de chercher dans le chapitre 4 si d'autres enzymes pouvaient avec ou sans bAKR1B1 être responsables de la production de PGF2α dans cette espèce.

Parmi les enzymes identifiées comme ayant un potentiel de PGFS dans l'endomètre bovin, AKR1A1 fut une surprise puisque aucune AKR1A n'avait été identifiée comme PGFS chez un mammifère auparavant. Il fut étonnant, est de constater que AKR1A1 autant humaine que bovine était aussi, sinon plus, efficace qu'AKR1B1 pour produire du PGF2α. La forte activité PGFS de AKR1B1 chez les deux espèces en présence de COX-1 ou de COX-2 a aussi été confirmée. Aucune des deux AKR n'a semblé avoir une préférence d'association avec l'une où l'autre des deux COX. Ceci n'exclut cependant pas la possibilité d'associations intracellulaires préférentielles pour ces enzymes in vivo.

Le deuxième objectif était d'étudier chez l'humain la régulation des deux synthases connues soit AKR1B1 et AKR1C3 et leur impact relatif dans la production de PGF2α. En vue des résultats précédemment obtenus, hAKR1A1 devait aussi être considérée. Le chapitre 5 a servi à approfondir les connaissances sur hAKR1B1 dans nos cellules immortalisées afin de vérifier l'impact potentiel des autres synthases vis-à-vis de hAKR1B1. L'induction de la production de prostaglandines dans différent types cellulaires démontre par ailleurs que l'augmentation de hAKR1B1 n'est pas toujours nécessaire pour produire une augmentation de PGF2α. En effet, on n'observe pas de forte augmentation dans les cardiomyocytes et les cellules HUVEC. Il est cependant à noter que hAKR1B1 y est tout de même présent à un niveau important. Ceci pourrait rejoindre les observations chez le bovin où l'on dénote une augmentation de prostaglandines en présence, mais sans augmentation de bAKR1B1.

Par ailleurs, le Ponalrestat (Statil), un inhibiteur sélectif de hAKR1B1 n'ayant pas d'impact sur hAKR1A1 (Ward, Sennitt et al. 1990) (Chapitre 6), inhibe presque complètement la production de PGF2α autant dans les cellules stromales qu'épithéliales humaines. Cette inhibition est cependant beaucoup moins forte chez le bovin même à une dose 5 fois plus élevée. Pourtant, le ponalrestat était aussi efficace chez le bovin que l'humain lors des essais avec enzymes recombinantes (Chapitre 6 et figure 48). Ceci pourrait nous amener à croire que bAKR1A1 dans ce système pourrait jouer un rôle plus important que hAKR1A1, qui chez l'humain semble jouer un rôle mineur. Les inhibiteurs ne démontrent pas une sélectivité à toute épreuve et l'utilisation de siRNA a démontré qu'une diminution de 50% de hAKR1B1 diminuait de 50% la stimulation de

production de PGF2α par l'IL-1β (Bresson, Boucher-Kovalik et al. 2011). Ces résultats ne peuvent cependant pas préciser si le reste du PGF2α produit en de telles conditions est dû ou non à la présence d'autres PGFS. Ainsi, pour confirmer que hAKR1A1 est moins importante dans les cellules humaines que bovines, une perte complète de fonction de hAKR1B1 était nécessaire.

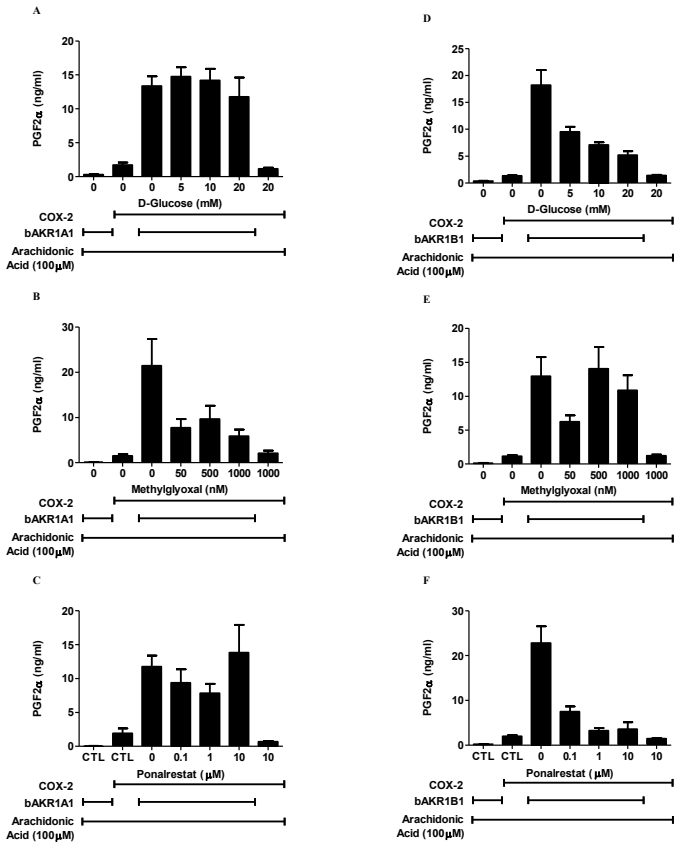


Figure 44 – Inhibition compétitive de l'activité PGFS de bAKR1A1 et bAKR1B1dans les mêmes conditions qu'à là figure 21.

Une approche permettant une complète absence de la protéine était donc de mise, afin de confirmer si hAKR1B1 était la seule enzyme pouvant faire du PGF2α dans l'utérus humain. Cette preuve requérait donc une invalidation génique par édition génomique dans les cellules d'intérêt. C'est ce qui a été tenté au chapitre 7.

Bien que l'approche ait été testée à la fois sur les cellules stromales et épithéliales humaines, seules les cellules stromales ont conduit à des lignées utilisables. À posteriori, la présence simultanée de trois PGFS,

hAKR1B1, hAKR1C3 et hAKR1A1 dans les cellules épithéliales aurait rendu plus difficile l'interprétation de nos résultats. Il serait cependant très intéressant de refaire la même expérience dans ces cellules afin de pouvoir comparer l'importance de hAKR1B1 dans les deux types cellulaires. De plus, malgré que la probabilité d'un impact d'AKR1A1 soit plus élevée chez le bovin, c'est chez l'humain que l'étude a débuté. La raison principale de ce choix était le faible taux de transfection des cellules épithéliales bovines, qui auraient autrement été des candidats de choix pour l'étude du PGF2α.

D'après les résultats obtenus, lors de stimulation par l'IL-1β, la presque totalité du PGF2α proviendrait d'une conversion du PGH2 par hAKR1B1. Une faible contribution de CBR1 qui peut convertir PGE2 en PGF2α ne peut cependant pas être exclue et pourrait expliquer le niveau basal qu'on continue d'observer en absence de hAKR1B1. La présence de hAKR1C3 et de hAKR1A1 dans les cellules épithéliales fait en sorte que dans ces dernières, plus d'un mécanisme de compensation pourrait être présent. Toutefois, aucun mécanisme ne semble pouvoir compenser la perte de hAKR1B1 pour la forte production de PGF2α induite par l'IL-1β au niveau du stroma. Ceci met l'emphase sur l'importance de cette enzyme dans la régulation du PGF2α et compte tenu de son activité envers plusieurs substrats, sur les interactions compétitives possibles avec le glucose en conditions diabétiques.

Le troisième objectif était de définir comment la communication via les récepteurs EP/FP influence les réponses subséquentes en prostaglandines ainsi que la communication entre les deux types cellulaires composant l'endomètre. En ce sens, on peut remarquer que la PGE2 semble toujours suivre la tendance du PGF2α, autant chez le bovin que chez l'humain (Parent, Villeneuve et al. 2003; Arosh, Banu et al. 2004; Krishnaswamy, Chapdelaine et al. 2009). Le fait que l'inhibition de la PGFS induise une diminution de PGE2, même en présence d'une augmentation de MPGES-1, combiné avec le fait que le PGE2 soit temporellement produit après le PGF2α lors de stimulation par l'IL-1β (chapitre 5) nous a poussés à croire qu'il pouvait exister une boucle de rétroaction entre PGE2 et PGF2α.

Les prostaglandines peuvent être considérées comme un mini système endocrinien local, réagissant aux changements afin de rétablir une condition optimale locale pour des phénomènes finement régulés tel que l'ovulation, la reconnaissance de la gestation et l'hémostase dans le système vasculaire. Il est démontré que l'utilisation simultanée de deux systèmes de régulation à action opposée permet un niveau de précision accru. Dans ces circonstances, les régulations observées entre les deux prostaglandines sont très probables. La boucle de rétroaction n'a cependant été observée que pour le récepteur FP. En effet, ce dernier semble contrôler les niveaux de PGE2 tandis que le récepteur EP ne semble pas avoir d'impact sur le PGF2α.

La boucle de rétroaction identifiée est fort probablement responsable des incidents cardiaques qui ont mené au retrait du Vioxx. En condition de glucose élevé, il y aura augmentation de hAKR1B1 mais le PGF2α demeurera faible étant donné la compétition entre le PGH2 et le glucose. Lorsque le glucose retombera à des niveaux plus bas, les forts niveaux de hAKR1B1 génèreront beaucoup de PGF2α qui sera heureusement compensée par la boucle de rétroaction et le relâchement de PGE2. Les tissus répondront cependant au PGE2 par de l'inflammation et de la douleur. En conséquence, une condition silencieuse résultant d'une compensation en PGE2 pour une surproduction de PGF2α engendrera la prise d'AINS. Dans ce cas, on peut émettre l'hypothèse que la prise d'un antidouleur puissant mais ayant peu d'effets secondaires tel que le rofecoxib (Vioxx), ciblant spécifiquement le PGE2 produit par MPGES-1, n'induira pas de diminution de PGF2α. Contrairement à ce qu'on aurait pu observer par la prise d'un inhibiteur d'AKR1B1. Donc, si quelqu'un prenant le Vioxx était dans une condition de surproduction de PGF2α, le mécanisme compensatoire PGE2/EP2 n'étant plus disponible pour contrecarrer les effets ischémiques, les risques de problèmes cardiaques augmenteront en flèche. La boucle de compensation étant seulement observée pour le récepteur FP, ceci apporte un point de vue nouveau sur le traitement des pathologies liées aux prostaglandines. Fait intéressant, même après ajustement, les patients diabétiques étaient 8 fois plus à risque de mourir d'accidents cardiaques sous Vioxx que les non-diabétiques (Baron, Sandler et al. 2008). En contraste, ceux prenant à la fois Vioxx et de l'aspirine (bloquant AKR1B1) étaient protégés.

L'une des principales perspectives suite à cette thèse est de valider l'impact relatif de bAKR1B1 et bAKR1A1 dans les cellules endométriales bovines. Une approche similaire à celle tentée au chapitre 6 pourrait s'avérer utile pour le démontrer. Cette approche permettrait très probablement aussi de mettre en lumière la présence ou non d'une boucle de rétroaction par le récepteur FP chez cette espèce et comment l'INF-τ peut affecter cette boucle et la (ou les) PGFS bovines.

En conclusion, la capacité d'AKR1B1 de relâcher du PGF2α et la boucle de rétroaction PGF2α/FP apportent un angle nouveau d'analyse de plusieurs conditions physiologiques ou pathologiques tant au niveau de la reproduction, que dans d'autres systèmes, en particulier en conditions diabétiques.

.

BIBLIOGRAPHIE

Abel, M. H. and D. T. Baird (1980). "The effect of 17 beta-estradiol and progesterone on prostaglandin production by human endometrium maintained in organ culture." Endocrinology **106**(5): 1599-606.

Abera, A. B., K. J. Sales, et al. (2010). "EP2 receptor mediated cAMP release is augmented by PGF 2 alpha activation of the FP receptor via the calcium-calmodulin pathway." Cell Signal **22**(1): 71-9.

Abramovitz, M., M. Adam, et al. (2000). "The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs." Biochim Biophys Acta **1483**(2): 285-93.

Abramovitz, M., Y. Boie, et al. (1994). "Cloning and expression of a cDNA for the human prostanoid FP receptor." J Biol Chem **269**(4): 2632-6.

Alexander, C. L., S. J. Miller, et al. (2002). "Prostaglandin analog treatment of glaucoma and ocular hypertension." Ann Pharmacother **36**(3): 504-11.

Anai, T., K. Urata, et al. (2002). "Pregnancy complicated with lactate dehydrogenase M-subunit deficiency: the first case report." J Obstet Gynaecol Res **28**(2): 108-11.

Anggard, E. (1966). "The biological activities of three metabolites of prostaglandin E 1." Acta Physiol Scand **66**(4): 509-10.

Arosh, J. A., S. K. Banu, et al. (2003). "Molecular cloning and characterization of bovine prostaglandin E2 receptors EP2 and EP4: expression and regulation in endometrium and myometrium during the estrous cycle and early pregnancy." Endocrinology **144**(7): 3076-91.

Arosh, J. A., S. K. Banu, et al. (2004). "Temporal and tissue-specific expression of prostaglandin receptors EP2, EP3, EP4, FP, and cyclooxygenases 1 and 2 in uterus and fetal membranes during bovine pregnancy." Endocrinology **145**(1): 407-17.

Arosh, J. A., S. K. Banu, et al. (2004). "Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function." Endocrinology **145**(5): 2551-60.

Arosh, J. A., S. K. Banu, et al. (2004). "Effect of interferon-tau on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2." Endocrinology **145**(11): 5280-93.

Arosh, J. A., J. Parent, et al. (2002). "Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle." Biol Reprod **67**(1): 161-9.

Asselin, E., F. W. Bazer, et al. (1997). "Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells." Biol Reprod **56**(2): 402-8.

Asselin, E., P. Drolet, et al. (1998). "In vitro response to oxytocin and interferon-Tau in bovine endometrial cells from caruncular and inter-caruncular areas." Biol Reprod **59**(2): 241-7.

Asselin, E. and M. A. Fortier (2000). "Detection and regulation of the messenger for a putative bovine endometrial 9-keto-prostaglandin E(2) reductase: effect of oxytocin and interferon-tau." Biol Reprod **62**(1): 125-31.

Asselin, E., A. K. Goff, et al. (1996). "Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium." Biol Reprod **54**(2): 371-9.

Asselin, E., D. Lacroix, et al. (1997). "IFN-tau increases PGE2 production and COX-2 gene expression in the bovine endometrium in vitro." Mol Cell Endocrinol **132**(1-2): 117-26.

Augustus Cory, E. (1840). "EXCITEMENT OF PREMATURE PARTURITION." The Lancet **35**(895): 149-152.

Balsinde, J. and E. A. Dennis (1997). "Function and inhibition of intracellular calcium-independent phospholipase A2." J Biol Chem **272**(26): 16069-72.

Bandeira-Melo, C., P. F. Weller, et al. (2011). "Identifying intracellular sites of eicosanoid lipid mediator synthesis with EicosaCell assays." Methods Mol Biol **717**: 277-89.

Banu, S. K., J. A. Arosh, et al. (2003). "Molecular cloning and spatio-temporal expression of the prostaglandin transporter: a basis for the action of prostaglandins in the bovine reproductive system." Proc Natl Acad Sci U S A **100**(20): 11747-52.

Banu, S. K., J. Lee, et al. (2008). "Molecular cloning and characterization of prostaglandin (PG) transporter in ovine endometrium: role for multiple cell signaling pathways in transport of PGF2alpha." Endocrinology **149**(1): 219-31.

Banu, S. K., J. Lee, et al. (2010). "Interferon tau regulates PGF2alpha release from the ovine endometrial epithelial cells via activation of novel JAK/EGFR/ERK/EGR-1 pathways." Mol Endocrinol **24**(12): 2315-30.

Baron, J. A., R. S. Sandler, et al. (2008). "Cardiovascular events associated with rofecoxib: final analysis of the APPROVe trial." Lancet **372**(9651): 1756-64.

Barski, O. A., S. M. Tipparaju, et al. (2008). "The aldo-keto reductase superfamily and its role in drug metabolism and detoxification." Drug Metab Rev **40**(4): 553-624.

Bartz, J. A. and E. Hollander (2008). "Oxytocin and experimental therapeutics in autism spectrum disorders." Prog Brain Res **170**: 451-62.

Bazer, F. W., R. C. Burghardt, et al. (2008). "Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways." Reprod Biol **8**(3): 179-211.

Beisswenger, P. J., S. K. Howell, et al. (1999). "Metformin reduces systemic methylglyoxal levels in type 2 diabetes." Diabetes **48**(1): 198-202.

Bera, T. K., C. Iavarone, et al. (2002). "MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer." Proc Natl Acad Sci U S A **99**(10): 6997-7002.

Binelli, M., A. Guzeloglu, et al. (2000). "Interferon-tau modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-A(2) from bovine endometrial cells." Biol Reprod **63**(2): 417-24.

Bingham, C. O., 3rd and K. F. Austen (1999). "Phospholipase A2 enzymes in eicosanoid generation." Proc Assoc Am Physicians **111**(6): 516-24.

Bito, L. Z. and R. A. Baroody (1975). "Impermeability of rabbit erythrocytes to prostaglandins." Am J Physiol **229**(6): 1580-4.

Borst, P., C. de Wolf, et al. (2007). "Multidrug resistance-associated proteins 3, 4, and 5." Pflugers Arch **453**(5): 661-73.

Bos, C. L., D. J. Richel, et al. (2004). "Prostanoids and prostanoid receptors in signal transduction." Int J Biochem Cell Biol **36**(7): 1187-205.

Bresson, E., S. Boucher-Kovalik, et al. (2011). "The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium." J Clin Endocrinol Metab **96**(1): 210-9.

Breyer, M. D., H. R. Jacobson, et al. (1993). "In situ hybridization and localization of mRNA for the rabbit prostaglandin EP3 receptor." Kidney Int **44**(6): 1372-8.

Breyer, R. M., C. K. Bagdassarian, et al. (2001). "Prostanoid receptors: subtypes and signaling." Annu Rev Pharmacol Toxicol **41**: 661-90.

Brownlee, M. (1995). "Advanced protein glycosylation in diabetes and aging." Annu Rev Med **46**: 223-34.

Calcutt, N. A., M. E. Cooper, et al. (2009). "Therapies for hyperglycaemia-induced diabetic complications: from animal models to clinical trials." Nat Rev Drug Discov **8**(5): 417-29.

Carson, D. D., E. Lagow, et al. (2002). "Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening." Mol Hum Reprod **8**(9): 871-9.

Catalano, R. D., M. R. Wilson, et al. (2011). "Comprehensive expression analysis of prostanoid enzymes and receptors in the human endometrium across the menstrual cycle." Mol Hum Reprod **17**(3): 182-92.

Chan, B. S., S. Endo, et al. (2002). "Identification of lactate as a driving force for prostanoid transport by prostaglandin transporter PGT." Am J Physiol Renal Physiol **282**(6): F1097-102.

Chan, B. S., J. A. Satriano, et al. (1998). "Mechanism of prostaglandin E2 transport across the plasma membrane of HeLa cells and Xenopus oocytes expressing the prostaglandin transporter "PGT"." J Biol Chem **273**(12): 6689-97.

Chandrasekharan, N. V., H. Dai, et al. (2002). "COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression." Proc Natl Acad Sci U S A **99**(21): 13926-31.

Chang, D. G., M. Sun, et al. (1981). "Prostaglandin 9-ketoreductase and type II 15-hydroxyprostaglandin dehydrogenase from swine kidney are alternate activities of a single enzyme protein." Biochem Biophys Res Commun **99**(2): 745-51.

Chang, D. G. and H. H. Tai (1981). "Prostaglandin 9-ketoreductase/type II 15-hydroxyprostaglandin dehydrogenase is not a prostaglandin specific enzyme." Biochem Biophys Res Commun **101**(3): 898-904.

Chang, K. C., K. S. Paek, et al. (2002). "Substrate-induced up-regulation of aldose reductase by methylglyoxal, a reactive oxoaldehyde elevated in diabetes." Mol Pharmacol **61**(5): 1184-91.

Charpigny, G., P. Reinaud, et al. (1997). "Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy." Endocrinology **138**(5): 2163-71.

Chen, Z. S. and A. K. Tiwari (2011). "Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases." FEBS J **278**(18): 3226-45.

Chi, Y., S. M. Khersonsky, et al. (2006). "Identification of a new class of prostaglandin transporter inhibitors and characterization of their biological effects on prostaglandin E2 transport." J Pharmacol Exp Ther **316**(3): 1346-50.

Cohen, I., P. Rider, et al. (2010). "Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation." Proc Natl Acad Sci U S A **107**(6): 2574-9.

Contassot, E., H. D. Beer, et al. (2012). "Interleukin-1, inflammasomes, autoinflammation and the skin." Swiss Med Wkly **142**: w13590.

Cooke, F. N., K. A. Pennington, et al. (2009). "Several fibroblast growth factors are expressed during pre-attachment bovine conceptus development and regulate interferon-tau expression from trophectoderm." Reproduction **137**(2): 259-69.

Coulter, A., K. McPherson, et al. (1988). "Do British women undergo too many or too few hysterectomies?" Soc Sci Med **27**(9): 987-94.

Crowston, J. G., J. D. Lindsey, et al. (2005). "Effect of bimatoprost on intraocular pressure in prostaglandin FP receptor knockout mice." Invest Ophthalmol Vis Sci **46**(12): 4571-7.

Dale, H. H. (1906). "On some physiological actions of ergot." J Physiol **34**(3): 163-206.

Danet-Desnoyers, G., C. Wetzels, et al. (1994). "Natural and recombinant bovine interferon tau regulate basal and oxytocin-induced secretion of prostaglandins F2 alpha and E2 by epithelial cells and stromal cells in the endometrium." Reprod Fertil Dev **6**(2): 193-202.

de Geest, K., M. Thiery, et al. (1985). "Plasma oxytocin in human pregnancy and parturition." J Perinat Med **13**(1): 3-13.

Dennis, E. A. (1994). "Diversity of group types, regulation, and function of phospholipase A2." J Biol Chem **269**(18): 13057-60.

Devost, D., P. Wrzal, et al. (2008). "Oxytocin receptor signalling." Prog Brain Res **170**: 167-76.

Dorniak, P., F. W. Bazer, et al. (2012). "Conceptus-derived prostaglandins regulate endometrial function in sheep." Biol Reprod **87**(1): 9, 1-7.

Draper, M. P., R. L. Martell, et al. (1997). "Indomethacin-mediated reversal of multidrug resistance and drug efflux in human and murine cell lines overexpressing MRP, but not P-glycoprotein." Br J Cancer **75**(6): 810-5.

Emond, V., E. Asselin, et al. (2000). "Interferon-tau stimulates granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes and endometrial stromal cells." Biol Reprod **62**(6): 1728-37.

Emond, V., L. A. MacLaren, et al. (2004). "Expression of cyclooxygenase-2 and granulocyte-macrophage colony-stimulating factor in the endometrial epithelium of the cow is up-regulated during early pregnancy and in response to intrauterine infusions of interferon-tau." Biol Reprod **70**(1): 54-64.

Farquhar, C. M., A. Lethaby, et al. (1999). "An evaluation of risk factors for endometrial hyperplasia in premenopausal women with abnormal menstrual bleeding." Am J Obstet Gynecol **181**(3): 525-9.

Fields, S. D., P. J. Hansen, et al. (2011). "Fibroblast growth factor requirements for in vitro development of bovine embryos." Theriogenology **75**(8): 1466-75.

Flint, A. P., W. M. Leat, et al. (1986). "Stimulation of phosphoinositide hydrolysis by oxytocin and the mechanism by which oxytocin controls prostaglandin synthesis in the ovine endometrium." Biochem J **237**(3): 797-805.

Fortier, M. A., K. Krishnaswamy, et al. (2008). "A postgenomic integrated view of prostaglandins in reproduction: implications for other body systems." J Physiol Pharmacol **59 Suppl 1**: 65-89.

Fujino, H., D. Srinivasan, et al. (2000). "Differential regulation of prostaglandin F(2alpha) receptor isoforms by protein kinase C." Mol Pharmacol **57**(2): 353-8.

Funk, C. D., L. Furci, et al. (1993). "Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype." J Biol Chem **268**(35): 26767-72.

Gabler, C., S. Odau, et al. (2008). "Exploring cumulus-oocyte-complex-oviductal cell interactions: gene profiling in the bovine oviduct." J Physiol Pharmacol **59 Suppl 9**: 29-42.

Gekeler, V., W. Ise, et al. (1995). "The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance." Biochem Biophys Res Commun **208**(1): 345-52.

Gimpl, G. and F. Fahrenholz (2001). "The oxytocin receptor system: structure, function, and regulation." Physiol Rev **81**(2): 629-83.

Ginther, O. J. (1981). "Local versus systemic uteroovarian relationships in farm animals." Acta Vet Scand Suppl **77**: 103-15.

Gislason, G. H., J. N. Rasmussen, et al. (2009). "Increased mortality and cardiovascular morbidity associated with use of nonsteroidal anti-inflammatory drugs in chronic heart failure." Arch Intern Med **169**(2): 141-9.

Gonzalez, R. G., P. Barnett, et al. (1984). "Direct measurement of polyol pathway activity in the ocular lens." Diabetes **33**(2): 196-9.

Griffin, B. W., P. Klimko, et al. (1999). "AL-8810: a novel prostaglandin F2 alpha analog with selective antagonist effects at the prostaglandin F2 alpha (FP) receptor." J Pharmacol Exp Ther **290**(3): 1278-84.

Grossman, E. M., W. E. Longo, et al. (2000). "Role of cytoplasmic and secretory phospholipase A2 in intestinal epithelial cell prostaglandin E2 formation." Int J Surg Investig **1**(6): 467-76.

Guzeloglu, A., F. Michel, et al. (2004). "Differential effects of interferon-tau on the prostaglandin synthetic pathway in bovine endometrial cells treated with phorbol ester." J Dairy Sci **87**(7): 2032-41.

Han, Y., E. Randell, et al. (2007). "Plasma methylglyoxal and glyoxal are elevated and related to early membrane alteration in young, complication-free patients with Type 1 diabetes." Mol Cell Biochem **305**(1-2): 123-31.

Hara, S., D. Kamei, et al. (2010). "Prostaglandin E synthases: Understanding their pathophysiological roles through mouse genetic models." Biochimie **92**(6): 651-9.

Hata, A. N. and R. M. Breyer (2004). "Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation." Pharmacol Ther **103**(2): 147-66.

Helmersson, J., B. Vessby, et al. (2005). "Cyclooxygenase-mediated prostaglandin F2alpha is decreased in an elderly population treated with low-dose aspirin." Prostaglandins Leukot Essent Fatty Acids **72**(4): 227-33.

Hinz, B., O. Cheremina, et al. (2008). "Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man." FASEB J **22**(2): 383-90.

Hofmann, G. E., C. V. Rao, et al. (1983). "Topography of human uterine prostaglandin E and F2 alpha receptors and their profiles during pathological states." J Clin Endocrinol Metab **57**(2): 360-6.

Hoque, M. T. and S. P. Cole (2008). "Down-regulation of Na+/H+ exchanger regulatory factor 1 increases expression and function of multidrug resistance protein 4." Cancer Res **68**(12): 4802-9.

Hoque, M. T., G. Conseil, et al. (2009). "Involvement of NHERF1 in apical membrane localization of MRP4 in polarized kidney cells." Biochem Biophys Res Commun **379**(1): 60-4.

Huang, J. C., D. Y. Liu, et al. (1998). "Interleukin-1 beta induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells." J Clin Endocrinol Metab **83**(2): 538-41.

Jabbour, H. N., R. W. Kelly, et al. (2006). "Endocrine regulation of menstruation." Endocr Rev **27**(1): 17-46.

Jabbour, H. N. and K. J. Sales (2004). "Prostaglandin receptor signalling and function in human endometrial pathology." Trends Endocrinol Metab **15**(8): 398-404.

Jakobsson, P. J., S. Thoren, et al. (1999). "Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target." Proc Natl Acad Sci U S A **96**(13): 7220-5.

JAMA (1928). "SEparation of the active principles of the posterior lobe of the pituitary gland." Journal of the American Medical Association **90**(8): 618-619.

Jedlitschky, G., K. Tirschmann, et al. (2004). "The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage." Blood **104**(12): 3603-10.

Jeng, Y. J., D. Liebenthal, et al. (2000). "Complementary mechanisms of enhanced oxytocin-stimulated prostaglandin E2 synthesis in rabbit amnion at the end of gestation." Endocrinology **141**(11): 4136-45.

Jones, R. L., M. A. Giembycz, et al. (2009). "Prostanoid receptor antagonists: development strategies and therapeutic applications." Br J Pharmacol **158**(1): 104-45.

Kabututu, Z., S. K. Martin, et al. (2003). "Prostaglandin production from arachidonic acid and evidence for a 9,11-endoperoxide prostaglandin H2 reductase in Leishmania." Int J Parasitol **33**(2): 221-8.

Kalapos, M. P. (1999). "Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications." Toxicol Lett **110**(3): 145-75.

Kalapos, M. P. (2008). "The tandem of free radicals and methylglyoxal." Chem Biol Interact **171**(3): 251-71.

Kalinski, P. (2012). "Regulation of immune responses by prostaglandin E2." J Immunol **188**(1): 21-8.

Kang, J., P. Chapdelaine, et al. (2005). "Expression of human prostaglandin transporter in the human endometrium across the menstrual cycle." J Clin Endocrinol Metab **90**(4): 2308-13.

Kasajima, H., S. Yamagishi, et al. (2001). "Enhanced in situ expression of aldose reductase in peripheral nerve and renal glomeruli in diabetic patients." Virchows Arch **439**(1): 46-54.

Kim, S., Y. Choi, et al. (2003). "Effects of the estrous cycle, pregnancy and interferon tau on expression of cyclooxygenase two (COX-2) in ovine endometrium." Reprod Biol Endocrinol **1**: 58.

Kimura, T., O. Tanizawa, et al. (1992). "Structure and expression of a human oxytocin receptor." Nature **356**(6369): 526-9.

Kiriyama, M., F. Ushikubi, et al. (1997). "Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells." Br J Pharmacol **122**(2): 217-24.

Komoto, J., T. Yamada, et al. (2004). "Crystal structure of human prostaglandin F synthase (AKR1C3)." Biochemistry **43**(8): 2188-98.

Kotani, M., I. Tanaka, et al. (2000). "Multiple signal transduction pathways through two prostaglandin E receptor EP3 subtype isoforms expressed in human uterus." J Clin Endocrinol Metab **85**(11): 4315-22.

Krishnaswamy, N., P. Chapdelaine, et al. (2009). "Development and characterization of a simian virus 40 immortalized bovine endometrial stromal cell line." Endocrinology **150**(1): 485-91.

Krishnaswamy, N., G. Danyod, et al. (2009). "Oxytocin receptor down-regulation is not necessary for reducing oxytocin-induced prostaglandin F(2alpha) accumulation by interferon-tau in a bovine endometrial epithelial cell line." Endocrinology **150**(2): 897-905.

Kruh, G. D., Y. Guo, et al. (2007). "ABCC10, ABCC11, and ABCC12." Pflugers Arch **453**(5): 675-84.

Kubata, B. K., M. Duszenko, et al. (2000). "Identification of a novel prostaglandin f(2alpha) synthase in Trypanosoma brucei." J Exp Med **192**(9): 1327-38.

Kubata, B. K., Z. Kabututu, et al. (2002). "A key role for old yellow enzyme in the metabolism of drugs by Trypanosoma cruzi." J Exp Med **196**(9): 1241-51.

Kuchinke, W., O. Barski, et al. (1992). "A lung type prostaglandin F synthase is expressed in bovine liver: cDNA sequence and expression in E. coli." Biochem Biophys Res Commun **183**(3): 1238-46.

Kudo, I. and M. Murakami (2002). "Phospholipase A2 enzymes." Prostaglandins Other Lipid Mediat **68-69**: 3-58.

Kuhla, B., H. J. Luth, et al. (2005). "Methylglyoxal, glyoxal, and their detoxification in Alzheimer's disease." Ann N Y Acad Sci **1043**: 211-6.

Lai, J., H. Jin, et al. (1996). "Prostaglandin F2 alpha induces cardiac myocyte hypertrophy in vitro and cardiac growth in vivo." Am J Physiol **271**(6 Pt 2): H2197-208.

Lee, J., S. K. Banu, et al. (2012). "Early pregnancy induced expression of prostaglandin E2 receptors EP2 and EP4 in the ovine endometrium and regulated by interferon tau through multiple cell signaling pathways." Mol Cell Endocrinol **348**(1): 211-23.

Lee, J., J. A. McCracken, et al. (2010). "Transport of Prostaglandin F2{alpha} Pulses from the Uterus to the Ovary at the Time of Luteolysis in Ruminants Is Regulated by Prostaglandin Transporter-Mediated Mechanisms." Endocrinology.

Leung, S. T., Z. Cheng, et al. (2001). "The effects of lipopolysaccharide and interleukins-1alpha, -2 and -6 on oxytocin receptor expression and prostaglandin production in bovine endometrium." J Endocrinol **168**(3): 497-508.

Lim, H., B. C. Paria, et al. (1997). "Multiple female reproductive failures in cyclooxygenase 2-deficient mice." Cell **91**(2): 197-208.

Loftin, C. D., D. B. Trivedi, et al. (2001). "Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2." Proc Natl Acad Sci U S A **98**(3): 1059-64.

Lu, J., E. Randell, et al. (2011). "Increased plasma methylglyoxal level, inflammation, and vascular endothelial dysfunction in diabetic nephropathy." Clin Biochem **44**(4): 307-11.

Lundstrom, V. and K. Green (1978). "Endogenous levels of prostaglandin F2alpha and its main metabolites in plasma and endometrium of normal and dysmenorrheic women." Am J Obstet Gynecol **130**(6): 640-6.

Madore, E., N. Harvey, et al. (2003). "An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium." J Biol Chem **278**(13): 11205-12.

Majewska, M., I. Woclawek-Potocka, et al. (2010). "Is interleukin-1alpha a luteotrophic or luteolytic agent in cattle?" Reproduction **139**(3): 665-72.

Mayoral, R., A. Fernandez-Martinez, et al. (2005). "Prostaglandin E2 promotes migration and adhesion in hepatocellular carcinoma cells." Carcinogenesis **26**(4): 753-61.

McCracken, J. A., E. E. Custer, et al. (1999). "Luteolysis: a neuroendocrine-mediated event." Physiol Rev **79**(2): 263-323.

McLellan, A. C., P. J. Thornalley, et al. (1994). "Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications." Clin Sci (Lond) **87**(1): 21-9.

McPherson, J. D., B. H. Shilton, et al. (1988). "Role of fructose in glycation and cross-linking of proteins." Biochemistry **27**(6): 1901-7.

Michael, D. D., I. M. Alvarez, et al. (2006). "Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-tau production in bovine trophectoderm." Endocrinology **147**(7): 3571-9.

Miller, S. B. (2006). "Prostaglandins in health and disease: an overview." Semin Arthritis Rheum **36**(1): 37-49.

Milne, S. A., G. B. Perchick, et al. (2001). "Expression, localization, and signaling of PGE(2) and EP2/EP4 receptors in human nonpregnant endometrium across the menstrual cycle." J Clin Endocrinol Metab **86**(9): 4453-9.

Ming, X. and D. R. Thakker (2010). "Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil." Biochem Pharmacol **79**(3): 455-62.

Molnar, M., J. Rigo, Jr., et al. (1999). "Oxytocin activates mitogen-activated protein kinase and up-regulates cyclooxygenase-2 and prostaglandin production in human myometrial cells." Am J Obstet Gynecol **181**(1): 42-9.

Moore, J. J., G. R. Dubyak, et al. (1988). "Oxytocin activates the inositol-phospholipid-protein kinase-C system and stimulates prostaglandin production in human amnion cells." Endocrinology **123**(4): 1771-7.

Moriuchi, H., N. Koda, et al. (2008). "Molecular characterization of a novel type of prostamide/prostaglandin F synthase, belonging to the thioredoxin-like superfamily." J Biol Chem **283**(2): 792-801.

Murakami, M. and I. Kudo (2004). "Recent advances in molecular biology and physiology of the prostaglandin E2-biosynthetic pathway." Prog Lipid Res **43**(1): 3-35.

Murakami, M., K. Nakashima, et al. (2003). "Cellular prostaglandin E2 production by membrane-bound prostaglandin E synthase-2 via both cyclooxygenases-1 and -2." J Biol Chem **278**(39): 37937-47.

Murakami, M., H. Naraba, et al. (2000). "Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2." J Biol Chem **275**(42): 32783-92.

Nakanishi, M., V. Gokhale, et al. (2010). "mPGES-1 as a target for cancer suppression: A comprehensive invited review "Phospholipase A2 and lipid mediators"." Biochimie **92**(6): 660-4.

Nakanishi, M. and D. W. Rosenberg (2013). "Multifaceted roles of PGE2 in inflammation and cancer." Semin Immunopathol **35**(2): 123-37.

Nies, A. T., G. Jedlitschky, et al. (2004). "Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain." Neuroscience **129**(2): 349-60.

Niesen, F. H., L. Schultz, et al. (2010). "High-affinity inhibitors of human NAD-dependent 15-hydroxyprostaglandin dehydrogenase: mechanisms of inhibition and structure-activity relationships." PLoS One **5**(11): e13719.

Nishigaki, N., M. Negishi, et al. (1996). "Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist." Mol Pharmacol **50**(4): 1031-7.

Nishihara, I., T. Minami, et al. (1995). "Effect of NMDA receptor antagonists on prostaglandin E2-induced hyperalgesia in conscious mice." Brain Res **677**(1): 138-44.

Nishimura, R., A. Bowolaksono, et al. (2004). "Possible role of interleukin-1 in the regulation of bovine corpus luteum throughout the luteal phase." Biol Reprod **71**(5): 1688-93.

Nishizawa, M., T. Nakajima, et al. (2000). "Close kinship of human 20alpha-hydroxysteroid dehydrogenase gene with three aldo-keto reductase genes." Genes Cells **5**(2): 111-25.

Nozaki, Y., H. Kusuhara, et al. (2007). "Species difference in the inhibitory effect of nonsteroidal anti-inflammatory drugs on the uptake of methotrexate by human kidney slices." J Pharmacol Exp Ther **322**(3): 1162-70.

Oates, P. J. and B. L. Mylari (1999). "Aldose reductase inhibitors: therapeutic implications for diabetic complications." Expert Opin Investig Drugs **8**(12): 2095-2119.

Obrosova, I. G. (2005). "Increased sorbitol pathway activity generates oxidative stress in tissue sites for diabetic complications." Antioxid Redox Signal **7**(11-12): 1543-52.

Okuda, K., Y. Miyamoto, et al. (2002). "Regulation of endometrial prostaglandin F(2alpha) synthesis during luteolysis and early pregnancy in cattle." Domest Anim Endocrinol **23**(1-2): 255-64.

Oliveira, L. M., A. Lages, et al. (2012). "Insulin glycation by methylglyoxal results in native-like aggregation and inhibition of fibril formation." BMC Biochem **12**: 41.

Parent, J. and M. A. Fortier (2005). "Expression and contribution of three different isoforms of prostaglandin E synthase in the bovine endometrium." Biol Reprod **73**(1): 36-44.

Parent, J., C. Villeneuve, et al. (2003). "Evaluation of the contribution of cyclooxygenase 1 and cyclooxygenase 2 to the production of PGE2 and PGF2 alpha in epithelial cells from bovine endometrium." Reproduction **126**(4): 539-47.

Pastel, E., J. C. Pointud, et al. (2012). "Aldo-Keto Reductases 1B in Endocrinology and Metabolism." Front Pharmacol **3**: 148.

Peterson, A. J., H. R. Tervit, et al. (1976). "Jugular levels of 13, 14-dihydro-15-keto-prostaglandin F and progesterone around luteolysis and early pregnancy in the ewe." Prostaglandins **12**(4): 551-8.

Petrash, J. M. (2004). "All in the family: aldose reductase and closely related aldo-keto reductases." Cell Mol Life Sci **61**(7-8): 737-49.

Pierce, K. L., T. J. Bailey, et al. (1997). "Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor." J Biol Chem **272**(2): 883-7.

Piper, P. J., J. R. Vane, et al. (1970). "Inactivation of prostaglandins by the lungs." Nature **225**(5233): 600-4.

Pollak, N., C. Dolle, et al. (2007). "The power to reduce: pyridine nucleotides--small molecules with a multitude of functions." Biochem J **402**(2): 205-18.

Poyser, N. L. (1995). "The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation." Prostaglandins Leukot Essent Fatty Acids **53**(3): 147-95.

Prentice, A. (1999). "Health care implications of dysfunctional uterine bleeding." Baillieres Best Pract Res Clin Obstet Gynaecol **13**(2): 181-8.

Priddy, A. R., S. R. Killick, et al. (1990). "The effect of prostaglandin synthetase inhibitors on human preovulatory follicular fluid prostaglandin, thromboxane, and leukotriene concentrations." J Clin Endocrinol Metab **71**(1): 235-42.

Pugliese, G., E. G. Spokas, et al. (1985). "Hepatic transformation of prostaglandin D2 to a new prostanoid, 9 alpha,11 beta-prostaglandin F2, that inhibits platelet aggregation and constricts blood vessels." J Biol Chem **260**(27): 14621-5.

Ramana, K. V. and S. K. Srivastava (2011). "Aldose reductase: a novel therapeutic target for inflammatory pathologies." Int J Biochem Cell Biol **42**(1): 17-20.

Ramasamy, R., S. J. Vannucci, et al. (2005). "Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation." Glycobiology **15**(7): 16R-28R.

Rees, M. C. (1989). "Heavy, painful periods." Baillieres Clin Obstet Gynaecol **3**(2): 341-56.

Rees, M. C. and R. W. Kelly (1986). "Prostaglandin D2 release by endometrium and myometrium." Br J Obstet Gynaecol **93**(10): 1078-82.

Reese, J., B. C. Paria, et al. (2000). "Coordinated regulation of fetal and maternal prostaglandins directs successful birth and postnatal adaptation in the mouse." Proc Natl Acad Sci U S A **97**(17): 9759-64.

Reid, G., P. Wielinga, et al. (2003). "Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5." Mol Pharmacol **63**(5): 1094-103.

Reid, G., P. Wielinga, et al. (2003). "The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs." Proc Natl Acad Sci U S A **100**(16): 9244-9.

Ricciotti, E. and G. A. FitzGerald (2011). "Prostaglandins and inflammation." Arterioscler Thromb Vasc Biol **31**(5): 986-1000.

Rizner, T. L. (2012). "Enzymes of the AKR1B and AKR1C Subfamilies and Uterine Diseases." Front Pharmacol **3**: 34.

Roberts, R. M. (2007). "Interferon-tau, a Type 1 interferon involved in maternal recognition of pregnancy." Cytokine Growth Factor Rev **18**(5-6): 403-8.

Roberts, R. M., Y. Chen, et al. (2008). "Interferons and the maternal-conceptus dialog in mammals." Semin Cell Dev Biol **19**(2): 170-7.

Robertson, R. P. (1983). "Prostaglandins, glucose homeostasis, and diabetes mellitus." Annu Rev Med **34**: 1-12.

Robinson, R. S., G. E. Mann, et al. (1999). "The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow." J Endocrinol **160**(1): 21-33.

Robinson, R. S., G. E. Mann, et al. (2001). "Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows." Reproduction **122**(6): 965-79.

Rodina, T. M., F. N. Cooke, et al. (2009). "Oxygen tension and medium type actions on blastocyst development and interferon-tau secretion in cattle." Anim Reprod Sci **111**(2-4): 173-88.

Russel, F. G., J. B. Koenderink, et al. (2008). "Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules." Trends Pharmacol Sci **29**(4): 200-7.

Sales, K. J., V. Grant, et al. (2008). "Prostaglandin E2 and F2alpha activate the FP receptor and up-regulate cyclooxygenase-2 expression via the cyclic AMP response element." Mol Cell Endocrinol **285**(1-2): 51-61.

Sales, K. J. and H. N. Jabbour (2003). "Cyclooxygenase enzymes and prostaglandins in pathology of the endometrium." Reproduction **126**(5): 559-67.

Sales, K. J., T. List, et al. (2005). "A novel angiogenic role for prostaglandin F2alpha-FP receptor interaction in human endometrial adenocarcinomas." Cancer Res **65**(17): 7707-16.

Sales, K. J., S. Maudsley, et al. (2004). "Elevated prostaglandin EP2 receptor in endometrial adenocarcinoma cells promotes vascular endothelial growth factor expression via cyclic 3',5'-adenosine monophosphate-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase 1/2 signaling pathways." Mol Endocrinol **18**(6): 1533-45.

Sales, K. J., S. A. Milne, et al. (2004). "Expression, localization, and signaling of prostaglandin F2 alpha receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the epidermal growth factor receptor and mitogen-activated protein kinase signaling pathways." J Clin Endocrinol Metab **89**(2): 986-93.

Salilew-Wondim, D., M. Holker, et al. (2010). "Bovine pretransfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after embryo transfer." Physiol Genomics **42**(2): 201-18.

Samuelsson, B., R. Morgenstern, et al. (2007). "Membrane prostaglandin E synthase-1: a novel therapeutic target." Pharmacol Rev **59**(3): 207-24.

Sassone-Corsi, P. "The cyclic AMP pathway." Cold Spring Harb Perspect Biol **4**(12).

Schafer, E. and K. Mackenzie (1911). "The Action of Animal Extracts on Milk Secretion." Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character (1905-1934) **84**(568): 6.

Schatz, F., L. Markiewicz, et al. (1987). "Differential effects of estradiol, arachidonic acid, and A23187 on prostaglandin F2 alpha output by epithelial and stromal cells of human endometrium." Endocrinology **120**(4): 1465-71.

Schuster, V. L. (1998). "Molecular mechanisms of prostaglandin transport." Annu Rev Physiol **60**: 221-42.

Schuster, V. L. (2002). "Prostaglandin transport." Prostaglandins Other Lipid Mediat **68-69**: 633-47.

Semer, D., K. Reisler, et al. (1991). "Responsiveness of human endometrial stromal cells to cytokines." Ann N Y Acad Sci **622**: 99-110.

Sharif, N. A., G. W. Williams, et al. (2001). "Bimatoprost and its free acid are prostaglandin FP receptor agonists." Eur J Pharmacol **432**(2-3): 211-3.

Shiri, R., J. Koskimaki, et al. (2006). "Effect of nonsteroidal anti-inflammatory drug use on the incidence of erectile dysfunction." J Urol **175**(5): 1812-5; discussion 1815-6.

Shrestha, H. K., M. A. Beg, et al. (2012). "Plasma clearance and half-life of prostaglandin F2alpha: a comparison between mares and heifers." Biol Reprod **87**(1): 18, 1-6.

Silvia, W. J., G. S. Lewis, et al. (1991). "Hormonal regulation of uterine secretion of prostaglandin F2 alpha during luteolysis in ruminants." Biol Reprod **45**(5): 655-63.

Simmons, D. L., R. M. Botting, et al. (2004). "Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition." Pharmacol Rev **56**(3): 387-437.

Sinha, C., A. Ren, et al. (2013). "Multi-drug resistance protein 4 (MRP4)-mediated regulation of fibroblast cell migration reflects a dichotomous role of intracellular cyclic nucleotides." J Biol Chem **288**(6): 3786-94.

Sirois, J. and J. S. Richards (1992). "Purification and characterization of a novel, distinct isoform of prostaglandin endoperoxide synthase induced by human chorionic gonadotropin in granulosa cells of rat preovulatory follicles." J Biol Chem **267**(9): 6382-8.

Sitter, T., B. Haslinger, et al. (1998). "High glucose increases prostaglandin E2 synthesis in human peritoneal mesothelial cells: role of hyperosmolarity." J Am Soc Nephrol **9**(11): 2005-12.

Skarzynski, D. J., G. Ferreira-Dias, et al. (2008). "Regulation of luteal function and corpus luteum regression in cows: hormonal control, immune mechanisms and intercellular communication." Reprod Domest Anim **43 Suppl 2**: 57-65.

Smith, S. K., M. H. Abel, et al. (1981). "Prostaglandin synthesis in the endometrium of women with ovular dysfunctional uterine bleeding." Br J Obstet Gynaecol **88**(4): 434-42.

Smith, W. L., Y. Urade, et al. (2011). "Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis." Chem Rev **111**(10): 5821-65.

Spencer, T. E. and F. W. Bazer (1996). "Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium." Endocrinology **137**(3): 1144-7.

Spencer, T. E. and F. W. Bazer (2004). "Conceptus signals for establishment and maintenance of pregnancy." Reprod Biol Endocrinol **2**: 49.

Spencer, T. E., G. A. Johnson, et al. (2007). "Fetal-maternal interactions during the establishment of pregnancy in ruminants." Soc Reprod Fertil Suppl **64**: 379-96.

Srivastava, S. K., K. V. Ramana, et al. (2005). "Role of aldose reductase and oxidative damage in diabetes and the consequent potential for therapeutic options." Endocr Rev **26**(3): 380-92.

Stock, S., K. Bremme, et al. (1991). "Plasma levels of oxytocin during the menstrual cycle, pregnancy and following treatment with HMG." Hum Reprod **6**(8): 1056-62.

Strakova, Z., J. A. Copland, et al. (1998). "ERK2 mediates oxytocin-stimulated PGE2 synthesis." Am J Physiol **274**(4 Pt 1): E634-41.

Sugimoto, Y., K. Hasumoto, et al. (1994). "Cloning and expression of a cDNA for mouse prostaglandin F receptor." J Biol Chem **269**(2): 1356-60.

Sugimoto, Y., A. Yamasaki, et al. (1997). "Failure of parturition in mice lacking the prostaglandin F receptor." Science **277**(5326): 681-3.

Sun, H. S., K. Y. Hsiao, et al. (2003). "Transactivation of steroidogenic acute regulatory protein in human endometriotic stromalcells is mediated by the prostaglandin EP2 receptor." Endocrinology **144**(9): 3934-42.

Suzawa, T., C. Miyaura, et al. (2000). "The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs." Endocrinology **141**(4): 1554-9.

Suzuki-Yamamoto, T., M. Nishizawa, et al. (1999). "cDNA cloning, expression and characterization of human prostaglandin F synthase." FEBS Lett **462**(3): 335-40.

Suzuki, T., Y. Fujii, et al. (1999). "cDNA cloning, expression, and mutagenesis study of liver-type prostaglandin F synthase." J Biol Chem **274**(1): 241-8.

Tabibzadeh, S. and X. Z. Sun (1992). "Cytokine expression in human endometrium throughout the menstrual cycle." Hum Reprod **7**(9): 1214-21.

Tai, H. H., C. M. Ensor, et al. (2002). "Prostaglandin catabolizing enzymes." Prostaglandins Other Lipid Mediat **68-69**: 483-93.

Takeuchi, K. (2012). "Pathogenesis of NSAID-induced gastric damage: importance of cyclooxygenase inhibition and gastric hypermotility." World J Gastroenterol **18**(18): 2147-60.

Takusagawa, F. (2013). "Microsomal prostaglandin E synthase type 2 (mPGES2) is a glutathione-dependent heme protein, and dithiothreitol dissociates the bound heme to produce active prostaglandin E2 synthase in vitro." J Biol Chem **288**(14): 10166-75.

Tang, L., K. Loutzenhiser, et al. (2000). "Biphasic Actions of Prostaglandin E2 on the Renal Afferent Arteriole : Role of EP3 and EP4 Receptors." Circulation Research **86**(6): 663-670.

Tanikawa, M., T. J. Acosta, et al. (2005). "Regulation of prostaglandin synthesis by interleukin-1alpha in bovine endometrium during the estrous cycle." Prostaglandins Other Lipid Mediat **78**(1-4): 279-90.

Tanikawa, M., H. Y. Lee, et al. (2008). "Regulation of prostaglandin biosynthesis by interleukin-1 in cultured bovine endometrial cells." J Endocrinol **199**(3): 425-34.

Tanikawa, N., Y. Ohmiya, et al. (2002). "Identification and characterization of a novel type of membrane-associated prostaglandin E synthase." Biochem Biophys Res Commun **291**(4): 884-9.

Tanioka, T., Y. Nakatani, et al. (2000). "Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis." J Biol Chem **275**(42): 32775-82.

Thornalley, P. J. (1993). "The glyoxalase system in health and disease." Mol Aspects Med **14**(4): 287-371.

Tilley, S. L., T. M. Coffman, et al. (2001). "Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes." J Clin Invest **108**(1): 15-23.

Toth, L., L. Muszbek, et al. (2013). "Mechanism of the irreversible inhibition of human cyclooxygenase-1 by aspirin as predicted by QM/MM calculations." J Mol Graph Model **40**: 99-109.

Trelle, S., S. Reichenbach, et al. (2011). "Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis." BMJ **342**: c7086.

Ulbrich, S. E., K. Schulke, et al. (2009). "Quantitative characterization of prostaglandins in the uterus of early pregnant cattle." Reproduction **138**(2): 371-82.

Urade, Y., K. Watanabe, et al. (1995). "Prostaglandin D, E, and F synthases." J Lipid Mediat Cell Signal **12**(2-3): 257-73.

van Aubel, R. A., P. H. Smeets, et al. (2002). "The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP." J Am Soc Nephrol **13**(3): 595-603.

Vander Jagt, D. L., B. Robinson, et al. (1992). "Reduction of trioses by NADPH-dependent aldo-keto reductases. Aldose reductase, methylglyoxal, and diabetic complications." J Biol Chem **267**(7): 4364-9.

Wallace, J. L., W. McKnight, et al. (2000). "NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2." Gastroenterology **119**(3): 706-14.

Wang, B. and A. K. Goff (2003). "Interferon-tau stimulates secretion of macrophage migration inhibitory factor from bovine endometrial epithelial cells." Biol Reprod **69**(5): 1690-6.

Wang, D. and R. N. DuBois (2004). "Cyclooxygenase 2-derived prostaglandin E2 regulates the angiogenic switch." Proc Natl Acad Sci U S A **101**(2): 415-6.

Wang, M., W. L. Song, et al. (2008). "Microsomal prostaglandin E synthase-1 inhibition in cardiovascular inflammatory disease." J Intern Med **263**(5): 500-5.

Wang, X., K. Desai, et al. (2004). "Increased methylglyoxal and advanced glycation end products in kidney from spontaneously hypertensive rats." Kidney Int **66**(6): 2315-21.

Ward, W. H., C. M. Sennitt, et al. (1990). "Ponalrestat: a potent and specific inhibitor of aldose reductase." Biochem Pharmacol **39**(2): 337-46.

Warden, S. J. (2010). "Prophylactic use of NSAIDs by athletes: a risk/benefit assessment." Phys Sportsmed **38**(1): 132-8.

Warner, T. D., F. Giuliano, et al. (1999). "Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis." Proc Natl Acad Sci U S A **96**(13): 7563-8.

Watanabe, K. (2002). "Prostaglandin F synthase." Prostaglandins Other Lipid Mediat **68-69**: 401-7.

Watanabe, K. (2011). "Recent reports about enzymes related to the synthesis of prostaglandin (PG) F(2) (PGF(2alpha) and 9alpha, 11beta-PGF(2))." J Biochem **150**(6): 593-6.

Watanabe, K., S. Ito, et al. (2008). "Studies on membrane-associated prostaglandin E synthase-2 with reference to production of 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT)." Biochem Biophys Res Commun **367**(4): 782-6.

Watanabe, K., R. Yoshida, et al. (1985). "Enzymatic formation of prostaglandin F2 alpha from prostaglandin H2 and D2. Purification and properties of prostaglandin F synthetase from bovine lung." J Biol Chem **260**(11): 7035-41.

Watanabe, T., A. Nakao, et al. (1994). "Prostaglandin F2 alpha enhances tyrosine phosphorylation and DNA synthesis through phospholipase C-coupled receptor via Ca(2+)-dependent intracellular pathway in NIH-3T3 cells." J Biol Chem **269**(26): 17619-25.

Weber, A., P. Wasiliew, et al. (2010). "Interleukin-1 (IL-1) pathway." Sci Signal **3**(105): cm1.

Wehbrink, D., M. Hassig, et al. (2008). "Immunohistochemical demonstration of cyclooxygenase-2 (COX-2) and prostaglandin receptors EP2 and FP expression in the bovine intercaruncular uterine wall around term." Anim Reprod Sci **106**(3-4): 241-54.

Wermuth, B. (1981). "Purification and properties of an NADPH-dependent carbonyl reductase from human brain. Relationship to prostaglandin 9-ketoreductase and xenobiotic ketone reductase." J Biol Chem **256**(3): 1206-13.

Woodward, D. F., D. J. Pepperl, et al. (1995). "6-Isopropoxy-9-oxoxanthene-2-carboxylic acid (AH 6809), a human EP2 receptor antagonist." Biochem Pharmacol **50**(10): 1731-3.

Wu, C. P., A. M. Calcagno, et al. (2005). "Modulatory effects of plant phenols on human multidrug-resistance proteins 1, 4 and 5 (ABCC1, 4 and 5)." FEBS J **272**(18): 4725-40.

Wu, C. P., A. Klokouzas, et al. (2005). "Interactions of mefloquine with ABC proteins, MRP1 (ABCC1) and MRP4 (ABCC4) that are present in human red cell membranes." Biochem Pharmacol **70**(4): 500-10.

Wu, P., M. Fritzo, et al. (1990). "Purification and characterization of an NAD(+)-dependent dehydrogenase that catalyzes the oxidation of thromboxane B2 at C-11 from porcine liver. Development and application of 11-dehydro-thromboxane B2 radioimmunoassay to enzyme assay." Biochim Biophys Acta **1036**(1): 55-63.

Xu, D., S. E. Rowland, et al. (2008). "MF63 [2-(6-chloro-1H-phenanthro[9,10-d]imidazol-2-yl)-isophthalonitrile], a selective microsomal prostaglandin E synthase-1 inhibitor, relieves pyresis and pain in preclinical models of inflammation." J Pharmacol Exp Ther **326**(3): 754-63.

Yabe-Nishimura, C. (1998). "Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications." Pharmacol Rev **50**(1): 21-33.

Yabe-Nishimura, C., T. Nishinaka, et al. (2003). "Up-regulation of aldose reductase by the substrate, methylglyoxal." Chem Biol Interact **143-144**: 317-23.

Yu, L., W. K. Wu, et al. (2009). "Prostaglandin E(2) promotes cell proliferation via protein kinase C/extracellular signal regulated kinase pathway-dependent induction of c-Myc expression in human esophageal squamous cell carcinoma cells." Int J Cancer **125**(11): 2540-6.

Yu, P. H. (2001). "Involvement of cerebrovascular semicarbazide-sensitive amine oxidase in the pathogenesis of Alzheimer's disease and vascular dementia." Med Hypotheses **57**(2): 175-9.

Yu, R., L. Xiao, et al. (2011). "Competitive enzymatic interactions determine the relative amounts of prostaglandins E2 and D2." J Pharmacol Exp Ther **339**(2): 716-25.

Zakharov, V., H. K. Lin, et al. (2010). "Suppressed expression of type 2 3alpha/type 5 17beta-hydroxysteroid dehydrogenase (AKR1C3) in endometrial hyperplasia and carcinoma." Int J Clin Exp Pathol **3**(6): 608-17.

Zarco, L., G. H. Stabenfeldt, et al. (1988). "Modification of prostaglandin F-2 alpha synthesis and release in the ewe during the initial establishment of pregnancy." J Reprod Fertil **83**(2): 527-36.

Zarco, L., G. H. Stabenfeldt, et al. (1988). "Release of prostaglandin F-2 alpha and the timing of events associated with luteolysis in ewes with oestrous cycles of different lengths." J Reprod Fertil **83**(2): 517-26.

Zhong, M., M. Yang, et al. (2003). "Extracellular signal-regulated kinase 1/2 activation by myometrial oxytocin receptor involves Galpha(q)Gbetagamma and epidermal growth factor receptor tyrosine kinase activation." Endocrinology **144**(7): 2947-56.

Ziegler, D., P. Mayer, et al. (1991). "One-year treatment with the aldose reductase inhibitor, ponalrestat, in diabetic neuropathy." Diabetes Res Clin Pract **14**(1): 63-73.

ANNEXE

Prostaglandin (PG) F2 alpha synthesis in human subcutaneous and omental adipose tissue: modulation by inflammatory cytokines and role of the human aldose reductase AKR1B1

Andréanne Michaud [1, 2], Nicolas Lacroix-Pépin [4], Mélissa Pelletier [1], Alain Veilleux [2], Suzanne Noël [3], Céline Bouchard [3], Picard Marceau [5], Michel A. Fortier [4], André Tchernof[1, 2]

Cette annexe est présentée sous forme d'article et publié dans la revue :
PLoS One. 2014 Mar 24;9(3):e90861.

1: Endocrinology and Nephrology, Laval University Medical Center, Quebec City, Canada;
2: Department of Nutrition, Laval University, Quebec City, Canada;
3: Gynecology Unit, Laval University Medical Center, Quebec City, Canada;
4: Reproduction and biology, Laval University Medical Center, Quebec City, Canada
5: Department of Surgery, Quebec Cardiology and Pulmonology Institute, Quebec City, Canada.

Résumé

Introduction: La PGF2α peut être impliquée dans la régulation de la fonction des tissus adipeux. Objectifs: 1) Examiner la libération du PGF2α par les pré-adipocytes primaires, les adipocytes matures et des explants de tissus entiers des compartiments de graisse sous-cutanée et omentales ; 2) évaluer la PGF synthase qui est la plus pertinente dans le tissu adipeux humain. Méthodes : Des échantillons de graisse ont été obtenus par chirurgie chez des femmes. La libération du PGF2α par les pré-adipocytes, les adipocytes et les explants sous stimulation par le TNF- α, IL- 1β, ou les deux a été mesurée. Les niveaux d'expression des messagers d'AKR1B1 et d'AKR1C3 ont été mesurés par RT- PCR dans le tissu adipeux et l'ensemble des pré-adipocytes traités par des cytokines. On a étudié l'effet de l'inhibiteur d'AKR1B1 ponalrestat sur la synthèse du PGF2α. Résultats: La libération du PGF2α a été induite de manière significative en réponse à des cytokines par rapport au témoin dans le tissus omental ($p = 0,01$) et dans une moindre mesure dans les pré-adipocytes sous-cutanés ($p = 0,02$). L'ARN messager de la COX- 2 est significativement plus élevé dans le tissu omental par rapport au pré-adipocytes sous-cutanés en réponse au TNF - α et IL-1β combinés ($p = 0,01$). Les cytokines inflammatoires ont augmenté l'expression de l'ARNm d'AKR1B1 et de la protéine ($p \leq 0,05$), mais n'ont pas réussi à augmenter les niveaux d'expression d'AKR1C3 dans les pré-adipocytes en culture. En conséquence, le ponalrestat diminue la synthèse de PGF2α par les pré-adipocytes au niveau basal et en conditions stimulées ($p \leq 0,05$). Les femmes dont le taux de PGF2α produit par les adipocytes omentaux était plus élevé avaient un IMC plus élevé ($p = 0,05$), un tour de taille ($p \leq 0,05$) et un indice Homa-IR ($p \leq 0,005$) plus élevés ainsi qu'une hausse d'expression de l'ARNm d'AKR1B1 dans le tissu omental ($p < 0,10$) et sous-cutanés ($p \leq 0,05$) par rapport aux femmes avec une libération de PGF2α plus faible dans les adipocytes omentaux. Des corrélations positives ont été observées entre l'IMC, le tour de taille, ainsi que l'indice Homa-IR et le niveau d'expression de l'ARNm de AKR1B1 dans les deux compartiments ($p \leq 0,05$ pour l'ensemble). Conclusion : La libération du PGF2α par les adipocytes matures omentaux est augmentée chez les femmes présentant une obésité abdominale. En outre, l'expression de COX- 2 et de relâchement de PGF2α est particulièrement sensible à la stimulation inflammatoire dans les pré-adipocytes omentaux. De plus, le blocage de la PGF synthase AKR1B1 inhibe la majorité de la libération de PGF2α.

ABSTRACT

Introduction: PGF2α may be involved in the regulation of adipose tissue function. Objectives: 1) To examine PGF2α release by primary preadipocytes, mature adipocytes and whole tissue explants from the subcutaneous and omental fat compartments; 2) To assess which PGF synthase is the most relevant in human adipose tissue. Methods: Fat samples were obtained by surgery in women. PGF2α release by preadipocytes, adipocytes and explants under stimulation by TNF-α, IL-1β or both was measured. Messenger RNA expression levels of AKR1B1 and AKR1C3 were measured by RT-PCR in whole adipose tissue and cytokine-treated preadipocytes. The effect of AKR1B1 inhibitor ponalrestat on PGF2α synthesis was investigated. Results: PGF2α release was significantly induced in response to cytokines compared to control in omental ($p=0.01$) and to a lesser extent in subcutaneous preadipocytes ($p=0.02$). Messenger RNA of COX-2 was significantly higher in omental compared to subcutaneous preadipocytes in response to combined TNF-α and IL-1β ($p=0.01$). Inflammatory cytokines increased AKR1B1 mRNA expression and protein levels ($p\leq0.05$), but failed to increase expression levels of AKR1C3 in cultured preadipocytes. Accordingly, ponalrestat blunted PGF2α synthesis by preadipocytes in basal and stimulated conditions ($p\leq0.05$). Women with the highest PGF2α release by omental adipocytes had a higher BMI ($p=0.05$), waist circumference ($p\leq0.05$) and HOMAir index ($p\leq0.005$) as well as higher mRNA expression of AKR1B1 in omental ($p<0.10$) and subcutaneous ($p\leq0.05$) adipose tissue compared to women with low omental adipocytes PGF2α release. Positive correlations were observed between mRNA expression of AKR1B1 in both compartments and BMI, waist circumference as well as HOMAir index ($p\leq0.05$ for all). Conclusion: PGF2α release by omental mature adipocytes is increased in abdominally obese women. Moreover, COX-2 expression and PGF2α release is particularly responsive to inflammatory stimulation in omental preadipocytes. Yet, blockade of PGF synthase AKR1B1 inhibits most of the PGF2α release.

INTRODUCTION

Expansion of body fat mass as seen in obesity is related to alterations of the metabolic and endocrine function of adipose tissue leading to poor handling of postprandial lipids, fatty acid spillover to other tissues and organs, macrophage infiltration and release of pro-inflammatory mediators [1–5]. This condition has been proposed as a potential mechanism linking obesity with metabolic diseases [4–6]. Among the many inflammatory factors secreted by adipose tissue, prostaglandins (PGs) have been proposed as contributing mediators of inflammation in obesity, hyperinsulinemia, hypertension and cardiovascular disease [7–9].

Whole tissue explants, isolated mature adipocytes and non-fat cells from the stromal-vascular fraction were used by other groups to study PG release or synthesis by the various cell fractions of adipose tissue [10–20]. Taken together, these studies indicate that mature adipocytes and stromal-vascular cells both contribute to the synthesis and release of PGs, the main secreting cells being the non-fat, stromal-vascular fraction of adipose tissue [10,11,15,21]. Mature adipocytes from humans, mice or rats are also known to release PGs including PGE2 and PGF2α as well as PGI2 and other metabolites [10,13,15]. Recent in vitro studies demonstrated that PGD2 and PGI2 enhance adipocyte differentiation [22,23], while others have shown that PGE2 and PGF2α inhibit this process through their specific receptors, the EP4 receptor [24] and the FP receptor respectively [25–28]. These findings suggest that PGF2α may have anti-adipogenic functions.

PGs are derived from arachidonic acid (AA) through the activity of two PGH synthases (PTGS), the constitutive cyclooxygenase (COX)-1 or the inducible COX-2, both converting AA consecutively into PGG2 and PGH2 [29]. PGF2α is mostly synthesized by the reduction of the 9,11-endoperoxide moiety of PGH2 [30]. PGF2α may also be formed through reduction of PGD2 by 11-keto reductase or PGE2 by 9-ketoreductase [31]. These reactions are catalyzed by enzymes of the aldo-keto reductase (AKRs) family [31], which are monomeric, soluble oxido-reductases dependent on NAD(P)H. In mammals, the first PGF synthase identified belongs to the AKR1C family (AKR1C3) [32]. We also demonstrated in other models including bovine and human endometrium or other tissues that enzymes of the AKR1B family exhibit PGF synthase activity [33–35]. In vitro studies have previously confirmed the PGF synthase activity of mouse Akr1b3 and Akr1b7 as well as human AKR1B1 [36]. A recent study also demonstrated that Akr1b3, the murine ortholog of human AKR1B1, acts as a functional PGF synthase and is involved in the suppression of adipogenesis through the FP receptor in the 3T3-L1 cell line [37]. The relevance of these data in humans remains to be established.

Considering that human body fat distribution is quite heterogeneous and that excess abdominal adipose tissue accumulation is associated with increased cardiometabolic risk independent of total body fat mass [38], depot-specific differences in PGF2α synthesis may have a pathophysiological role in the development of visceral obesity-related comorbidities. Until now, whether and how PGF2α release by mature adipocytes from the subcutaneous and omental fat depot is affected in human obesity has never been clearly established. The aim of this study was to examine PGF2α release by primary preadipocytes, mature adipocytes and whole tissue explants from the subcutaneous and visceral (omental) fat compartments. We tested the hypothesis that preadipocytes from the omental fat compartment release more PGF2α than those from the subcutaneous depot and that abdominal obesity is related to alterations in PGF2α release by the mature cell fraction from the omental fat compartment. Since previous literature did not allow determining which PGF synthase is the most relevant in human adipose tissue, we also focused on AKR1B1 and AKR1C3 in response to inflammatory cytokines in human preadipocyte cultures, and investigated the impact of an AKR1B1 inhibitor on PGF2α synthesis. We tested the hypothesis that AKR1B1 inhibition significantly impairs the synthesis of PGF2α by human cultured preadipocytes.

MATERIALS AND METHODS

Subjects and ethics statement

The study sample included lean to obese women recruited through the elective surgery schedule of the Gynecology Unit at Laval University Medical Center. Women were undergoing gynecological surgery for total or subtotal abdominal hysterectomies. The study was approved by the Research Ethics Committees of Laval University Medical Center (protocol C09-08-086). We also included cultures from women undergoing biliopancreatic diversion for the treatment of morbid obesity (n=2, aged 42 and 53 years, BMI 40 and 52.7 kg/m2) with approval from the Research Ethics Committees of the Quebec Cardiology and Pulmonology Institute (protocol CERHL 1142). All subjects provided written informed consent before their inclusion in the study.

Clinical parameters and glucose homeostasis measurements

Body weight, height, body mass index (BMI) and waist circumference were measured on the morning of the surgery according to a standard protocol. Fasting glucose and insulin were measured in pre-surgery blood samples collected after a 12h-overnight fast. Plasma glucose was measured using the glucose oxidase method and plasma insulin levels were measured by ELISA (Millipore, St.Charles, MO, USA). The HOMA insulin resistance index (HOMAir) was calculated as described [39].

Adipose tissue sampling

Subcutaneous and omental adipose tissue samples were respectively collected at the site of the surgical incision and greater omentum and immediately carried to the laboratory. A portion of the fresh sample was used for adipocyte and preadipocyte isolation and pieces of fresh adipose tissue samples were fixed in 10% formalin for 24-48 hours at room temperature prior processing for routine paraffin wax embedding. A proportion of 30 mg fresh adipose tissue was cut into 5-10 mg pieces and placed in serum-free Medium 199. Adipose tissue explants were kept in culture at 37°C under a 5% CO_2 atmosphere. The remaining portion of the sample was immediately frozen and kept for future analyses.

Adipocyte isolation and adipocyte size measurement

A portion of each fresh tissue sample was digested 45 min at 37oC with type I collagenase in Krebs-Ringer-Henseleit (KRH) buffer supplemented with glucose, adenosine, ascorbic acid and BSA according to a modified version of the Rodbell method [40]. Digested tissues were filtered through nylon mesh and mature adipocytes were separated from the stromal-vascular fraction by floatation. Cells were washed 3 times and mature adipocyte suspensions were visualized using a phase contrast microscope attached to a camera and computer interface. Pictures of the suspensions were taken and the Scion Image software was used to measure the size (diameter) of 250 adipocytes for each tissue sample. Average adipocyte size of each sample was used in analyses.

Preadipocyte isolation and primary cultures

Preadipocytes were isolated using a modification of the Van Harmelen method [41]. The residual KRH buffer of the adipocyte isolation, which contained the stromal-vascular fraction, was centrifuged and the pellet was washed in DMEM-F12 culture medium supplemented with 10% calf serum, 2.5µg/ml amphotericin B and 50µg/ml gentamicin. Stromal-vascular cells were then filtered through 140µm nylon mesh to remove endothelial/mesothelial cells, placed in culture plates and cultured at 37 ºC under a 5% CO_2 atmosphere. Medium was changed every 2–3 days.

PGF2α measurements

$PGF_{2\alpha}$ release by isolated subcutaneous and omental mature adipocytes was measured in suspensions of approximately 5000 cells incubated for 2h at 37 ºC in KRH buffer. The $PGF_{2\alpha}$ response of mature adipocytes to inflammatory cytokine stimulation was tested by incubating suspensions with TNF-α (1ng/ml) and/or IL-1β (1ng/ml) or vehicle for 2 hours. The $PGF_{2\alpha}$ response of primary preadipocytes and primary organ cultures to inflammatory cytokines was assessed by incubating the cells/explants with TNF-α (1ng/ml) and/or IL-1β (1ng/ml) or vehicle for 24 hours. The incubation time with inflammatory stimuli was established according to a time-course experiment (0, 3h, 6h, 16h and 24h) and the dose of inflammatory cytokines was established according to a dose-response experiment (0.01ng/ml, 0.1ng/ml, 1ng/ml, 10ng/ml and 100ng/ml). Taking into consideration that mature fat cells cannot be kept more than a few hours in suspension, a short incubation (2 hours) was performed with this fraction. The $PGF_{2\alpha}$ response of primary preadipocytes was also assessed by incubating cells with IL-1β (1ng/ml) or vehicle in the presence or absence of the aldose reductase inhibitor ponalrestat (0.05, 0.5, 5, 10 or 20μM) [42] for 24 hours. Aldose reductase inhibitor ponalrestat was from Tocris Bioscience (Ellisville, MO, USA). Cytotoxicity was assessed by the measurement of adenylate kinase release in the medium using ToxiLight Non-destructive cyclooxygenase bioassay kit (Lonza, Rockland, ME, USA). $PGF_{2\alpha}$ content in the media was measured by enzyme immunoassay, and acetylcholinesterase-linked $PGF_{2\alpha}$ tracer (Cayman) as previously described [43]. Considering the nature and cultivability of each cell type, $PGF_{2\alpha}$ release by omental and subcutaneous mature adipocytes was expressed as pg/10^6 cells*2h. $PGF_{2\alpha}$ release by cultured primary subcutaneous and omental preadipocytes was expressed as pg/ml*μg protein*24h and $PGF_{2\alpha}$ release by omental and subcutaneous adipose tissue explants was expressed as pg/ml*mg tissue*24h. Recombinant human TNF-α and IL-1β were purchased from PeproTech (Rocky Hill, NJ, USA).

Messenger RNA expression by quantitative real-time RT-PCR

Total RNA was extracted using the RNeasy lipid tissue extraction kit and on-column DNase treatment (Qiagen, Valencia, CA, USA) from whole subcutaneous and omental adipose tissue or from preadipocyte cultures treated with TNF-α and/or IL-1β or vehicle. RNA quality and concentration was assessed using the Agilent Technologies 2100 bioanalyzer (Agilent, Santa Clara, CA, USA). Complementary DNA was generated from total RNA using random hexamers, oligo dT_{18} and Superscript III Rnase H-RT (Invitrogen Life Technologies, Burlington, ON, Canada) and purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Real-time cDNA amplification was performed in duplicate using the LightCycler 480 (Roche Diagnostics, Mannheim, DE, USA) and the SYBR Green I Master (Roche Diagnostics, Indianapolis, IN, USA). The conditions for PCR reactions were: 45 cycles, denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, elongation at 72°C for 14 sec and then 74°C for 5 sec (reading). A melting curve was generated to assess non-specific signal. Calculation of the number of copies of each mRNA was performed according to Luu-The et al. [44] using second derivative method and a standard curve of Cp versus logarithm of the quantity. The standard curve was established using known amounts of purified PCR products and the LightCycler 480 v1.5 program provided by the manufacturer (Roche Diagnostics, Mannheim, DE, USA). PCR amplification efficiency was verified. Target gene amplifications were normalized using housekeeping gene expression levels of ATP synthase O subunit (*ATP5O*) for whole tissue extracts or Glucose-6-phosphate dehydrogenase (*G6PD*) for stimulated preadipocytes. Expression levels of *ATP5O* were not different in omental versus subcutaneous adipose tissue and were not associated with adiposity measurements in our study sample. *G6PD* mRNA expression was not significantly modulated during inflammatory cytokine stimulation in preadipocytes. The transcripts examined were *COX-2* and the two putative PGF synthases, *AKR1B1* and *AKR1C3*. Primer sequences were designed using GeneTools (Biotools Inc., Jupiter, FL, USA) and are listed in **Table 1**. Quantitative realtime PCR measurements were performed by the CHU de Québec Research Center Gene Expression Platform (Quebec, Canada).

Western blot analysis

Cultures were harvested in lysis buffer containing protease inhibitors. For immunoblotting, 12 µg of protein homogenate diluted in sodium dodecyl sulfate (SDS) buffer was heated at 37°C for 3 minutes and separated on a 10% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (1 hour at 100 V), and unspecific sites were blocked with 5% nonfat milk diluted in wash solution for 1 hour at room temperature. Membranes were then incubated overnight at 4°C with the primary antibody against AKR1B1 (dilution 1/1000, provided by Dr Fortier [45]), AKR1C3 (dilution 1/1000, Acris Antibody Inc., Sandiego, CA, USA), COX-2 (dilution 1/3000, provided by Dr S Kargman (Merck, Quebec, Canada)) or β-tubulin (Cell Signaling Technology, Danvers, MA, USA) as loading control, washed 4 × 15 minutes, and incubated for 1 hour with anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. Finally, membranes were washed 4 × 15 minutes and proteins were visualized by chemiluminescence. Densitometric analysis of protein levels was performed using Image J software (NIH, USA).

Immunohistochemistry

Adipose tissue macrophage infiltration was quantified by fluorescence immunostaining on formalin-fixed and paraffin-embedded adipose tissue samples, as previously described [1]. Immunostaining for CD68 (mouse anti-human CD68 antibody, Cedarlane, Burlington, Ontario, Canada) was performed and the number of cells infiltrated by macrophages was counted (identified as CD68+ cells) in a blinded manner. A minimum of 400 adipocytes were examined for each sample. The number of macrophages was normalized for 100 adipocytes.

Statistical analyses

Repeated-measures analysis of variance in each fat compartment was used to compare mean PGF2α release by preadipocytes, isolated mature adipocytes or adipose tissue explants in response to TNF-α and/or IL-1β and in the presence or absence of increasing concentrations (0-20µM) of ponalrestat. Repeated-measures analysis of variance was also performed to compare mean COX-2, ARK1B1 and AKR1C3 expression levels by preadipocytes in response to TNF-α and/or IL-1β. Pearson correlation coefficients were computed to quantify associations between adipose tissue mRNA expression of AKR1B1 and adiposity as well as metabolic measurements. PGF2α release by mature adipocytes was high in approximately a third of the patients (n=12) in each fat compartment and relatively low in the remaining patients. For this reason, PGF2α release by mature cells was analyzed in categorical fashion. Indeed, women were subdivided in two subgroups with either low (n=23) or high (n=12) PGF2α release in each fat compartment. Patients with undetectable PGF2α release by mature adipocytes (n=5 patients for both compartments and in n=2 patients in one of the compartments) were included in the group characterized with low PGF2α release. Adiposity measurements and metabolic outcomes were compared in women with low vs. high PGF2α release using Student's t tests. All data were presented as mean ± SEM. Log10 and box-cox transformations were used for non-normally distributed variables. Statistical analyses were performed with the JMP 4.0 software (SAS Institute, Cary, NC).

RESULTS

PGF2α release by primary preadipocytes, mature adipocytes and adipose tissue explants from the subcutaneous and omental compartments

Figure 1 shows PGF2α release by subcutaneous and omental preadipocyte primary cultures over 24 hours, collagenase-isolated mature adipocyte suspensions over 2 hours or explants over 24 hours. Figures 1A shows the release of PGF2α by subcutaneous and omental primary preadipocyte cultures following stimulation with TNF-α and/or IL-1β. PGF2α release was significantly induced in response to TNF-α and/or IL-1β

compared to control in omental (p=0.01) and to a lesser extent in subcutaneous preadipocytes (p=0.02). PGF2α release by omental preadipocytes was significantly higher in response to IL-1β and combined TNF-α and IL-1β compared to that of subcutaneous preadipocytes, with a significant treatment-by-depot interaction (p≤0.05).

Using repeated-measures analysis of variance, PGF2α release was not significantly increased in response to short-term TNF-α and/or IL-1β treatments compared to control in omental or subcutaneous mature adipocytes. No significant depot difference was observed in PGF2α release by omental and subcutaneous mature adipocytes in all conditions tested suggesting that the effect of IL-1β and TNF-α may be transcriptional and may require longer incubation times (Figure 1B).

Similar to preadipocyte cultures, Figures 1C shows that PGF2α release was significantly induced in response to IL-1β or combined TNF-α and IL-1β compared to control in explants from both fat compartments (p=0.01). Omental explants tended to have a higher PGF2α release in response to TNF-α and IL-1β compared to subcutaneous explants, but this difference did not reach significance.

COX-2 expression and PGF synthase expression in primary preadipocytes

Considering that inflammatory cytokines significantly induced PGF2α release by cultured preadipocytes (Figure 1), we also examined the expression of COX-2 and the two putative terminal PGF synthases, AKR1B1 and AKR1C3, in cultured preadipocytes in response to inflammatory cytokines (Figure 2). Messenger RNA expression (Figure 2A) and protein levels (Figure 2B) of COX-2 in cultured preadipocytes were significantly increased in response to IL-1β and combined TNF-α and IL-1β compared to control (p≤0.05). Interestingly, abundance of COX-2 mRNA was significantly higher in omental compared to subcutaneous preadipocytes in response to combined TNF-α and IL-1β (p=0.01) (Figure 3). No significant depot difference was observed in protein levels of COX-2 (data not shown). Messenger RNA expression (Figure 2C) and protein levels (Figure 2D) of AKR1B1 in cultured preadipocytes were also significantly increased in response to TNF-α and/or IL-1β compared to control (p≤0.05). No significant depot difference was observed in mRNA expression and protein levels of AKR1B1 (data not shown). Interestingly, TNF-α and/or IL-1β treatment in cultured preadipocytes failed to increase expression of the other putative terminal PGF synthase AKR1C3 and although subcutaneous mRNA expression of this gene tended to be higher compared to omental cultured preadipocytes, this trend did not reach significance (Figure 2E). Furthermore, even if we detected the recombinant protein of AKR1C3 (positive control), protein levels of AKR1C3 were not detected in cultured preadipocytes (data not shown). Similar to results of Figure 1, PGF2α release was also significantly induced in response to TNF-α and/or IL-1β compared to control in these cultures (p=0.02) (data not shown).

Effect of aldose reductase inhibitor on PGF2α release by human primary preadipocytes

Considering that inflammatory cytokines increased the expression of AKR1B1 at the mRNA and protein levels, but failed to increase the expression of AKR1C3, and that protein levels of AKR1C3 were not detected in preadipocytes, we examined the effect of ponalrestat, an aldose reductase inhibitor developed to inhibit the conversion of glucose to sorbitol by AKR1B1, on PGF2α release by human primary preadipocytes. Figure 4 illustrates that ponalrestat completely reversed the stimulatory effect of IL-1β on PGF2α release by human primary preadipocytes, in a dose-dependant manner (p≤0.05). Pronalrestat also significantly inhibited the production of PGF2α by cultured preadipocytes in basal conditions (p≤0.05). Ponalrestat had no effect on cell viability.

Expression levels of AKR1B1 in whole subcutaneous vs. omental adipose tissue

Considering that AKR1B1 seems to have a predominant role in PGF2α synthesis by human preadipocytes in response to inflammatory cytokines, we measured whole subcutaneous and omental adipose tissue expression of AKR1B1 in a sample of 46 women exhibiting lean to severely obese phenotypes. Table 2 summarizes the characteristics of the study sample. Messenger RNA levels of AKR1B1 were detectable in tissues from both fat compartments and were higher in omental compared to subcutaneous adipose tissue ($p \leq 0.01$, data not shown). Positive and significant correlations were observed between whole tissue AKR1B1 mRNA expression in both compartments, and BMI, waist circumference as well as HOMAir index ($p \leq 0.05$, for all) (Table 3).

PGF2α release by subcutaneous and omental mature adipocytes in relation with body fatness, glucose homeostasis and adipose tissue macrophage infiltration

PGF2α release by subcutaneous and omental mature adipocyte suspensions was measured in a subsample of the study (35 women exhibiting lean to severely obese phenotypes) for which we have prepared isolated mature adipocytes from the omental and subcutaneous fat compartments. Women were subdivided in two subgroups with either low (n=23) or high (n=12) omental adipocyte PGF2α release. According to this stratification, women with the highest PGF2α release by omental adipocytes had significantly higher omental adipocyte PGF2α release compared to women with low omental adipocyte PGF2α release ($p \leq 0.0001$) (Figure 5A). Women with the highest PGF2α release by omental adipocytes also had significantly higher PGF2α release by subcutaneous adipocytes ($p \leq 0.05$) (Figure 5B) compared to women with low PGF2α release by omental adipocytes. These women also tended to have higher omental adipose tissue mRNA expression of AKR1B1 ($p < 0.10$) and had significantly higher subcutaneous adipose tissue mRNA expression of AKR1B1 ($p \leq 0.05$) (Figure 5C and D).

Women with the highest PGF2α release by omental adipocytes had significantly higher BMI ($p = 0.05$) (Figure 6A), waist circumference ($p \leq 0.05$) (Figure 6B) and omental adipocyte diameter ($p \leq 0.005$) (data not shown) compared to women with low omental PGF2α release. Regarding glucose homeostasis, women with the highest PGF2α release by omental adipocytes had significantly higher fasting glycemia ($p \leq 0.005$) (data not shown), fasting insulinemia ($p \leq 0.05$) (data not shown) and HOMAir index ($p \leq 0.005$) (Figure 6C) compared to women with low omental adipocytes PGF2α release. Finally, regarding adipose tissue macrophage infiltration, women with the highest omental PGF2α release had significantly more CD68+cells in both omental ($p \leq 0.05$) (Figure 6D) and subcutaneous adipose tissue ($p \leq 0.05$) (Figure 6E) compared to women with low PGF2α release. PGF2α release by subcutaneous mature adipocytes was not significantly related to metabolic parameters. Only statistical trends were observed.

DISCUSSION

To our knowledge, this is the first study to clearly examine fat depot-specific differences in PGF2α release by human subcutaneous and omental adipose tissue cell fractions. We found that PGF2α release was significantly induced in response to TNF-α and/or IL-1β compared to control in omental and to a lesser extent in subcutaneous preadipocytes. Higher mRNA expression levels of COX-2 were observed in omental compared to subcutaneous stimulated preadipocytes, suggesting that the change in COX-2 expression in response to inflammatory cytokines is an important regulator of PGF2α production in preadipocytes. We also observed that inflammatory cytokines stimulated AKR1B1 mRNA and protein levels, but failed to increase expression levels of AKR1C3 in preadipocytes, suggesting that AKR1B1 is a likely candidate for cytokine-stimulated PGF2α synthesis in preadipocytes, as opposed to AKR1C3. Accordingly, our experiment with ponalrestat demonstrated that AKR1B1 may have a predominant role in the production of PGF2α synthesis by cultured preadipocytes in response to basal and inflammatory conditions. We also found that PGF2α release

by omental mature adipocytes and whole tissue mRNA expression of AKR1B1 is increased in abdominally obese women with altered glucose homeostasis.

One important finding in this study is that AKR1B1 has a predominant role in PGF2α synthesis by human preadipocytes in response to inflammatory cytokines compared to AKR1C3. AKR1C3 is known to exhibit ketosteroid reductase activity (type 5 17β-HSD), which mostly inactivates progesterone into 20α-hydroxyprogesterone [46]. AKR1C3 also displays 17β-HSD and 3α-HSD activities [46,47]. Theses studies demonstrated that AKR1C3 is highly expressed in subcutaneous adipose tissue and seems to have steroid reductase activities in human abdominal adipose tissue samples [48]. In the present study, we observed that cytokine treatment stimulated PGF2α release as well as the mRNA and protein levels of AKR1B1 and COX-2, but failed to increase expression levels of AKR1C3 in preadipocytes. Furthermore, AKR1C3 protein levels were not detected in cultured preadipocytes treated with cytokines or vehicle while AKR1C3 mRNA expression levels were very low, further excluding a potential role of AKR1C3 in PGF2α synthesis by preadipocytes. We also found that ponalrestat, an inhibitor of AKR1B1, significantly decreased the inflammatory effect of IL-1β on PGF2α production by primary preadipocytes. These results further suggest that AKR1BI may be relevant for PGF synthase in human preadipocytes. Consistent with our results, Kabututu et al. demonstrated that recombinant AKR1B1, Akr1b3 and Akr1b7 had better PGF synthase activities than previously-characterized PGF synthases (AKR1C family members) in mammals [36]. We also previously showed that AKR from the 1B family exhibited PGF synthase activity, first in the bovine model [33], but also in human endometrium [34] and other tissues [35], which is consistent with the present results. Indeed, we previously demonstrated that the reduction of AKR1B1 by specific siRNA knockdown was related to a significant decrease in PGF2α release [34]. The PGF synthase activity of AKR1B1 has also been established by others [49–51]. Fujimori et al. also demonstrated that siRNA for Akr1b3 suppresses PGF2α synthesis in the 3T3-L1 cell line, indicating that Akr1b3 is the primary PGF synthase in mouse preadipocytes [37]. Consistent with these studies, we establish for the first time in human adipose tissue that preadipocyte release of PGF2α is responsive to inflammatory stimulation and that AKR1B1 may be largely responsible for this response.

We demonstrated that preadipocytes from the omental fat compartment released more PGF2α in response to inflammatory stimuli compared to those from subcutaneous fat. The higher mRNA expression of COX-2 in omental compared to subcutaneous preadipocytes after inflammatory stimulation very likely explains these depot differences. Indeed, we found no significant depot differences in the expression of AKR1B1 in stimulated preadipocytes. Consistent with our results, previous studies had demonstrated that COX-2 is induced by inflammatory stimulation and is the rate-limiting enzyme in the synthesis of PG [51]. Even if COX-2 explains depot differences and is implicated in PGF2α production, our experiment with ponalrestat demonstrated that blockade of AKR1B1 still inhibits most of the PGF2α synthesis by cultured preadipocytes either in the basal state or in response to inflammatory stimulation. Our results indirectly suggest a predisposition of omental fat cells to respond to inflammation through this mechanism. The particular sensitivity of preadipocytes from the omental fat depot in response to inflammatory stimulation possibly plays a pathophysiological role in visceral obesity.

We also demonstrated that women with elevated omental adipocyte PGF2α release have a significantly higher BMI and waist circumference. Consistently, obesity level was significantly and positively related to adipose tissue mRNA expression levels of AKR1B1. The physiological consequences of these observations remain unclear. McQuaid et al. recently demonstrated that abdominally obese subjects had significantly lower adipose tissue blood flow in the fasting and postprandial states compared to lean subjects [2]. More specifically, they demonstrated that abdominal obesity is associated to adipose tissue adaptation in terms of systemic non-esterified fatty acid (NEFA) delivery and vascular functions of the tissue, which seem to be involved in fat storage dysfunction and ectopic fat deposition [2]. Farb et al. also recently demonstrated that cyclooxygenase-derived vasoconstrictor prostanoids may contribute to endothelial dysfunction of visceral adipose arterioles [52]. Considering that PGF2α is an important vasoconstrictor [31], high release of PGF2α by adipose cells of women with abdominal obesity may contribute to these phenomena and may represent an

indicator of adipose tissue dysfunction. We also observed that women with elevated omental adipocyte PGF2α release have increased adipose tissue macrophage infiltration. In addition, mature omental adipocytes that released the highest amounts of PGF2α were those with the largest size, indirectly suggesting that omental adipocyte hypertrophy may be a determinant of this secretory function of the cell. Accordingly, other studies have demonstrated that adipocyte hypertrophy creating local hypoxic conditions may be involved in the attraction of macrophages by stimulating inflammatory pathways such as JNK1-regulated chemokine release [5,53,54]. The elevated release of PGF2α by mature fat cells of women with abdominal obesity may either reflect or contribute to these phenomena as a mediator of inflammation.

The main PG-secreting cells are in the stromal-vascular fraction of adipose tissue [10,11,15]. In the present study, we could neither confirm nor contradict this notion due to methodological differences in culture conditions. We used mature fat cell suspensions normalized for the amount of cells on the one hand, and adherent preadipocytes in primary cultures on the other, with data expressed as a function of protein level. The reasons for this methodological discrepancy relates to the nature and cultivability of each cell type. Mature fat cells cannot be kept more than a few hours in suspensions. Conversely, we could not study prostaglandin release in freshly isolated stromal-vascular fractions due to the small cell numbers present in our samples. These methodological limitations in the culture models may explain why the depot difference in PGF2α release was not significant in some of the conditions tested. Short incubation times (2 hours) of the mature adipocyte preparations may explain the lack of stimulation by inflammatory cytokines. Furthermore, the lack of a significant depot difference in the release of PGF2α by adipose tissue explants may relate to the small number of cultures. We also failed to observe depot difference in COX-2 protein levels in our samples, but we suggest that this discrepancy may be related to the smaller number of samples examined in western blot experiments. Quantitative realtime PCR measurements are generally more sensitive to assess fat depot differences with relatively small sample sizes compared to protein measurements. We also observed that PGF2α release by subcutaneous mature adipocytes was not significantly related to all metabolic parameters. We suggested that the wider range of variability in visceral adipose tissue area and omental adipocyte size in that sample favored stronger correlations of a given parameter measured in visceral fat compared to the same parameter in subcutaneous adipose tissue. Furthermore, power analysis suggests that a much larger sample size would have been required to observe significant differences in metabolic parameters between women with either low or high PGF2α release by subcutaneous adipocytes. In spite of these limitations, we show that omental preadipocytes have a higher capacity to release PGF2α and respond to inflammatory stimuli compared to subcutaneous preadipocytes. Another acknowledged limitation of the study is the absence of male subjects. The main reason for not including men is the difficulty to obtain comparable samples from generally healthy lean to moderately obese subjects. We also assessed total macrophage infiltration using CD68 as the sole marker. Other markers of macrophage infiltration may have generated different results.

In conclusion, we found that omental preadipocyte release of PGF2α is particularly responsive to inflammatory stimulation. Higher expression levels of COX-2 observed in omental compared to subcutaneous stimulated preadipocytes suggests that the changes in COX-2 expression are an important regulator of PGF2α production in preadipocytes. This study also demonstrates for the first time that AKR1B1 may have a predominant role in PGF2α synthesis by human preadipocytes in response to inflammatory cytokines compared to AKR1C3. In this context, further studies are needed to examine the role of human AKR1B1 as a PGF synthase in various cells of adipose tissue and how it might modulate adipose tissue homeostasis in humans.

REFERENCES

1. Michaud A, Drolet R, Noel S, Paris G, Tchernof A (2012) Visceral fat accumulation is an indicator of adipose tissue macrophage infiltration in women. Metabolism 61: 689-698.

2. McQuaid SE, Hodson L, Neville MJ, Dennis AL, Cheeseman J, et al. (2011) Downregulation of adipose tissue fatty acid trafficking in obesity: a driver for ectopic fat deposition? Diabetes 60: 47-55.

3. Wellen KE, Hotamisligil GS (2003) Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 112: 1785-1788.

4. Frayn KN (2002) Adipose tissue as a buffer for daily lipid flux. Diabetologia 45: 1201-1210.

5. Bluher M (2009) Adipose tissue dysfunction in obesity. Exp Clin Endocrinol Diabetes 117: 241-250.

6. Berg AH, Scherer PE (2005) Adipose tissue, inflammation, and cardiovascular disease. Circ Res 96: 939-949.

7. Curtis-Prior PB, Jenner M, Smethurst M, Woodward JW (1979) Plasma prostaglandin levels in fed and starved lean, normal and obese women. Experientia 35: 911-912.

8. Axelrod L, Levine L (1983) Inhibitory effect of insulin on prostacyclin production by isolated rat adipocytes. Prostaglandins 25: 571-579.

9. Axelrod L, Shulman GI, Blackshear PJ, Bornstein W, Roussell AM, et al. (1986) Plasma level of 13,14-dihydro-15-keto-PGE2 in patients with diabetic ketoacidosis and in normal fasting subjects. Diabetes 35: 1004-1010.

10. Chatzipanteli K, Rudolph S, Axelrod L (1992) Coordinate control of lipolysis by prostaglandin E2 and prostacyclin in rat adipose tissue. Diabetes 41: 927-935.

11. Borglum JD, Pedersen SB, Ailhaud G, Negrel R, Richelsen B (1999) Differential expression of prostaglandin receptor mRNAs during adipose cell differentiation. Prostaglandins Other Lipid Mediat 57: 305-317.

12. Fain JN, Kanu A, Bahouth SW, Cowan GS, Jr., Hiler ML, et al. (2002) Comparison of PGE2, prostacyclin and leptin release by human adipocytes versus explants of adipose tissue in primary culture. Prostaglandins Leukot Essent Fatty Acids 67: 467-473.

13. Richelsen B (1987) Factors regulating the production of prostaglandin E2 and prostacyclin (prostaglandin I2) in rat and human adipocytes. Biochem J 247: 389-394.

14. Yan H, Kermouni A, Abdel-Hafez M, Lau DC (2003) Role of cyclooxygenases COX-1 and COX-2 in modulating adipogenesis in 3T3-L1 cells. J Lipid Res 44: 424-429.

15. Fain JN, Buehrer B, Bahouth SW, Tichansky DS, Madan AK (2008) Comparison of messenger RNA distribution for 60 proteins in fat cells vs the nonfat cells of human omental adipose tissue. Metabolism 57: 1005-1015.

16. Chou WL, Chuang LM, Chou CC, Wang AHJ, Lawson JA, et al. (2007) Identification of a novel prostaglandin reductase reveals the involvement of prostaglandin E2 catabolism in regulation of peroxisome proliferator-activated receptor gamma activation. J Biol Chem 282: 18162-18172.

17. Nishimura K, Setoyama T, Tsumagari H, Miyata N, Hatano Y, et al. (2006) Endogenous prostaglandins E2 and F 2alpha serve as an anti-apoptotic factor against apoptosis induced by tumor necrosis factor-alpha in mouse 3T3-L1 preadipocytes. Biosci Biotechnol Biochem 70: 2145-2153.

18. Hetu PO, Riendeau D (2007) Down-regulation of microsomal prostaglandin E2 synthase-1 in adipose tissue by high-fat feeding. Obesity 15: 60-68.

19. Aprikian O, Reynaud D, Pace-Asciak C, Leone P, Blancher F, et al. (2007) Neonatal dietary supplementation of arachidonic acid increases prostaglandin levels in adipose tissue but does not promote fat mass development in guinea pigs. Am J Physiol Regul Integr Comp Physiol 293: R2006-R2012.

20. Madan AK, Tichansky DS, Coday M, Fain JN (2006) Comparison of IL-8, IL-6 and PGE(2) formation by visceral (omental) adipose tissue of obese Caucasian compared to African-American women. Obes Surg 16: 1342-1350.

21. Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW (2004) Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 145: 2273-2282.

22. Fujimori K, Aritake K, Urade Y (2007) A novel pathway to enhance adipocyte differentiation of 3T3-L1 cells by up-regulation of lipocalin-type prostaglandin D synthase mediated by liver X receptor-activated sterol regulatory element-binding protein-1c. J Biol Chem 282: 18458-18466.

23. Vassaux G, Gaillard D, Darimont C, Ailhaud G, Negrel R (1992) Differential response of preadipocytes and adipocytes to prostacyclin and prostaglandin E2: physiological implications. Endocrinology 131: 2393-2398.

24. Tsuboi H, Sugimoto Y, Kainoh T, Ichikawa A (2004) Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. Biochem Biophys Res Commun 322: 1066-1072.

25. Casimir DA, Miller CW, Ntambi JM (1996) Preadipocyte differentiation blocked by prostaglandin stimulation of prostanoid FP2 receptor in murine 3T3-L1 cells. Differentiation 60: 203-210.

26. Liu L, Clipstone NA (2007) Prostaglandin F2alpha inhibits adipocyte differentiation via a G alpha q-calcium-calcineurin-dependent signaling pathway. J Cell Biochem 100: 161-173.

27. Miller CW, Casimir DA, Ntambi JM (1996) The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin F2alpha. Endocrinology 137: 5641-5650.

28. Serrero G, Lepak NM (1997) Prostaglandin F2alpha receptor (FP receptor) agonists are potent adipose differentiation inhibitors for primary culture of adipocyte precursors in defined medium. Biochem Biophys Res Commun 233: 200-202.

29. Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271: 33157-33160.

30. Watanabe K, Yoshida R, Shimizu T, Hayaishi O (1985) Enzymatic formation of prostaglandin F2 alpha from prostaglandin H2 and D2. Purification and properties of prostaglandin F synthetase from bovine lung. J Biol Chem 260: 7035-7041.

31. Watanabe K (2002) Prostaglandin F synthase. Prostaglandins Other Lipid Mediat 68-69: 401-407.

32. Hayaishi O, Watanabe K, Fujii Y, Nakayama K, Ohkubo H, et al. (1988) Prostaglandin F synthetase, a dual function enzyme. Prog Clin Biol Res 274: 577-587.

33. Madore E, Harvey N, Parent J, Chapdelaine P, Arosh JA, et al. (2003) An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin f2 alpha in the bovine endometrium. J Biol Chem 278: 11205-11212.

34. Bresson E, Boucher-Kovalik S, Chapdelaine P, Madore E, Harvey N, et al. (2011) The human aldose reductase AKR1B1 qualifies as the primary prostaglandin F synthase in the endometrium. J Clin Endocrinol Metab 96: 210-219.

35. Bresson E, Lacroix-Pepin N, Boucher-Kovalik S, Chapdelaine P, Fortier MA (2012) The Prostaglandin F Synthase Activity of the Human Aldose Reductase AKR1B1 Brings New Lenses to Look at Pathologic Conditions. Front Pharmacol 3:98.

36. Kabututu Z, Manin M, Pointud JC, Maruyama T, Nagata N, et al. (2009) Prostaglandin F2alpha synthase activities of aldo-keto reductase 1B1, 1B3 and 1B7. J Biochem 145: 161-168.

37. Fujimori K, Ueno T, Nagata N, Kashiwagi K, Aritake K, et al. (2010) Suppression of adipocyte differentiation by aldo-keto reductase 1B3 acting as prostaglandin F2alpha synthase. J Biol Chem 285: 8880-8886.

38. Despres JP, Lemieux I (2006) Abdominal obesity and metabolic syndrome. Nature 444: 881-887.

39. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, et al. (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28: 412-419.

40. Rodbell M (1964) Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239: 375-380.

41. van Harmelen V, Rohrig K, Hauner H (2004) Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. Metabolism 53: 632-637.

42. Sochor M, Kunjara S, McLean P (1988) The effect of aldose reductase inhibitor Statil (ICI 128436) on the glucose over-utilization in kidney of diabetic rats. Biochem Pharmacol 37: 3349-3356.

43. Asselin E, Goff AK, Bergeron H, Fortier MA (1996) Influence of sex steroids on the production of prostaglandins F2 alpha and E2 and response to oxytocin in cultured epithelial and stromal cells of the bovine endometrium. Biol Reprod 54: 371-379.

44. Luu-The V, Paquet N, Calvo E, Cumps J (2005) Improved real-time RT-PCR method for high-throughput measurements using second derivative calculation and double correction. Biotechniques 38: 287-293.

45. Chapdelaine P, Kang J, Boucher-Kovalik S, Caron N, Tremblay JP, et al. (2006) Decidualization and maintenance of a functional prostaglandin system in human endometrial cell lines following transformation with SV40 large T antigen. Mol Hum Reprod 12: 309-319.

46. Dufort I, Rheault P, Huang XF, Soucy P, Luu-The V (1999) Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase. Endocrinology 140: 568-574.

47. Zhang Y, Dufort I, Rheault P, Luu-The V (2000) Characterization of a human 20alpha-hydroxysteroid dehydrogenase. J Mol Endocrinol 25: 221-228.

48. Blouin K, Richard C, Belanger C, Dupont P, Daris M, et al. (2003) Local androgen inactivation in abdominal visceral adipose tissue. J Clin Endocrinol Metab 88: 5944-5950.

49. Nagata N, Kusakari Y, Fukunishi Y, Inoue T, Urade Y (2011) Catalytic mechanism of the primary human prostaglandin F2alpha synthase, aldo-keto reductase 1B1--prostaglandin D2 synthase activity in the absence of NADP(H). FEBS J 278: 1288-1298.

50. Watanabe K (2011) Recent reports about enzymes related to the synthesis of prostaglandin (PG) F(2) (PGF(2alpha) and 9alpha, 11beta-PGF(2)). J Biochem 150: 593-596.

51. Smith WL, Urade Y, Jakobsson PJ (2011) Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis. Chem Rev 111: 5821-5865.

52. Farb MG, Tiwari S, Karki S, Ngo DT, Carmine B, et al. (2013) Cyclooxygenase inhibition improves endothelial vasomotor dysfunction of visceral adipose arterioles in human obesity. Obesity 10.

53. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, et al. (2003) Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112: 1796-1808.

54. Ye J, Gao Z, Yin J, He Q (2007) Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. Am J Physiol Endocrinol Metab 293: E1118-E1128.

FIGURE

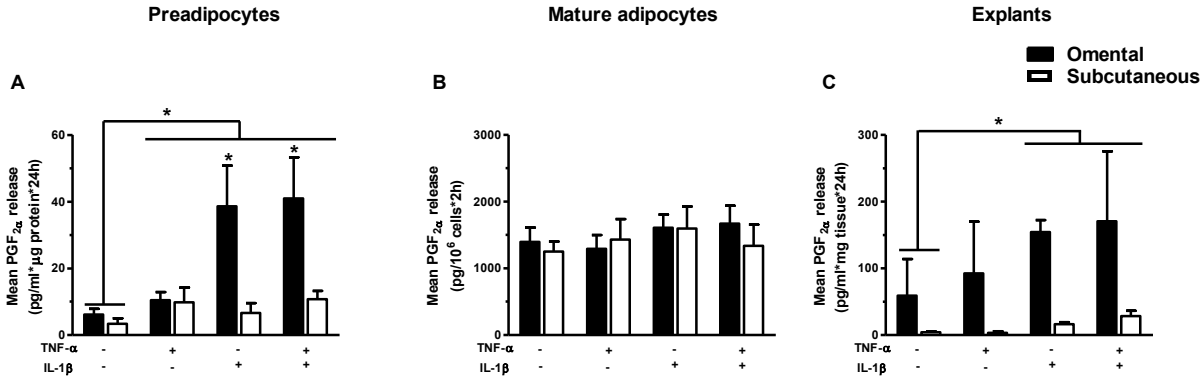


Figure 45: $PGF_{2\alpha}$ release by omental and subcutaneous fat cells:

(A) PGF2α release by subcutaneous and omental primary preadipocytes in response to TNF-α and/or IL-1β (preadipocytes stimulated for 24h with 1ng/ml TNF-α, 1ng/ml IL-1β or both). Results are expressed as pg/ml*µg protein*24h (n=14), (B) PGF2α release by isolated subcutaneous and omental mature adipocytes in response to TNF-α and/or IL-1β (isolated mature adipocytes stimulated for 2h with 1ng/ml TNF-α, 1ng/ml IL-1β or both). Results are expressed as pg/106cells*2h (n=12), (C) PGF2α release by subcutaneous and omental adipose tissue explants in response to TNF-α and/or IL-1β (explants stimulated for 24h with 1ng/ml TNF-α, 1ng/ml IL-1β or both). Results are expressed as pg/ml*mg tissue*24h. Data are presented as mean ± SEM. $p \leq 0.05$ for treatment-by-depot interaction in panel A and $p \leq 0.05$ for treatment effect in panels A and C. * $p \leq 0.05$

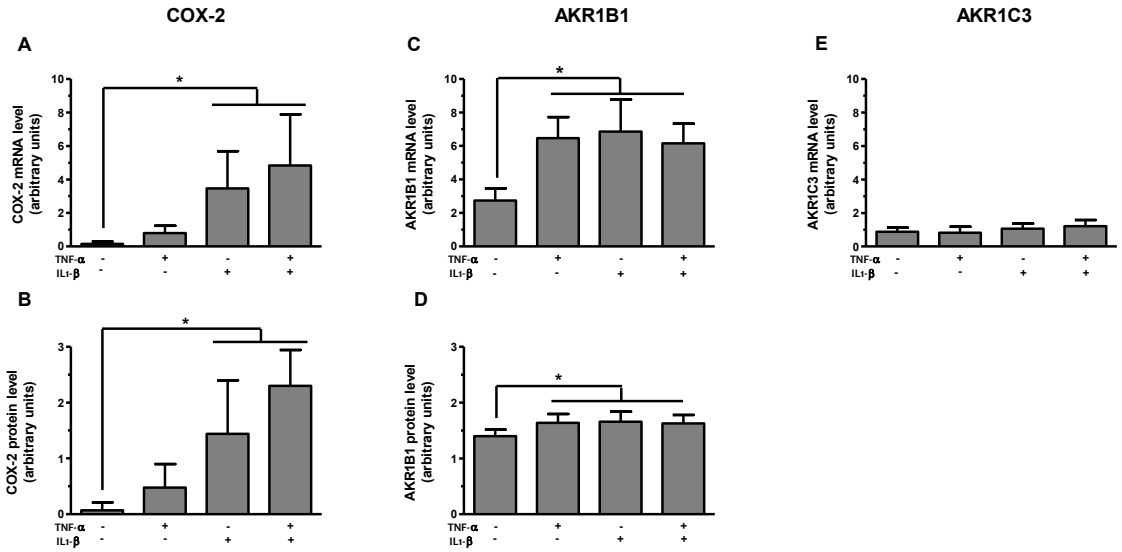


Figure 46: COX-2 and PGF synthase expression in primary preadipocytes:

Messenger RNA expression and protein levels of COX-2 (A and B, respectively), mRNA expression and protein levels of AKR1B1 (C and D, respectively) and mRNA expression of AKR1C3 (E) in subcutaneous and omental preadipocytes (n=4) stimulated for 24h with 1ng/ml TNF-α, 1ng/ml IL-1β or both. The data are presented as mean ± SEM (* p≤0.05 for treatment effect in panels A, B, C and D). Expression levels relative to G6PD mRNA expression. The western blot data were quantified by densitometric analysis and values were normalized to β-tubulin.

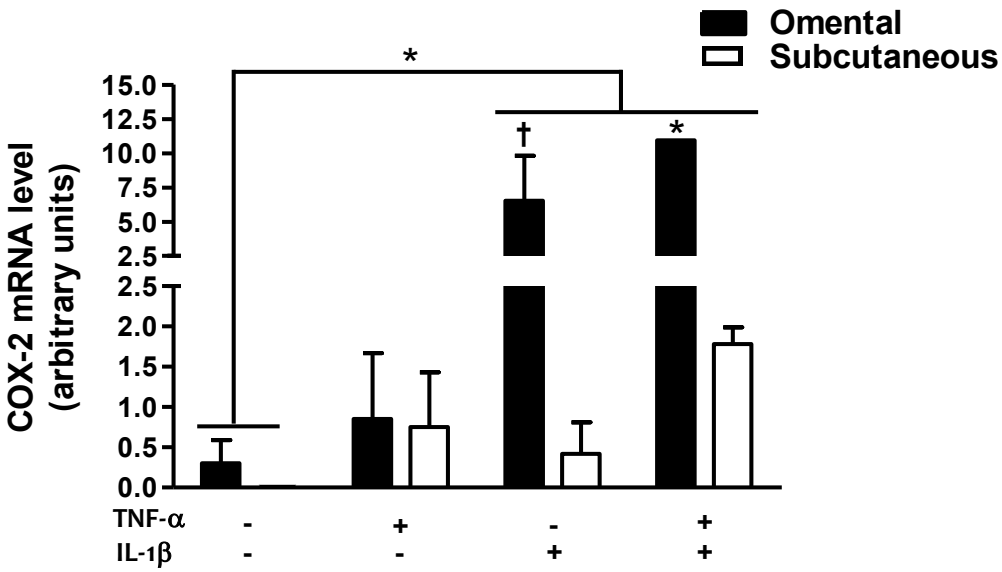


Figure 47 : Depot differences in COX-2 expression in primary preadipocytes:

Messenger RNA expression of COX-2 in subcutaneous (n=2) and omental (n=2) preadipocytes stimulated for 24h with 1ng/ml TNF-α, 1ng/ml IL-1β or both. Data are presented as mean ± SEM * p≤0.05, † p≤0.08. Expression levels relative to G6PD mRNA expression.

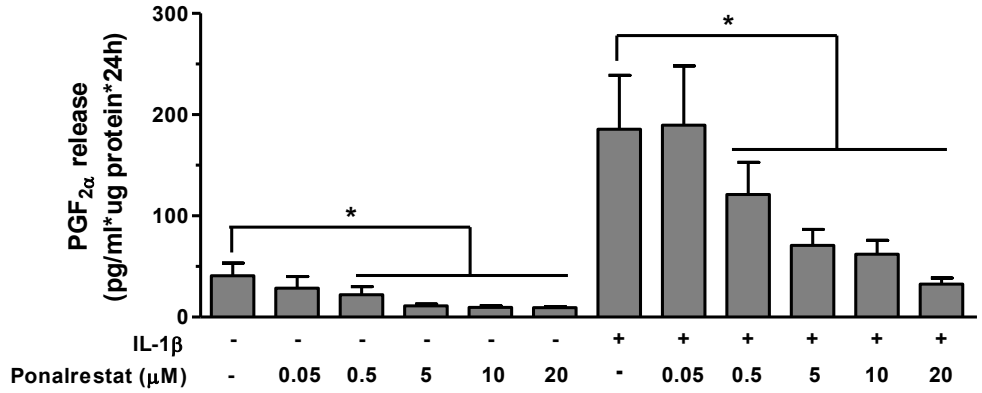


Figure 48 : Effect of aldose reductase inhibitor on $PGF_{2\alpha}$ release by human primary preadipocytes:

PGF2α release by subcutaneous and omental preadipocytes treated for 24h with 1ng/ml IL-1β in the presence or absence of increasing concentrations (0-20µM) of ponalrestat. Data are presented as mean ± SEM. Results are expressed as pg/ml*µg protein*24h (* p≤0.05, n=7 for all conditions).

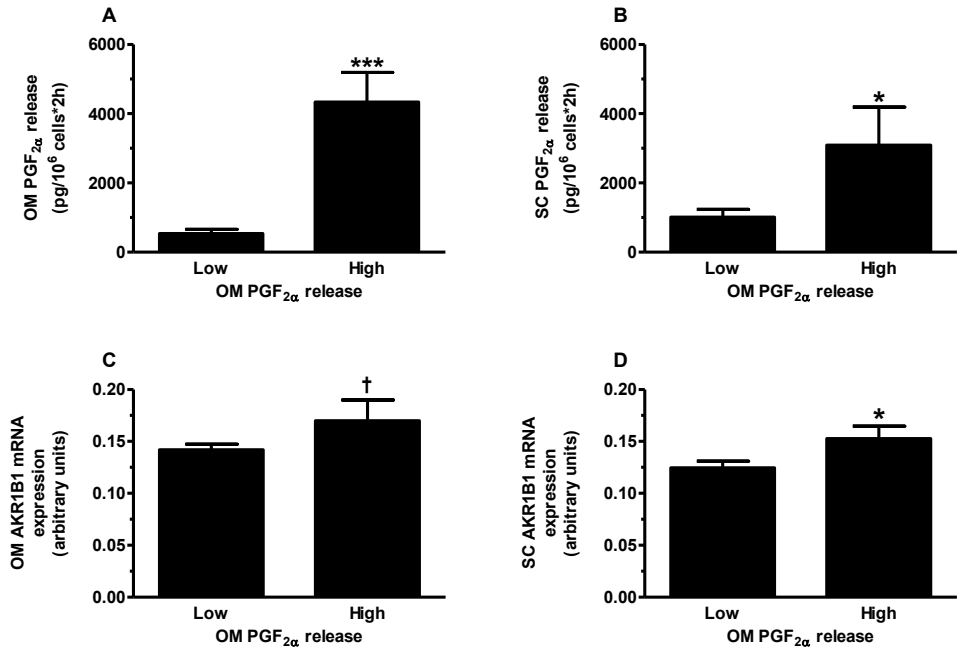


Figure 49: $PGF_{2\alpha}$ release by omental mature adipocytes:

Comparison of (A) omental adipocyte PGF2α release; (B) subcutaneous adipocyte PGF2α release; (C) omental AKR1B1 mRNA expression; and (D) subcutaneous AKR1B1 mRNA expression in women with low or high omental adipocyte PGF2α release. Data are presented as mean ± SEM. † $p < 0.10$, *$p \leq 0.05$, **$p \leq 0.005$, *** $p \leq 0.0001$. Expression levels relative to ATP5O mRNA expression. OM: omental; SC: Subcutaneous.

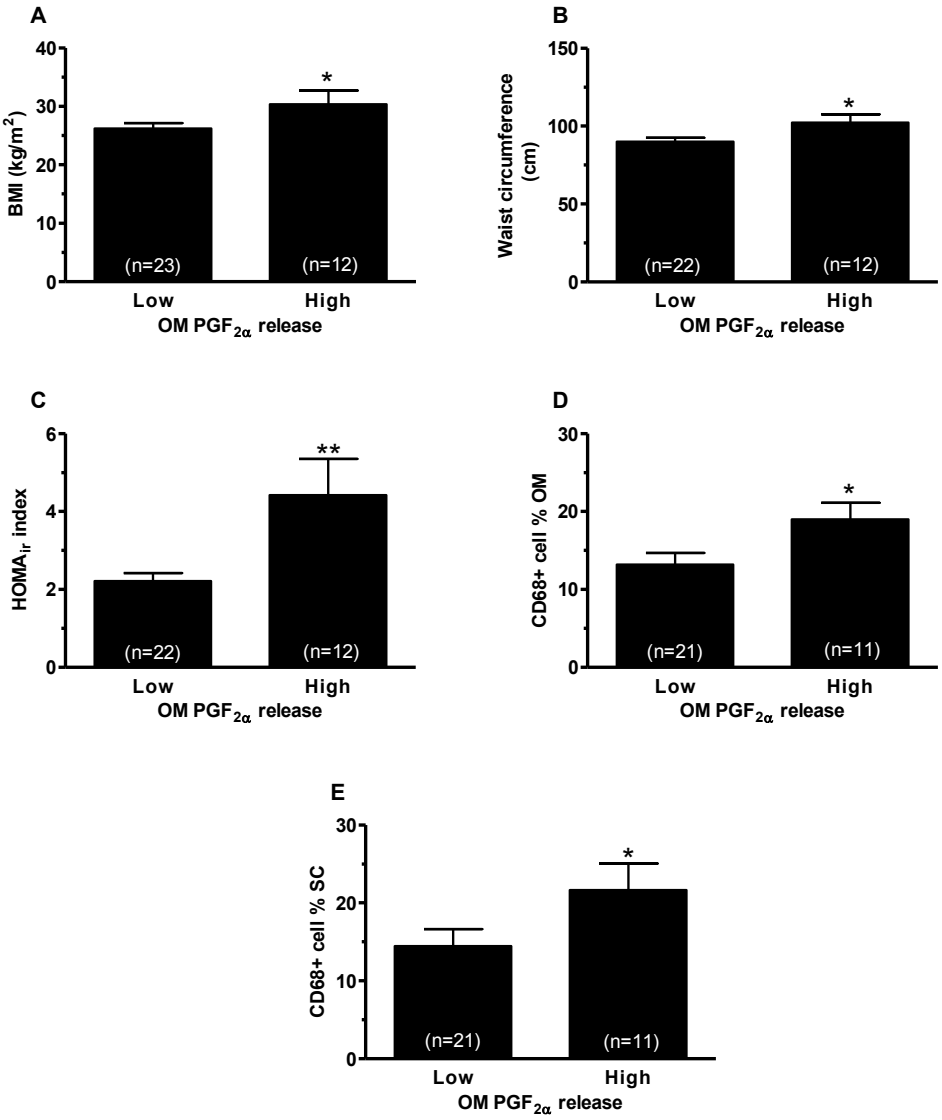


Figure 50: $PGF_{2\alpha}$ release by omental mature adipocytes in relation with body fatness and metabolic variables:

Comparison of (A) BMI; (B) waist circumference; (C) HOMAir index; (D) omental and (E) subcutaneous adipose tissue CD68+ cell percentage in women with low or high omental adipocyte PGF2α release. Data are presented as mean ± SEM. *p ≤ 0.05, ** p ≤ 0.005

Gene Symbol	Description	GenBank	Oligonucleotide Sequence 5'→ 3' Forward/Reverse
AKR1B1	Aldo-keto reductase family 1B1	NM_001628	GATCGCAGCCAAGCACAATAA/ACAGCTCAACAAGGCACAGAC
AKR1C3	Aldo-keto reductase family 1C3	NM_003739	CAACCAGGTAGAATGTCATCCGTAT/ACCCATCGTTTGTCTCGTTGA
COX-2	Cyclooxygenase 2	NM_000963	ATGGGTAATGTTATATGTTCTCCTGC/TGGTGACTGTTTTAATGAGCTCTG
ATP5O	ATP synthase O subunit	NM_001697	ATTGAAGGTCGCTATGCCACAG/AACGACTCCTTGGGTATTGCTTAA
G6PD	Glucose-6-phosphate dehydrogenase	NM_000402	GATGTCCCCTGTCCCACCAACTCTG/GCAGGGCATTGAGGTTGGGAG

Table 9: Oligonucleotides used in real-time RT-PCR quantification

Variables	Mean	±	SD	Range (min-max)
Age (yrs)	46.8	±	4.0	37.6-54.5
Body weight (kg)	74	±	17	48-133
Waist circumference (cm) [a]	94	±	15	72 -147
BMI (kg/m²)	28.0	±	6.4	19.5-50.1

[a] n=45

Table 10: Characteristics of the sample (n=46)

Variables	*AKR1B1*	
	OM	SC
BMI	0.30*	0.46**
Waist circumference[a]	0.29†	0.51**
$HOMA_{ir}$ Index	0.34*	0.58**

Table 11: Pearson correlation coefficients between AKR1B1 mRNA expression level in subcutaneous (SC) or omental (OM) adipose tissue and anthropometric measurements or HOMAir Index (n=46)

AKR1B1 mRNA expression in whole tissue from each site. Expression levels relative to ATP5O mRNA expression, an=45, ** p≤0.005, *p ≤ 0.05, †p ≤ 0.10