Effects of aldosterone on the human placenta: Insights from placental perfusion studies

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ARTICLE INFO

ABSTRACT

Introduction: In pregnancy, aldosterone is linked to maternal plasma volume expansion, improved fetal and placental growth/angiogenesis and reduced maternal blood pressure. Aldosterone levels are low in women with pre-eclampsia. Given the placental growth properties of aldosterone in pregnancy, we hypothesised that increased aldosterone improves placental function *ex vivo*. We applied aldosterone in the dual human placenta perfusion model and analysed specific regulatory markers.

Methods: A single cotyledon was perfused using a trimodal perfusion setup consisting of a control phase (CP; basic perfusion medium (BPM) alone) and two consecutive experimental phases (EP1/EP2; BPM supplemented with 1.5×10^{-9}M and 1.5×10^{-7}M aldosterone, respectively). CP and EP1/EP2 were conducted in closed circuits lasting 2 h each. Quality/time control perfusions using BPM alone were performed for 360 min to distinguish time-dependent effects from aldosterone-related effects. Perfusates were assessed for control parameters (pH/pO_2/pCO_2/glucose/lactate/creatinine/antipyrine). Maternal perfusates were analysed for placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1), interleukin-10 (IL-10) and tumour necrosis factor-alpha (TNF-α) using ELISAs. mRNA expression of abovementioned factors was measured by qPCR in post-perfusion tissue.

Results: Data from quality/time control perfusions indicated that TNF-α and IL-10 release continuously increased over time. Contrary, in the trimodal perfusion setup the application of aldosterone decreased TNF-α secretion ($P < 0.05$, EP1/EP2 vs CP, 120 min) and increased PlGF release ($P < 0.05$, EP1 vs CP, 90/120 min) into the maternal perfusates. mRNA expression followed similar trends, but did not reach significance.

Discussion: Our *ex vivo* placental perfusion data suggest that increasing aldosterone promotes anti-inflammatory and pro-angiogenic factors, which could positively contribute to healthy pregnancy outcomes.

1. Introduction

High blood pressure in pregnancy affects 10% of women worldwide [1] and is a key component of pre-eclampsia, a potentially fatal pregnancy-specific condition associated with maternal multi-organ dysfunction and fetal morbidity and perinatal mortality [2–5]. Clinically, pre-eclampsia is defined as increased blood pressure, renal disease with proteinuria and/or signs of organ damage after 20 weeks' gestation [6]. Women who develop pre-eclampsia are at increased risk of hypertension, metabolic disorders, end stage renal disease and cardiovascular disease/death in later life [3–5]. Current theory suggests that placental ischaemia/reperfusion injury leads to a release of a range of mediators responsible for systemic endothelial dysfunction into the maternal circulation [7].

Table 1
Clinical parameters of mothers, newborns and placentae (n = 6).

| Clinical data							
Mother							
						Blood pressure (mmHg)	
Age (years)	Weight at birth (kg)	Height (cm)	BMI at birth (kg/m^2)	Gestational age (weeks)	Smoker	Systolic	Diastolic
34 ± 3.5	80 ± 8.3	165 ± 8.0	29 ± 3.5	39 ± 0.8	none	126 ± 11	75 ± 7.8
Placenta				**Newborn**			
Weight (g)[a]	Umbilical cord length (cm)			Gender		Weight (g)	
602 ± 114	26 ± 14			4♂, 2♀		3198 ± 385	

[a] Including placental weight including the membranes and the cord.

It is not known whether pre-eclampsia arises partly because of underlying anomalies (e.g. in the renin-angiotensin-aldosterone system (RAAS) and its regulation of sodium (Na$^+$)-water balance), which are critical for pregnancy-induced plasma volume expansion [8–10]. Increased salt retention is a consequence of elevated aldosterone concentrations, which in pregnancy is linked to a very early increased renin-angiotensin system, yet also to an ever-increasing angiogenic drive [11,12]. Maintained aldosterone sensitivity and conformational changes of the epithelial sodium channel (ENaC) in the renal distal tubule favour Na$^+$ retention during normal pregnancy and are necessary for optimal fetal development [13,14]. In contrast to non-pregnant adults, hyperaldosteronism in pregnancy does not lead to an increase in blood pressure and insufficient aldosterone availability compromises pregnancy outcome [15,16]. This suggests aldosterone to have important extrarenal effects, including a contribution to placental trophoblast proliferation, placental growth and fetal development [17,18]. The homeostatic mechanisms both controlled by and controlling aldosterone synthesis in pregnancy are already known. However, in established pre-eclampsia, aldosterone levels are suppressed because the angiogenic drive is compromised [11,15], reducing the pregnancy-associated expansion of circulating fluid volume. Apart from maintaining the salt-water balance, aldosterone is also involved in maintaining inflammatory responses [19]. In this context, the placenta also functions as an immunogenic organ, by secreting certain pro- and anti-inflammatory cytokines, important for the fetal development and protecting the fetus from maternal infections.

Our recent *in vitro* cell culture data suggest a positive role for aldosterone in up-regulating placental growth factor (PlGF), a clinical useful marker of placental angiogenic properties in pregnancy [20]. However, further exploration of this and its antiangiogenic soluble receptor, soluble fms-like tyrosine kinase-1 (sFlt-1), is required in more complex and highly physiological models. The dual perfusion of the human placental cotyledon has proved a useful physiological system for investigating a wide range of functions of this organ; it enables retention of a high level of structural organisation of placental tissue, more accurately approximating to the *in vivo* state compared to other *in vitro* experimental systems. We thus aimed to test the effect of aldosterone using this technique. Given the placental growth properties of aldosterone in pregnancy, such a feature might be of importance for adaptive angiogenesis. We hypothesised that increased aldosterone would improve placental function and positively affect the release of inflammatory markers. To investigate this hypothesis, we determined the effect of aldosterone on the secretion of selected regulatory markers using the *ex vivo* perfusion model in human placenta from normotensive pregnancy.
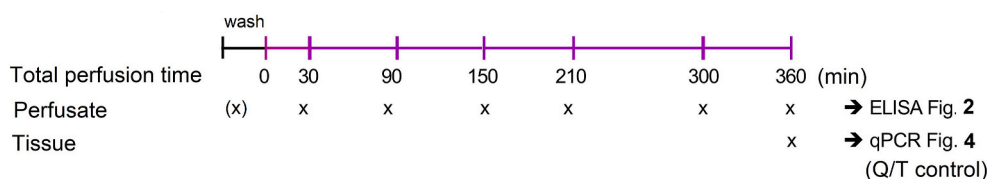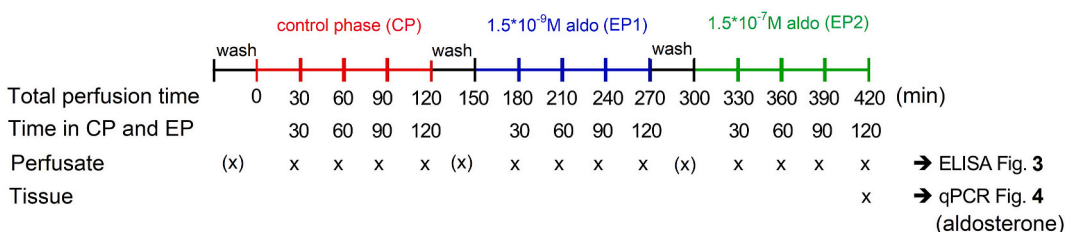

Fig. 1. Experimental setup of the placental perfusion studies. (A) Quality/time control perfusions. They were performed for 360 min in closed circuit for both the maternal and fetal circulations to evaluate the intrinsic effects of time on the placental release of selected markers. Initially a 30 min open wash phase was carried out. Maternal perfusates were collected at the indicated time points and analysed for different parameters using ELISA. Samples of the perfused cotyledon were collected at the end of the experiment and analysed by quantitative PCR. **(B)** Trimodal aldosterone (aldo) perfusions. They consisted of three consecutive phases: i) a control phase (CP), where the placenta was perfused with the basic perfusion medium (BPM) alone (2h, control phase, red label), ii) an experimental phase (EP) 1 during which the placenta was perfused with the BPM supplemented with 1.5 x 10^{-9}M aldosterone (2 h, EP1, blue label), and iii) an EP2 where the placenta was perfused with the BPM supplemented with 1.5 x 10^{-7}M aldosterone (2 h, EP2, green label). The perfusions were performed in closed circuits for the maternal and fetal circulation. Sampling time points are indicated and sample processing/measurements are identical to **(A)**. Maternal and fetal perfusates, collected during the wash phases are indicated as (x), and were used for the assessment of quality control parameters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
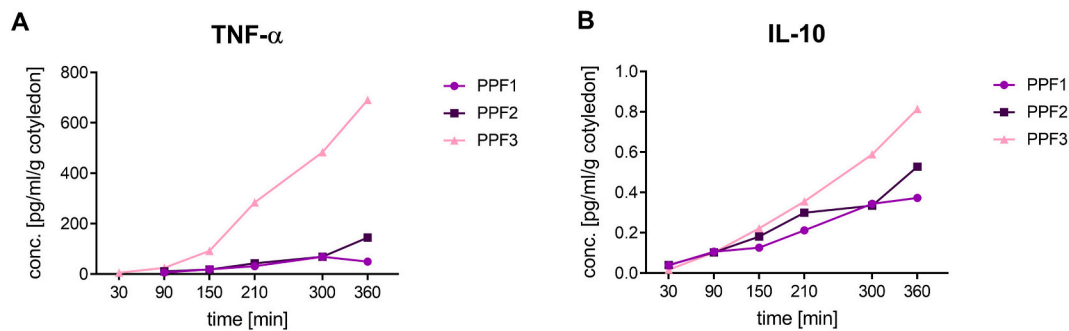
Fig. 2. Time-dependent secretion of tumour necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10) in quality/time control perfusions. Placentae (n = 3) were perfused in closed circuits for 360 min with basic perfusion medium. Secreted TNF-α **(A)** and IL-10 **(B)** were measured in the maternal perfusate by ELISA and concentrations (pg/ml) were normalised to the cotyledon weight. TNF-α and IL-10 accumulated over time in the maternal perfusate. Three individual perfusions are shown and labelled as placental perfusion (PPF)1 (violet), PPF2 (dark violet) and PPF3 (light violet). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Methods

2.1. Placenta collection

Human placental tissues used in this study were collected at the Division of Obstetrics and Gynecology, Lindenhofgruppe Bern, Switzerland. Placentae were obtained from uncomplicated pregnancies following elective Caesarean section beyond 37 weeks of gestation without prior labour upon patients request or due to breech presentation. The project was approved by the ethical commission of the Canton of Bern (approval No Basec 2016-00250). Written informed consent was obtained from all participants. Table 1 shows a summary of the subject characteristics. All experiments were carried out in accordance with the relevant guidelines and regulations.

2.2. Placenta perfusion

2.2.1. General perfusion setup

For the current *ex vivo* studies, the single cotyledon placenta dual perfusion system as described by Schneider et al. [21] was used. Briefly, the identified parallel fetal artery and vein on the chorionic plate were cannulated to set up a vascular circuit. The perfusion assays were carried-out using a basic perfusion medium (BPM), consisting of Dulbecco's modified Eagle medium (DMEM Thermofisher, Cat # 11800) and Earle's balanced salt solution (EBSS) in a ratio of 1:1, supplemented with 10 g/L Dextran FP40 (Serva Cat: 18660.02, Germany), 40 g/L bovine serum albumin (BSA, Sigma Cat # A9647, USA), 1.5 g/L D-Glucose (Sigma Cat #G7021, USA) and Liquemin® (Drossapharm AG). The final concentrations of glucose and Liquemin® were 2 g/L and 2500 IE/L, respectively.

After a routine confirmation of the circulatory integrity of the fetal circuit, the cannulated cotyledon including a portion of its surrounding placental tissue was transferred into a temperature-controlled (37 °C) chamber. To obtain steady pH and O_2 levels in maternal and fetal circuits, BPM in each circuit was passed through an in house-made membrane oxygenator throughout the perfusion. On the maternal side, the gas composition consisted of 19% O_2, 5% CO_2 and 76% N_2, while on fetal side it was 5% CO_2 and 95% N_2.

The original perfusion method established by Schneider et al. [21] had been modified by Soydemir et al. [22], who, instead of three to five cannulae, used 22 cannulae for the maternal circuit. The cannulae were inserted into the intervillous space at alternating depths of 1 and 2 cm. This modification allowed a better distribution of maternal perfusate inside the intervillous space. The resulting improvement of oxygenation of the trophoblast was reflected by the profile of secreted cytokine [23].

In the present study, the perfused cotyledons were cannulated with 11–25 cannulae depending on their size. The flow rates were set at 30 mL/h/cannula in the maternal circuit, and at 15 mL/h/cannula in the fetal circuit, respectively. The pressure in maternal and fetal arteries was monitored using a Millar instrument (Millar instruments, USA).

2.2.2. Quality/time control perfusions

In the framework of our study, we initially carried out quality/time control perfusions (n = 3) to monitor the effect of the perfusion time on the release of specific markers such as cytokines (Fig. 1A). Herein the placentae were continuously perfused during 360 min with BPM alone in closed maternal and fetal circuits. Maternal and fetal perfusate samples were collected in 60 min intervals and used for monitoring the general perfusion control parameters (see Figs. 1 and 2) during the perfusion time. Furthermore, in maternal perfusates the levels of the specific marker proteins were measured (see section "PlGF, sFlt-1, TNF-α and IL-10 measurements"). These samples were stored at −80 °C until analysis.

2.2.3. Aldosterone perfusions

Aldosterone perfusions (n = 3) were performed by using a trimodal perfusion setup (Fig. 1B). This setup consisted of a control phase (CP) where only BPM was used and two consecutive experimental phases (EP) where BPM was supplemented with 1.5×10^{-9}M (EP1) and 1.5×10^{-7}M (EP2) aldosterone, respectively. CP, EP1 and EP2 were conducted in closed maternal and fetal circuits and lasted 2 h each. This setup has the advantage that each perfusion has its own control phase and thus biases due to potentially high inter-placental differences can be avoided. On the other side, it has to be considered that the duration of the perfusion time affects the release of marker proteins and thus time-dependent effects have to be clearly discriminated from aldosterone-related effects. The concentrations were chosen according to previously reported aldosterone concentrations in the maternal [24–27] and fetal blood compartments [28].

Based on both data from the quality/time control perfusions, technical and logistic reasons, the duration of CP, EP1 and 2 was set to 120 min each. As depicted in Fig. 1B, in the trimodal perfusion setup each EP was preceded by a 30 min open wash phase. For the three wash phases, the BPM was supplemented with 80 mg/L of antipyrine and 150 mg/L of creatinine to assess the overlap of the maternal and fetal circulation throughout the perfusion time.

Perfusate samples used for the assessment of secreted protein markers were stored at −80°C until analysis.

2.2.4. Assessment of control parameters for validation

As indicated in Fig. 1 A and 1B, perfusate samples were collected in duplicates at various time points for assessment of specific control parameters (described below) from both maternal artery and maternal vein as well as from fetal artery and fetal vein. All collected samples

Table 2

Summary of control parameters for quality/time (QT) control perfusions (n = 3).

Phase/time point (min)	pH				O₂ consumption		Glucose consumption		Lactate production		Antipyrine clearance		Creatinine clearance	
	Maternal artery		Fetal artery		µL/min/g		µmol/min/g		µmol/min/g		mL/min/g		mL/min/g	
0/30 (open)	**7.43**	**± 0.02**	**7.37**	**± 0.06**	**1.09**	**± 0.55**	**0.33**	**± 0.12**	**0.86**	**± 0.41**	**0.075**	**±0.048**	**0.028**	**±0.011**
1/60	7.38	±0.04	7.34	±0.01	1.11	±0.31	0.37	±0.17	0.72	±0.35	0.064	±0.039	0.030	±0.011
1/120	7.38	±0.07	7.34	±0.01	1.11	±0.41	0.16	±0.41	0.61	±0.29	0.056	±0.034	0.030	±0.010
1/180	7.38	±0.09	7.33	±0.02	0.97	±0.22	0.32	±0.11	0.68	±0.30	0.054	±0.024	0.036	±0.015
1/240	7.37	±0.10	7.32	±0.04	1.01	±0.39	0.29	±0.07	0.39	±0.17	0.021	±0.009	0.022	±0.003
1/300	7.35	±0.11	7.31	±0.02	0.98	±0.32	0.30	±0.07	0.49	±0.12	0.016	±0.015	0.022	±0.000
1/360	7.36	±0.11	7.29	±0.03	0.95	±0.27	0.38	±0.10	0.40	±0.22	0.012	±0.011	0.014	±0.004

Phase O: 30 min wash phase in open perfusion starting after cannulation of the fetal artery and vein. The sample was taken after 30 min, i.e at the end of the wash phase. **Phase 1**: perfusion with basic perfusion medium in closed perfusion circuits over 360 min; samples were taken in 1 h intervals. For experimental setup see text and Fig. 1 A.

Table 3

Summary of control parameters for Aldosterone perfusions (n = 3).

End of 30 min open wash phase preceding	pH				O₂ consumption		Glucose consumption		Lactate production		Antipyrine clearance		Creatinine clearance	
	Maternal artery		Fetal artery		µL/min/g		µmol/min/g		µmol/min/g		mL/min/g		mL/min/g	
CP	7.41	±0.11	7.38	±0.03	0.70	±0.43	0.26	±0.07	0.40	±0.13	0.090	±0.047	0.023	±0.009
EP1	7.54	±0.11	7.43	±0.03	0.72	±0.35	0.27	±0.11	0.47	±0.13	0.078	±0.036	0.021	±0.009
EP2	7.65	±0.18	7.43	±0.01	0.73	±0.39	0.30	±0.14	0.48	±0.15	0.085	±0.042	0.024	±0.008

The aldosterone perfusions were performed in a trimodal setup and consisted of three consecutive phases: i) a control phase (CP), where the placenta was perfused with the basic perfusion medium (BPM) alone (2 h, control phase, CP), ii) an experimental phase (EP) 1 during which the placenta was perfused with the BPM supplemented with 1.5×10^{-9}M aldosterone (2 h, EP1), and iii) an EP2 where the placenta was perfused with the BPM supplemented with 1.5×10^{-7}M aldosterone (2 h, EP2). CP, EP1 and EP2 were performed in closed circuits for the maternal and fetal circulation. For assessing the control parameters, samples were taken at the end of the 30 min lasting open circuit wash phases preceding CP, EP1 and EP2. For experimental setup see text and Fig. 1 B.

were centrifuged at $1100 \times g$ for 10 min at 4°C to remove potentially eluted residual blood.

In order to validate the perfusions, selected control parameters, including the volume of the fetal perfusate, pH, pressure, oxygen consumption, glucose consumption, lactate production, antipyrine and creatinine transfer [29], were evaluated at the end of quality/time control perfusions (Table 2) and prior to running CP, EP1 and EP2 in the aldosterone perfusions (Table 3). The pH, O₂, glucose and lactate concentrations in collected perfusates were measured by using a Radiometer ABL 800. Antipyrine and creatinine contents in perfusate collected during the wash phase were assessed by using capillary electrophoresis on a Beckman Coulter PA800 system as described in Melhem et al. [30]. The formulas used for the calculation of these parameters are shown in Table 4.

In the present study, all established placental cotyledon circulations that exhibited a leakage secondary to poor vascular integrity or those that did not achieve a stable pressure, were abolished.

2.3. PlGF, sFlt-1, TNF-α and IL-10 measurements

Maternal placental perfusion samples were analysed using ELISAs according to manufacturer's instructions. PlGF (#DPG00, Bio-Techne, detection limit of 7 pg/ml) was measured in undiluted maternal arterial perfusates. sFlt-1 (#SVR100B, Bio-Techne, detection limit of 3.5 pg/ml) was assayed in 60-fold diluted maternal arterial perfusates. IL-10 (#HS100C, Bio-Techne, detection limit of 0.09 pg/ml) was measured in undiluted maternal venous perfusates and TNF-α (#BMS223HS, Thermo Fisher Scientific, detection limit of 0.13 pg/ml) was analysed in 200-fold diluted maternal venous perfusates.

2.4. RNA extraction and quantitative real-time PCR (qPCR)

At the end of the quality/time control perfusions (360 min perfusion with BPM) and aldosterone perfusions (3 consecutive perfusion phases à 120 min each, plus wash phases) placental tissue samples were collected (Fig. 1). Total RNA was extracted from all collected tissue specimens using TriZOL reagent and the total RNA concentrations were assessed with NanoDrop ND-1000.1 µg RNA was reverse transcribed with PrimeScript™ RT Reagent Kit (#RR037A Takara Bio Inc.). The Universal ProbeLibrary Assay Design Center software from Roche was used to design qPCR assays for PlGF, Flt-1, IL-10, HPRT1 and YWHAZ (Table S1). Primers were synthesised by Microsynth AG (Balgach, Switzerland); Universal ProbeLibrary hydrolysis probes were purchased from Sigma-Aldrich. qPCR assay for TNF-α (#Hs.PT.58.45380900) was purchased from Integrated DNA technologies and qPCR assay for Cyclophilin A (#4326316E) from Thermo Fisher Scientific. qPCR reactions (25 ng cDNA/reaction) were performed in duplicate using GoTaq® Probe qPCR Master Mix (#A6102 Promega) and measured with 7500 FAST Real-Time PCR System (Thermo Fisher Scientific). A no-reverse transcriptase control (NRT) and a no-template control (NTC) were included and had an undetected quantification cycle (Cq). Results were normalised to the geometric mean of the reference genes Cyclophilin A, HPRT1 and YWHAZ and are presented as fold-change ($2^{-\Delta\Delta Cq}$) relative to the mean of the control.

2.5. Statistics

All graphs and data are presented as mean ± standard deviation (SD). ELISA data were analysed by two-way ANOVA and Tukey's multiple comparisons test. qPCR results were analysed by unpaired *t*-test. The Null Hypothesis was rejected at p < 0.05. All statistical analyses were performed using GraphPad Prism (version 9; GraphPad Software, USA).

Table 4
Formulas for calculation of placenta perfusion parameters.

Constant O_2 factor

O_2 Consumption

$$O_2\,Factor\left(F_{O_2}\right) = \frac{\alpha\left(O_2\right)\times 100}{\left(pB - pH_2O\right)} \quad : \quad [1]$$

O2 Consumption (Cs_{O_2}) (Unit = ml/min/g)

$$O_2\,consumption = \frac{\left[\left(pO_2MaA - pO_2MaV\right)\times F_{O_2}\times Q_{Ma}\right] - \left[\left(pO_2FeV - pO_2FeA\right)\times F_{O_2}\times Q_{Fe}\right]}{100\, x\, m(Tissue)}$$

$\alpha(O_2) = 0.0239$ $pH_2O = 47\,mmHg$

Ma = **Ma**ternal

Fe = **Fe**tal

A = **A**rterial

V = **V**enous

Q = Flow rate

Glucose Consumption

Total Glucose consumption normalized to perfused cotyledon (Unit = µmol/min/g)

$$Glucose\,consumption = \frac{\left[\left(c_{Glu}MaA - c_{Glu}MaV\right)\times Q_{Ma}\right] - \left[\left(c_{Glu}FeV - c_{Glu}FeA\right)\times Q_{Fe}\right]}{m(Tissue)}$$

Lactate Production

Total Lactate Production normalized to perfused cotyledon (nP_{Lac}) (Unit = µmol/min/g)

$$Lactate\,Production = \frac{\left[\left(c_{Lac}MaV - c_{Lac}MaA\right)\times Q_{Ma}\right] + \left[\left(c_{Lac}FeV - c_{Lac}FeA\right)\times Q_{Fe}\right]}{m(Tissue)}$$

Antipyrine Clearance

Antipyrine Clearance (C_{AP}) (Unit = ml/min)

$$Antipyrine\,Clearance = \frac{\left(\rho^*_{AP}FeV - \rho^*_{AP}FeA\right)\times Q_{Fe}}{\rho^*_{AP}MaA} \quad [2]$$

Creatinine Clearance

Creatinine Clearance (C_{Cre}) (Unit = ml/min)

$$Creatinine\,Clearance = \frac{\left(\rho^*_{Cre}FeV - \rho^*_{Cre}FeA\right)\times Q_{Fe}}{\rho^*_{Cre}MaA} \quad [2]$$

[1] α represents the coefficient for physical solubility of oxygen in buffer (0.0239 ml/ ml buffer; see *Cotes et. al., 2009*)
[2] ρ* represents the amount of substance dissolved in solution (m/v)

Ref.: *Cotes, J. E., Chinn, D. J. and Miller, M. R. (2009). Transfer of Gases into Blood in Alveolar Capillaries. In Lung Function (eds J. E. Cotes, D. J. Chinn and M. R. Miller). doi: 10.1002/9781444312829*

3. Results

3.1. Validation of the perfusion system

We used the *ex vivo* dual perfusion of the healthy human placenta as highly physiological system to determine the magnitude of the secreted marker proteins into the maternal circulation. In the present study, depending on the size and area of the chosen cotyledon 11 to 25 cannulae have been inserted; the weight of the perfused cotyledons varied between 13.8 g and 91.4 g. To demonstrate integrity and viability of the perfused placental tissue, several parameters, including the volume of the fetal perfusate, pH, the stability of the pressure (not shown), consumption of oxygen and glucose and production of lactate were monitored (Tables 2 and 3). For the quality/time control perfusions all parameters are shown in 2 h intervals (Table 2). In the aldosterone perfusions (Table 3) antipyrine and creatinine were not added in the experimental phases. Therefore, all data shown in Table 3 are derived from the end (30 min) of the wash phases, which were performed prior to CP, EP1 and EP2.

The pH values in the maternal and fetal perfusate ranged between 7.3 and 7.4 in most measurements and remained unchanged. The pH values in the maternal artery were slightly increased during the aldosterone perfusions, up to a maximum of on average 7.65 ± 0.18. Within the aldosterone perfusions both oxygen consumption (0.70 ± 0.43 and 0.73 ± 0.39 µL/min/g after 30 and 300 min, respectively) and glucose consumption (0.26 ± 0.07 and 0.30 ± 0.14 µmol/min/g after 30 and 300 min, respectively) were constant throughout the experiments. Lactate production was also stable (0.40 ± 0.13 to 0.48 ± 0.15 µmol/min/g after 30 and 300 min). The clearance of antipyrine and creatinine served to determine the efficient establishment and matching between maternal and fetal circuits. Both parameters remained unchanged throughout the duration of the perfusions (Table 3).

During quality/time control perfusions, pH, O_2 and glucose consumption (Table 2) were also stable over the entire duration of the perfusion. Lactate production was similar in the first (after 30 min) and final (after 390 min, not shown) wash phase (0.86 ± 0.41 vs 0.84 ± 0.48 µmol/min/g). Similarly, antipyrine and creatinine clearances were comparable during the wash phases.

3.2. TNF-α and IL-10 secretion over time in control perfusions

To assess the intrinsic effect of the perfusion time on the placental secretion of selected marker proteins, we performed quality/time control perfusions and analysed the levels of TNF-α and IL-10 by ELISA in the maternal perfusates collected at different time points. The measured concentrations were normalised to the cotyledon weight. We observed a strong and steady accumulation of both TNF-α and IL-10 throughout the course of the perfusion (Fig. 2).

3.3. Effect of aldosterone on secretion of PlGF, sFlt-1, TNF-α and IL-10

Since the quality/time control experiments revealed a marked effect of the perfusion time on the secretion of marker proteins (see above), it was important to consider the factor time in the evaluation of the aldosterone perfusions. Therefore, we expressed all the measured concentrations throughout each 2 h-lasting phase (CP without aldosterone; EP1 and EP2 with different aldosterone concentrations) in relation to the first time point of the respective phase. IL-10 concentrations were below
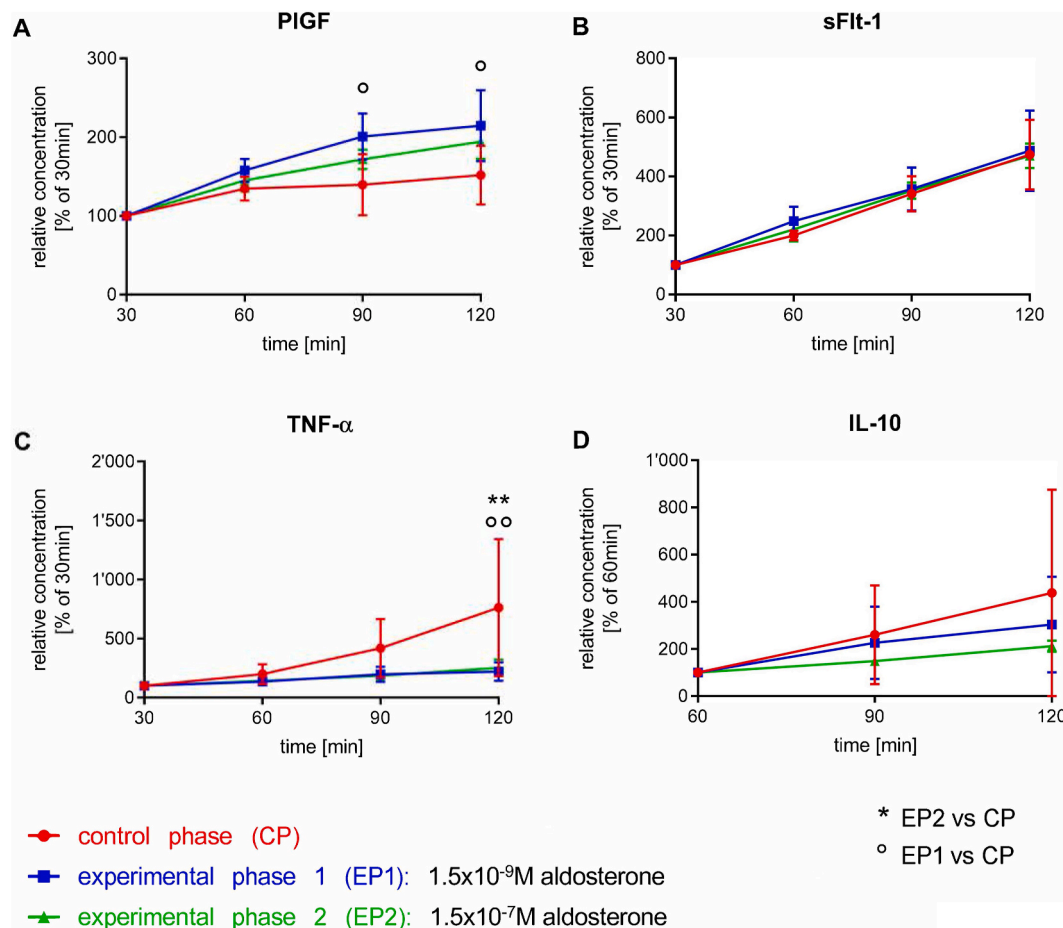
Fig. 3. Effect of aldosterone exposition on placental secretion of placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1), tumour necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10). Placentae (n = 3) were perfused in closed circuits consecutively with i) basic perfusion medium (BPM) alone (control phase; CP), ii) BPM supplemented with 1.5 x 10^{-9}M aldosterone (experimental phase 1; EP1), and iii) BPM supplemented with 1.5 x 10^{-7}M aldosterone (experimental phase 2; EP2). Each phase (CP, EP1, EP2) lasted 120 min, and was preceded by a 30 min open wash phase. PlGF, sFlt-1, TNFα and IL-10 were measured in the maternal perfusate by ELISA. All concentrations measured throughout each 2 h-lasting phases are expressed in relation to the first time point of the respective phase. IL-10 concentrations were below the limit of detection in most of the 30 min samples; therefore, only data from 60, 90 and 120 min are shown. For the remaining parameters (PlGF, sFlt-1, TNF-α) the concentrations measured at 30 min during CP, EP1 and EP2, respectively, were used as reference values to calculate relative concentrations. PlGF secretion increased significantly during EP1 **(A)**. sFlt-1 secretion was not affected by aldosterone exposition **(B)**. TNF-α **(C)** and IL-10 **(D)** showed a reduced secretion pattern during EP1 and EP2 compared to CP. The decrease in TNF-α both in EP1 and EP2 as compared to CP was statistically significant at 120 min **(C)**. Data are presented as mean ± SD. * or ° p < 0.05; ** or °° p < 0.01; * shows significances in marker release between CP and EP2. ° shows significances in marker release between CP and EP1.

the limit of detection in most of the 30 min samples; therefore, only data from 60, 90 and 120 min are shown. For the remaining parameters (PlGF, sFlt-1, TNF-α) the concentrations measured at 30 min during CP, EP1 and EP2, respectively, were used as reference values to calculate relative concentrations (Fig. 3). The placental secretion of angiogenic PlGF increased significantly when exposed to 1.5 x 10^{-9}M aldosterone (EP1; *P* < 0.05), but not with 1.5 x 10^{-7}M aldosterone (EP2; *P* > 0.05) as compared to no aldosterone exposition (CP) (Fig. 3A). In contrast, the secretion of the antiangiogenic sFlt-1 was similar in CP, EP1 and EP2 (Fig. 3B; *P* > 0.05).

Furthermore, both TNF-α and IL-10 showed a reduced secretion pattern during EP1 and EP2 compared to CP (Fig. 3C and D). The decrease in TNF-α both in EP1 and EP2 as compared to CP was statistically significant at 120 min (Fig. 3C). These findings are strongly supported by the results of the quality/time control perfusions performed in the absence of aldosterone which revealed that the placental release of TNF-α and IL-10 normally increases during the perfusion time (see Fig. 2).

3.4. Effect of aldosterone on mRNA expression of PlGF, Flt-1, TNF-α and IL-10

Placental mRNA levels of PlGF showed a trend towards an increase after aldosterone exposition as compared to the placentae perfused for approximately the same time with perfusion medium only (quality/time control perfusions, no addition of aldosterone) (Fig. 4 A). Placental sFlt-1, TNF-α and IL-10 mRNA expression showed a trend towards a decrease upon aldosterone exposition as compared to placentae which were perfused with perfusion medium only (Fig. 4B–D). However, these trends did not reach statistical significance (Fig. 4A–D).

4. Discussion

In this study we used the dual perfusion model of a single placental cotyledon to investigate the effect of increasing aldosterone levels on the human placenta *ex vivo*. We aimed to determine the release of pro- and anti-inflammatory factors in the perfusion medium, and some of these factors are secreted only in small amounts. Therefore, we applied the closed circuit perfusion setup and a consecutive perfusion with two
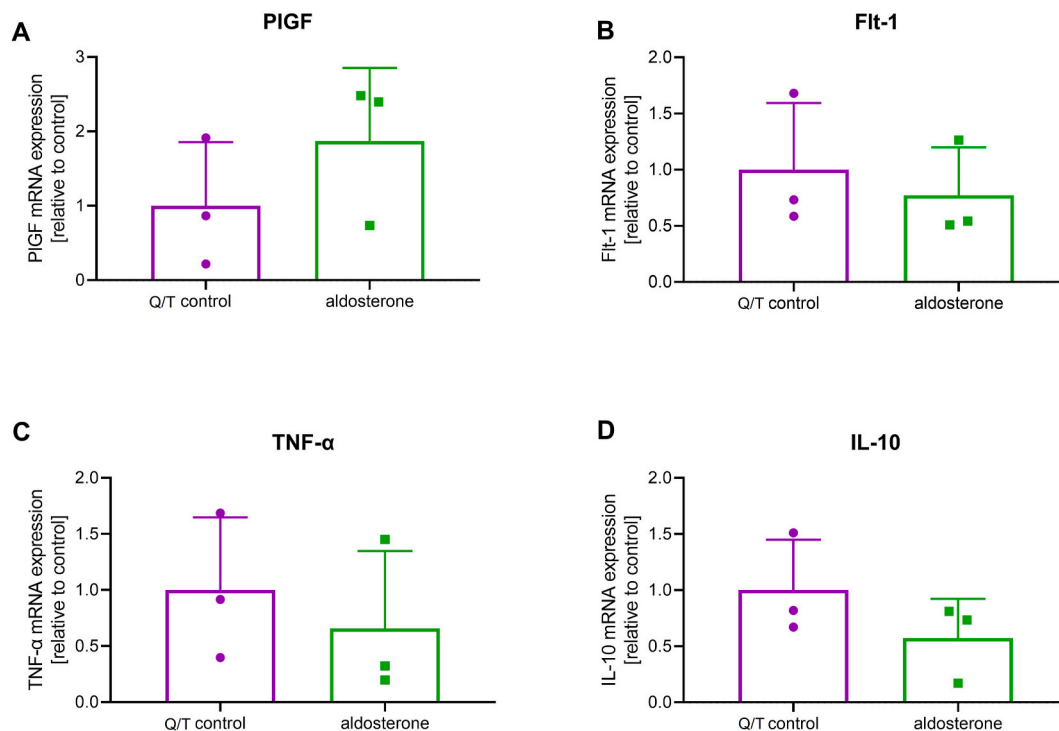
Fig. 4. Effect of aldosterone exposition on placental mRNA expression of placental growth factor (PlGF), soluble fms-like tyrosine kinase-1 (sFlt-1), tumour necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10). RNA was extracted from placental tissue specimens collected either at the end (360 min) of the quality/time (Q/T) control perfusions (labelled as QT control, left bars) or at the end of the aldosterone perfusions (labelled as aldosterone, right bars). The experimental setup of both perfusion sets is explained in Fig. 1 and in the text. The mRNA expression of PlGF, Flt-1, TNF-α and IL-10 (n = 3 for all) was measured by qPCR. Although not statistically significant, PlGF **(A)** mRNA levels tended to be higher in aldosterone perfused tissues, whereas Flt-1 **(B)**, TNF-α **(C)** and IL-10 **(D)** mRNA levels tended to be lower.

different aldosterone concentrations previously measured in the serum of pregnant women and pre-eclamptic patients [31].

As expected, during the 6 h perfusions, an increased release of specific cytokines such as TNF-α and IL-10 was detected. This is in line with previous findings where pro-inflammatory cytokine concentrations were reported to increase over perfusion time in both maternal and fetal venous perfusion samples, even under normoxic conditions [23]. In order to separate the effect of the perfusion time from the physiological effect that aldosterone exerts on the release of cytokines, it was necessary to control for the perfusion time. Therefore, we related all data within a specific treatment period (2 h without aldosterone, 2 h with 1.5 x 10^{-7}M aldosterone, 2 h with 1.5 x 10^{-9}M aldosterone) to an early time point (30 or 60 min) within the respective perfusion period.

The novel data generated by using this placenta perfusion and adapted analysis approach confirm our hypothesis of increased aldosterone concentrations contributing to improved placental hormone secretion. Previously published data suggested a clear role for TNF-α and IL-10 in pre-eclampsia, with TNF-α being increased while IL-10 being reduced [32,33]. In our *ex vivo* model, aldosterone treatment resulted in a decreased secretion of both TNF- α and IL-10 compared to control perfusion. These findings further suggest the expanded role of aldosterone in modulating inflammation in pregnancy [18,34]. Interestingly, in several non-pregnant clinical conditions including primary aldosteronism, the anti-inflammatory cytokine IL-10 remained unaffected by aldosterone, again in line with our data [35]. In addition to these rather short-term observations, we also determined the effect of aldosterone exposure on the mRNA expression of these cytokines. Although the duration of aldosterone treatment was rather short (4 h in total), the trend of reduced TNF-α and increased PlGF is in agreement with the ELISA data and further supports suggests a pro-angiogenic role for aldosterone that we have reported previously [11,36].

We have previously reported that aldosterone increases PlGF

expression in isolated trophoblasts [36]. Our current findings support this assumption and underline the regulatory role of aldosterone. Given the stimulatory capacity of aldosterone on PlGF, a regulator central to maternal angiogenic signalling, these findings are crucial. The systemic role for aldosterone is supported by the finding that sFlt-1 efflux from the placenta is unaltered. This precludes a potentially adverse anti-angiogenic event, which is contributed to aldosterone in other vascular beds in the non-pregnant condition [37,38]. The idea that there is a stimulatory effect on PlGF was supported by a tendency for higher transcription of PlGF within the perfused cotyledon. Our findings agree with previous studies that have demonstrated that exposure of vessels to aldosterone in models of atherosclerosis show enhanced PlGF expression, mediated by a mineralocorticoid responsive element in the promoter region of *plgf* [39,40].

It is well described that low maternal PlGF and high sFlt-1 levels are associated with a range of placentally-related pregnancy complications, such as pre-eclampsia [41]. This as well as low aldosterone observed in pre-eclampsia [15,42] and low birth weight [43], strengthens a mechanistic link between these factors and placental function. These data from the current study adds to the notion that helps explain an effective coordinated action of aldosterone on placental growth and adaptive angiogenesis [36]. Moreover, our perfusion model concurs with previous studies from placental vessels, suggesting that the placenta is the major source of PlGF [44].

An important technical finding from our data comes from our observation that both TNF-α and IL-10 both accumulated over time in the control perfusates. Thus, specifically in the context of measuring inflammatory markers released into the perfusion medium, the time effect should be considered when analysing perfusions where different treatments are applied consecutively.

In conclusion, our data in an *ex vivo* placental perfusion model suggest that increasing aldosterone, promotes anti-inflammatory and pro-

angiogenic factors, which could positively contribute to improved placental function and ultimately a healthy pregnancy outcome.

Funding

Declarations of competing interest

None.

Declaration of competing interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2022.03.129.

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