Université de Sherbrooke

Effects of DP and CRTH2 activation on osteoblast function

Mihai Nedelcescu

Departement de Pharmacologie

Mémoire présenté à la Faculté de Médecine

en vue de l'obtention du grade de

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Résumé

"Les effets de l'activation des récepteurs DP and CRTH2 sur la fonction des ostéoblastes"

Par
Mihai Nedelcescu
Departement de Pharmacologie
Mémoire présenté à la Faculté de médecine et des sciences de la santé en vue de l'obtention du grade de Maître en science (M.Sc.) en Pharmacologie, 2010, Université de Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4.

La modulation de PG par l'inhibition ou la stimulation de leur production peut être un facteur a considérer dans la gestion des différentes conditions pathologiques osseusses. Sur la base de résultats récents de nos laboratoires ainsi que sur la littérature, nous émettons l'hypothèse que la prostaglandine D_2 (PGD_2) est un important agent anabolisant pour les ostéoblastes. Nos résultats montrent que la PGD_2 diminue la prolifération des ostéoblastes agissant probablement par l'intermédiaire du récepteur CRTH2. Curieusement, alors que le DK-PGD_2 (agoniste spécifique CRTH2) a été utilisé seul ou avec le Naproxène, bien que la prolifération diminue avec la dose, l'effet semblait être restauré au niveau de contrôle avec les concentrations plus élevées de DK-PGD_2. Ainsi, nous envisageons l'hypothèse de l'existence d'autres mécanismes de compensation. La PGD_2 n'a pas eu aucun effet utilisée seule ou lorsqu'elle est utilisée avec le Naproxène, mais semblait réduire la différenciation ostéoblastique lorsqu'elle est utilisée avec Diclofénac à une concentration plus élevée seulement. Lorsque la vitamine D a été ajouté à toutes les conditions, PGD_2 a eu un effet inhibiteur sur la différenciation (dose-réponse). Lors d'un essai de compétition avec PGD_2 et des antagonistes DP/CRTH2, le blocage des récepteurs DP n'a pas donné aucun effet, et en bloquant le récepteur CRTH2 on a observé une diminution signifiante à une forte concentration de PGD_2. L'effet est semblable au test fait avec PGD_2 et antagoniste PPARγ ce qui suggère que celui-ci pourrait avoir un role compensatoire qui a renversé l'activation du DP. La PGD_2 a un effet légèrement positif sur la minéralisation des ostéoblastes, mais pas par le biais de ses récepteurs. Lorsque nous avons utilisé la PGD_2, en présence d'un antagoniste PPARγ, la calcification diminue de manière significative, indiquant que l'effet positif de la PGD_2 sur la calcification fonctionne plutôt à travers ce récepteur.

Mots-clefs: PGD_2, ostéoblastes, os, récepteurs, prostaglandins.

Abstract

"Effects of DP and CRTH2 activation on osteoblast function"

Modulation of PGs by inhibition or stimulation is a promising approach for the management of pain and inflammation in patients with rheumatic disease. Based on recent results from our laboratories as well as on the literature, we hypothesise that Prostaglandin D_2 (PGD_2) is an important anabolic agent for osteoblasts. Our results show that the PGD_2 decreases the osteoblasts proliferation acting probably through the CRTH2 receptor. Surprisingly, when DK-PGD_2 was used alone or with Naproxen, although the proliferation decreased with the dose, it seemed to be restored to the control level at higher concentrations of DK-PGD_2. Thus, we hypothesise the existence of other compensatory mechanisms. The PGD_2 had no relevant effect alone or when used with Naproxen, but seemed to decrease the osteoblast differentiation when used with Diclofenac at a higher concentration only. When vitamin D was added to all conditions, PGD_2 had an inhibitory effect on the differentiation (dose-response), but this could not be replicated when Naproxen was used. In a test with Diclofenac, we can assume a decreasing trend-line for differentiation when augmenting the PGD_2 dose, but the effect is not statistically relevant. In a competition test with PGD_2 and DP/CRTH2 antagonists, blocking DP receptor yielded no effect on differentiation, and blocking the CRTH2 receptor showed a relevant decrease at high concentration of PGD_2. The effect was similar in a test with PGD_2 and PPARγ antagonist suggesting that it might have a compensatory, positive effect that reversed DP activation. The PGD_2 has a slight positive effect on the osteoblast matrix mineralisation (with Naproxen), but not through its receptors since use of DP/CRTH2 antagonists did not abrogate this. In a competition test with PGD_2 and DP/CRTH2 antagonists we had no response. When we used the PGD_2 in the presence of PPARγ antagonist, the calcification decreased significantly, indicating that the positive effect of PGD_2 on calcification works rather through this receptor.

Keywords: PGD_2, osteoblasts, bone, receptors, prostaglandins.

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List of terms and abbreviations

15d-PGJ$_2$ 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$

AlkP Alkaline phosphatase

cAMP Cyclic Adenosine monophosphate

BSA Bovine serum albumin

BW 245C (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid

BW A868C 3-[(2-cyclohexyl-2-hydroxyethyl)amino]-2,5-dioxo-1-(phenylmethyl)-4-imidazolidine-heptanoic acid

Cbfa1 Cbfa1/Runx2 is a key transcription factor associated with osteoblast differentiation.

COX Cyclooxygenase

CRTH2 Chemoattractant homologous receptor expressed on Th2 cells

Diclofenac Benzeneacetic acid, 2-[(2,6-dichlorophenyl)amino]- monosodium salt. Nonsteroidal anti-inflammatory drug (NSAID)

DK-PGD$_2$ 13,14-dihydro-15-keto Prostaglandine D$_2$

DMEM Dulbecco's Modified Eagle's Medium

BMD Bone mineral density

DP PGD$_2$ receptor

FBS Fetal bovine serum

GPCR G protein-coupled receptors

hOB Human osteoblasts

H-PGDS Hematopoietic Prostaglandin D synthetase

IGF-1 Insulin-like growth factor-1

IL-1 Interleukine-1

IP$_3$ Inositol 1,4,5-trisphosphate

L-PGDS Lipocalin Prostaglandine D synthetase

M-CSF Macrophage colony-stimulating factor

Naproxen 2-Naphthaleneacetic acid, 6-methoxy-a-methyl-, (S)-. Nonsteroidal anti-inflammatory drug (NSAID)

NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerine
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLA_2	Phospholipase A_2
RA	Rheumatoid arthritis
PTH	Parathormone
PTX	Pertussis toxin
RANK	Receptor activator of NFkB
RANKL	Receptor activator of NFkB (RANK) ligand
TNF-α	Tumour necrosis factor-alpha
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular endothelial growth factor
VitD	1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]

I. Introduction

The bone matrix is continuously regenerated through the process of bone turnover when the "old" bone is replaced with "new" functional bone in the remodelling process. The lack of equilibrium between these two processes, the lysis and the deposition, is triggering the rheumatic disease.

Until recently the mainstay in their treatment has been the use of general measures without specificity. Such drugs as prednisone and other corticosteroids were used in the treatment of most of the diseases to suppress the inflammatory process and a usually over-active immune system. The effect was nonspecific and the side effects were often life-threatening.

Prostaglandins (PGs) are active biologic substances that are involved in a wide range of physiologic processes and their imbalance could be a factor in pathologies. The overproduction of PGs is responsible for pathologic inflammation, trauma and injury in rheumatologic and non-rheumatologic diseases. Restoring the PG balance could bring back the homeostasis in a more physiological way by acting over signalling mechanisms of the PG receptors as soon as these pathways are deciphered.

The new therapies include potentially safer (although not more effective with pain control) cyclooxygenase-2 specific nonsteroidal anti-inflammatory drugs (NSAIDs), leflunomide, tumour necrosis factor (TNF) inhibitors, etanercept and infliximab.

In this work, we will describe the bone modelling, remodelling, the factors involved, and we will underline the role of the PGD_2 receptors on the bone turnover.

1.1. Bone structure and architecture

Bone is a specialised connective tissues that serves three important functions in the body:

1. mechanical support in locomotion
2. protective function of vital organs
3. metabolic function as a reserve of calcium and phosphate.

Bones are extremely dense connective tissue that, in various shapes, constitute the skeleton. They are the hardest structures in the body, maintaining nevertheless a degree of elasticity because of their structure and composition. Hollow tube like, they provide great resistance and durability against axial compression forces having at the same time a very low weight. The ultimate tensile strength of bone approaches that of cast iron, and its capacity to absorb and release energy is twice that of oak, yet the weight of bone is only one third that of steel (Martin, 1989; Lee et al., 2002).

Bone is enclosed, except for the articular regions, in a fibrous outer membrane called the *periosteum*. Periosteum is composed of two layers, an outer fibrous layer and a deeper elastic layer containing osteoblasts that are capable of proliferating rapidly when a fracture occurs. In the interior of the long bones is a cylindrical cavity filled with bone marrow and lined with a membrane composed of highly vascular tissue called the endosteum (Sambrook, 2001).

1.1.1. Macroscopical structure

At the macroscopic level there are two major types of bone: compact or cortical bone and trabecular or cancellous bone. Cortical bone is located in the diaphyses of long bones and on the surfaces of flat bones. There is also a thin cortical shell at the epiphyses and metaphyses of long bones. Trabecular bone is limited to the epiphyseal and metaphyseal regions of long bones and is present within the cortical coverings in the smaller flat and short bones.

The morphology of cortical and cancellous bone is arranged to accommodate the stresses and strains during weight bearing. The functional differences are a conse-

quence of the structural differences (Fig. 1), cortical bone having mainly a mechanical and protective function and the trabecular bone a metabolic and mechanical function (Marc C. Hochberg, 2003).

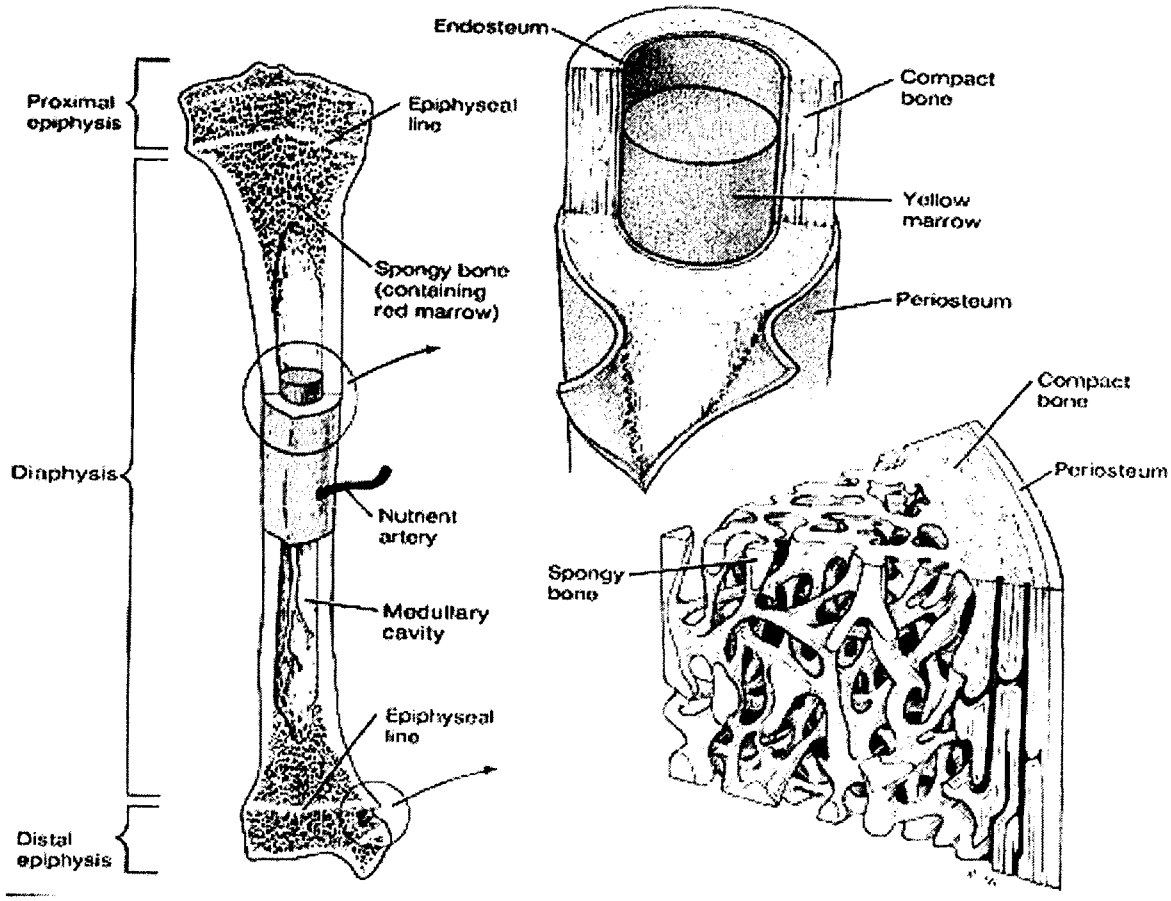

Fig. 1: Bone macroscopical structure (Alexander Spence, 1990).

I.1.2. Microscopic structure

Microscopically, bone is made up of two distinct phenotypes: woven and lamellar. Woven bone is characteristic of embryonic and foetal development, but it is also found in the healthy adult skeleton at ligament and tendon insertions and under pathologic conditions. Architecturally, it has an irregular, disorganised pattern of collagen fiber orientation and osteocyte distribution. Mechanical stimulation can cause rapid production of woven bone, which ultimately remodels into dense, lamellar bone

(Rubin et al., 1995), indicating that the woven bone response is a strategic means of rapidly responding to changes in functional activity.

Lamellar or mature bone is found in both cortical and trabecular bone. The structural subunits, the *lamellae*, run parallel to the *trabeculae* or, as is the case in cortical bone, are arranged in *osteons*, which are composed of up to 20 concentric lamellar plates forming a cylinder with a diameter of 200-300μm. A central capillary runs through the osteon, and up to seven concentric rings of osteocytes are incorporated into its wall (Albright J, 1987).

1.1.3. Bone matrix and minerals

Calcified bone contains about 25% organic matrix, including cells (2-5%), 5% water, and 70% hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$. The osteoid, the freshly synthesised matrix prior to its mineralisation consists primarily of fibers (approximately 94%) of collagen type I oriented in a preferential direction. It is now accepted that the backbone of the gene for type I collagen is identical in all connective tissues throughout the body. Therefore, it is most probable that the genetic specificity of the bone extracellular matrix resides within the non-collagenous constituents of the tissue. These proteins, however, are not spatially isolated within bone but are intimately associated with the bone collagen, forming a composite structure fulfilling all the biochemical, biomechanical and homeostatic requirements demanded of the bone matrix throughout life (Cowin, 2004).

The non-collagenous proteins (10% to 15% of the bone protein content), some of them unique to bone such as osteocalcin, are embedded in the extracellular matrix and may have important signalling functions (bone morphogeneic proteins, growth factors, cytokines, adhesion molecules) or play a role during the mineralisation process (osteopontin, osteonectin, matrix-gla protein). These highly anionic complexes have a high ion-binding capacity and are thought to play an important role in the calcification process.

A. Collagen

The collagen in bone is type I, which is the same in skin and tendon. Except for collagen V, no other forms of collagen are found in the bone matrix. The insoluble fibrils of collagen in bone are formed from the individual soluble tropocollagen molecules and are stabilised by intermolecular cross-links derived from aldehyde forms of hydroxylysine and lysine. These intermolecular cross-linking compounds of collagen are only present in its mature form and are specific for bone and cartilage. Therefore, it is believed that they represent a sensitive and specific marker for bone resorption. Urinary hydroxyproline, commonly used as marker for bone resorption in the past, is not specific to bone (Marc C. Hochberg, 2003).

B. Non-collagenous proteins

The proteoglycans consist of a central protein core to which are bound polysaccharide chains - glycosaminoglycans - which are strongly polyanionic due to carboxyl and sulphate groups. Studies in vitro and histochemical localisation in situ showed that proteoglycans are found in close association with collagen fibers and that they affect both the rate of fiber growth and the diameter of collagen fibers; it is possible that they influence the collagen scaffolding (Marc C. Hochberg, 2003).

C. Bone Gla-containing proteins (BGP) - osteocalcin

The name osteocalcin derives from the abundance of this protein in osseous tissue (10-20% of the non-collagenous protein) and its affinity for Ca^{2+}. Osteocalcin has also been called "the vitamin K-dependent protein of bone". Serum osteocalcin increases in situations where the bone formation rate is elevated or where bone turnover is increased. Osteocalcin binds tightly to hydroxyapatite and is believed to have a function in the assembly of mineralised bone, perhaps by participating in the regulation of hydroxyapatite crystal growth. The synthesis of osteocalcin is stimulated severalfold when 1,25-dihydroxyvitamin D_3 is added in osteoblast culture or in vivo (Marc C. Hochberg, 2003).

D. Sialoproteins

Two sialoproteins, osteopontin and bone sialoprotein (BSP), previously called sialoproteins I and II, are both cell adhesion molecules, mediating cell attachment of a number of cell types in vitro, including bone cells. Both proteins, however, behave differently in vitro and probably have a different function in vivo. The synthesis of BSP is inhibited by 1,25-dihydroxyvitamin D_3, whereas it is stimulated by dexamethasone added to the cultured osteoblasts. In contrast, the synthesis of osteopontin by osteoblasts is stimulated by 1,25-dihydroxyvitamin D_3 (Atkins et al., 2007).

E. Alkaline phosphatases

The classic vertebrate alkaline phosphatases are a group of isozymic membrane-bound glycoproteins with molecular weights of 100-200 kDa. The wide organ and tissue distribution of alkaline phosphatase activity suggests some type of generalised function. That this glycoprotein is primarily located in the plasma membrane implies either a carrier or a signal transducer function. Several possible actions of alkaline phosphatase in biomineralisation have been proposed:

- increasing local concentrations of inorganic phosphate (Pi);
- local destruction of mineral crystal growth inhibitors via expression of phosphohydrolase activity;
- Pi-transporter;
- Ca-binding protein;
- Ca-pump (Ca2+-ATPase) in cells or vesicle membranes;
- regulator of cellular division or differentiation, by acting as a tyrosine-specific phosphoprotein phosphatase.

Although there are supportive data for each, no singular function appears to be the principal action of the enzyme (Rodan, 1998).

F. Bone minerals

Bone mineral is generically referred to as hydroxyapatite [$Ca_{10}(PO4)_6(OH)_2$], a plate-like crystal 20-80 nm in length and 2-5 nm thick. Because it is four times smaller than naturally occurring apatites and less perfect in structure, it is more reactive and soluble and facilitates chemical turnover. The crystals are oriented in the same direction as the collagen fibers.

In 10 to 15 days after the organic matrix has been synthesised, the mineralisation process starts. During the 10 to 15 days, the mineral content increases to 70% of its final amount, whereas deposition of the final 30% takes several months. Other glycoproteins such as matrix-gla protein and glycosaminoglycans appear to play a role in the inhibition of excessive mineralisation (Sommerfeldt & Rubin, 2001) .

I.1.4. Bone cells

Three distinctly different cell types can be found within bone: the matrix-producing osteoblast, the tissue-resorbing osteoclast, and the osteocyte, the last accounting for 90% of all cells in the adult skeleton. Osteocytes can be viewed as highly specialised and fully differentiated osteoblasts; similarly, osteoblasts have recently been described as sophisticated fibroblasts (Huang et al., 2009). Fibroblasts, osteoblasts, osteocytes, and adipocytes derive from pluripotent mesenchymal stem cells, whereas osteoclasts are of hematopoietic descent and their precursors are located in the monocytic fraction of the bone marrow.

A. Osteoblasts

Osteoblasts are mono-nucleated cells that originate from mesenchymal stromal cells. Since osteoblasts arise from mesenchymal cells, there are critical developmental mechanisms appropriately activated to control the cell cycle and ensure that the phenotype of the osteoblast differs from that of other cells arising from the same origin, such as chondrocytes and adipocytes.

Osteoblasts are recruited to a site of bone formation where they are responsible for synthesising, secreting, organising, and mineralising the bone matrix, or os-

teoid. Osteoblasts secrete a variety of other non-collagenous proteins that are incorporated into the developing osteoid including fibronectin, biglycan, decorin, and galectin (Watkins et al., 2001) (Fig. 2).

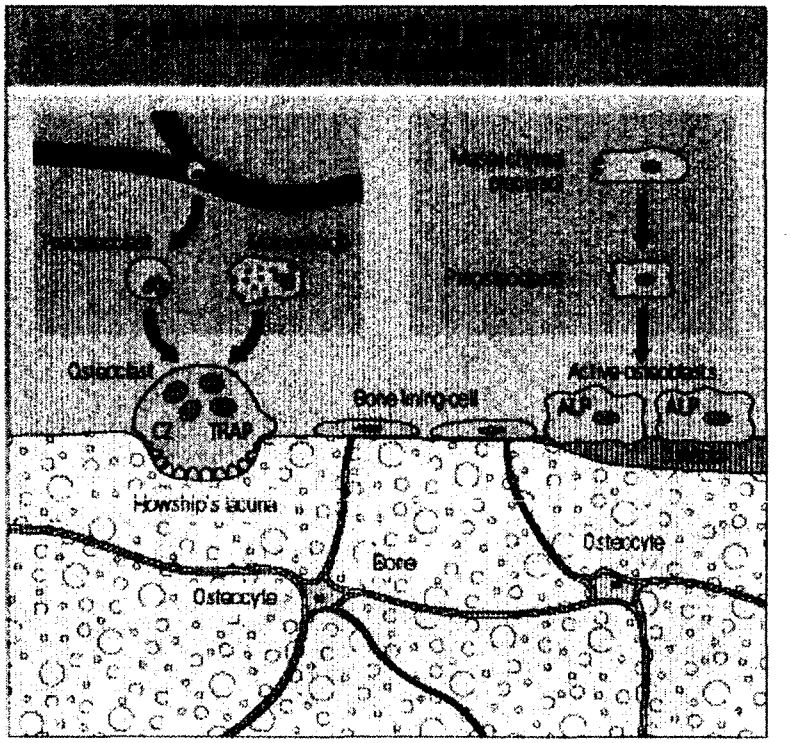

Fig. 2: Bone cells (Marc C. Hochberg, 2003).

Morphologically, these cells are cuboidal in shape and located at the bone surface together with their precursors, where they form a tight layer of cells. The plasma membrane of the osteoblast is characteristically rich in alkaline phosphatase, whose presence in the serum alkaline phosphatase is used as an index of bone formation (Marc C. Hochberg, 2003).

Osteoblasts are highly anchorage dependent and rely on extensive cell-matrix and cell-cell contacts via a variety of transmembranous proteins (integrins, connexins, cadherins) and specific receptors (for cytokines, hormones, growth factors) in order to maintain cellular function and responsiveness to metabolic and mechanical stimuli (Ferrari et al., 2000; Lecanda et al., 1998).

Contributors to osteoblast growth and differentiation include the bone morphogenic proteins, cell growth factors and cytokines (IGF-1, TGF-b, others), hormones (PTH, GH, insulin, glucocorticoids, 1,25 $(OH)_2$-vitamin D_3), and biomechanical forces (Watkins et al., 2001).

The lifespan of an osteoblast reaches up to 8 weeks in humans (Parfitt et al., 2000), during which time it lays down 0.5–1.5 μm osteoid per day (Owen, 1972). Glucocorticoid use reduces the osteoblast lifespan, increasing its apoptosis.

There are four commonly accepted stages in the osteoblast life span: the preosteoblast, which demonstrates alkaline phosphatase (ALP) activity and is located within bone; the bone matrix protein producer or the mature osteoblast; the osteocyte transformed osteoblast; the post-proliferative osteoblasts or the bone-lining cells.

B. Osteoclasts

Osteoclasts are large multi-nucleated bone-resorbing cells that form at skeletal sites from the fusion of hemopoietic precursors of the monocyte-macrophage lineage that arrive via the circulatory system. The M-CSF stimulation is necessary for the differentiation, proliferation and survival of the cells of the macrophage lineage.

The hemopoietic precursors express also a receptor known as receptor activator of NFkB (RANK). By activating this receptor, the transcriptional factor NFkB translocates to the nucleus and appears responsible for expression of genes that lead to the osteoclast phenotype. This receptor interacts with a ligand found on cells of the osteoblast/stromal lineage termed osteoclast differentiation factor (ODF, also known as RANKL) and with TRANCE (TNF-related activation-induced cytokine). The interaction of RANK with ODF keys the generation of the mature and active osteoclast (Watkins et al., 2001). A soluble receptor, osteoprotegerin (OPG, which leads to another alias for ODF - OPGL), has been shown to block the ODF/RANK interaction. Therefore, the ratio of ODF/OPG is an important regulatory mechanism in bone resorption (Fig. 3).

The mature osteoclast is a multinucleate cell, containing up to 20 nuclei, staining for tartrate-resistant acid phosphatase (TRAP).

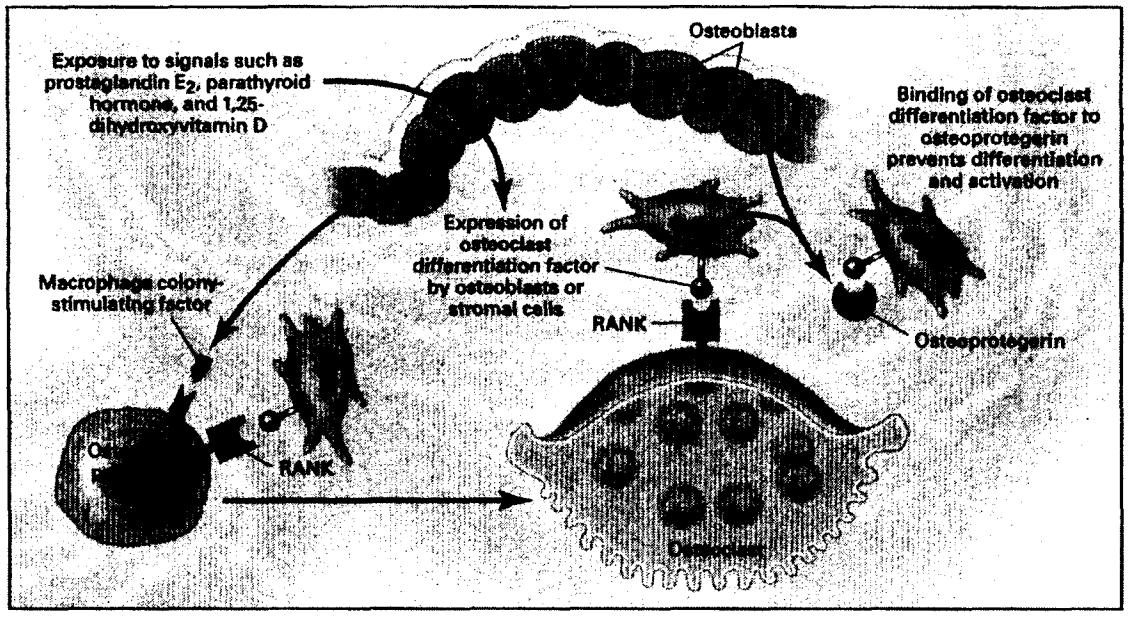

Fig. 3: Differentiation and Activation of Osteoclasts. Exposure of marrow stromal cells and osteoblasts to substances such as prostaglandin E2, parathyroid hormone, and 1,25-dihydroxy- vitamin D stimulates the expression of osteoclast differentiation factor, a transmembrane protein (Krane, 2002).

The main feature of osteoclasts is their ability to resorb fully mineralised bone at sites called Howship's lacunae. Both macrophages and osteoclasts are derived from hematopoietic stem cells and, similar to macrophages, osteoclasts are highly migratory, multi-nucleated, and polarised cells which carry an arsenal of lysosomal enzymes (Teitelbaum, 2000).

They have to be highly specialised to fulfill their task and possess several unique ultrastructural characteristics, such as pleomorphic mitochondria, vacuoles, and lysosomes (Walker, 1972).

An activated osteoclast is able to resorb 200,000 μm3/day, an amount of bone formed by seven to ten generations of osteoblasts with an average lifespan of 15–20 days (Albright J, 1987).

C. Osteocytes

Derived from osteoblasts yet distinctly different in morphology and function, osteocytes are the most abundant cells in bone. They are smaller in size than osteoblasts, contain less cell organelles such as ribosomes and endoplasmatic reticula, and have an increased nucleus to cytoplasm ratio. There is a higher number of filopodia, or cytoplasmatic extensions, which serve to interconnect the osteocytes and to connect them with the bone-lining cells, creating a three-dimensional *syncitium* (Curtis et al., 1985). Considering that it is osteocytes that are the principal cell in adult bone and that neither osteoclasts nor osteoblasts are evident in any significant numbers in a skeleton with low turnover, this osteocyte construct may actually orchestrate the spatial and temporal recruitment of the cells that form and resorb bone (Burger & Klein-Nulend, 1999).

The osteocytes remain connected with other similar cells but also with bone-lining cells, inactive osteoblasts (Miller and Jee, 1987) at the bone's surface, creating an extensive network of intercellular communication. There is accumulating evidence for a functional role of these cellular connections in sensing the need for and directing the site of new bone formation (Donahue et al., 1995) (Mosley, 2000) .The death of osteocytes by apoptosis in oestrogen deficiency, in corticosteroid therapy, in advancing age, or after damage to bone, is associated with a loss of bone strength before any bone loss (Seeman, 2008).

1.2. Bone modelling

The bone matrix is secreted by osteoblasts that lie at the surface of the existing matrix and deposit fresh layers of bone onto it. Some of the osteoblasts remain free at the surface, while others gradually become embedded in their own secretion. This freshly formed material (consisting chiefly of type 1 collagen) is called osteoid. It is rapidly converted into hard bone matrix by the deposition of calcium phosphate crystals in it. Once imprisoned in hard matrix, the original bone-forming cell, now called an osteocyte, has no opportunity to divide, although it continues to secrete further matrix in small quantities around itself. Since the networks of osteocytes do not se-

crete or erode substantial quantities of matrix, they probably play a part in controlling the activities of the cells that do. Hence, the first step in remodelling is unlikely to be bone resorption.

Osteoclasts must first be formed and then be told where to go and how much bone to resorb. These instructions are likely to arise from signals produced by the deformation or death of osteocytes, which define the location and amount of resorption needed (Seeman, 2008). While bone matrix is deposited by osteoblasts, it is eroded by osteoclasts. The precursor cells are released as monocytes into the bloodstream and collect at sites of bone resorption, where they fuse to form the multi-nucleated osteoclasts, which then cling to surfaces of the bone matrix and erode it (Fujikawa et al., 1996). Osteoclasts are capable of tunnelling deep into the substance of compact bone, forming cavities that are then invaded by other cells. A blood capillary grows down the center of such a tunnel, and the walls of the tunnel become lined with a layer of osteoblasts. To produce the plywood-like structure of compact bone, these osteoblasts lay down concentric layers of new bone, which gradually fill the cavity, leaving only a narrow canal surrounding the new blood vessel. Many of the osteoblasts become trapped in the bone matrix and survive as concentric rings of osteocytes. At the same time as some tunnels are filling up with bone, others are being bored by osteoclasts, cutting through older concentric systems.

The area causing matrix resorption is composed of three different domains: the attachment zone, ruffled border and the remainder. At the contact zone with the bone, proton pumps lower the pH to values between 2 and 4, activating the secreted enzymes such as tartrate-resistant acid phosphatase (Blair et al., 1989). In the attachment zone, a highly organised actin filament network, form dot-shaped, F-actin rich adhesion sites (Akisaka et al., 2006), so-called podosomes (Chabadel et al., 2007) and the actin ring (Luxenburg et al., 2006).

Vacuolar-type proton ATPase (V-ATPase) in osteoclasts is a ruffled border-associated enzyme responsible for the proton secretion, the acidity being the main factor in the Ca solubilisation (Blair et al., 1989; Okumura et al., 2006). The PH on the resorbtion pit reaches a value of 2-4, activating the secreted enzymes such as tartrate-resistant acid phosphatase (Sommerfeldt & Rubin, 2001). This resorptive phase is then

followed by a bone formation phase where osteoblasts fill the lacuna with osteoid. The latter is subsequently mineralised to form new bone matrix (Sambrook, 2001).

1.3. Regulation of bone remodelling

The skeletal system is involved in the body's homeostasis, it is an active system. To achieve this, there must be a constant interaction responding to hormones, to the physical demand, to stress, to the need of repairing.

To all these needs, the response is achieved trough the balance of two major processes: bone formation and bone resorption, the former controlled by the osteoblasts and the latter by osteoclasts. The endocrine, autocrine and paracrine systems also have a role in this interplay, acting on the mentioned cells on different stages of their development or as conjugated factors of their activities. They add to the roles of the bone specific functions like the homeostatic, hematopoietic and mechanical functions. The regulation of bone remodelling is both systemic and local (Hadjidakis & Androulakis, 2006).

1.3.1. Factors acting on osteoblasts and osteoclasts

Remodelling is a continuous, dynamic activity driven by humoral and biofunctional cues, the result being that about 25% of trabecular bone and about 3% of cortical bone are removed and replaced each year (Parfitt, 1994) (Fig 4). As we age, the balance between osteoblastic formation and osteoclastic resorption becomes asynchronous: bone loss occurs and results in the clinical disease (Parfitt et al., 1995).

The plasma concentration of calcium is one of the homeostatic triggers of bone remodelling, acting through a feedback mechanism which includes the liver, the kidneys and the parathyroid glands. The serum ions of calcium activate the humoral system changing the balance for 1,25-dihydroxy vitamin D_3, androgen, calcitonin, estrogen, glucocorticoids, GH, PTH, and thyroid hormone (Raisz, 1999; Bilezikian et al., 1996).

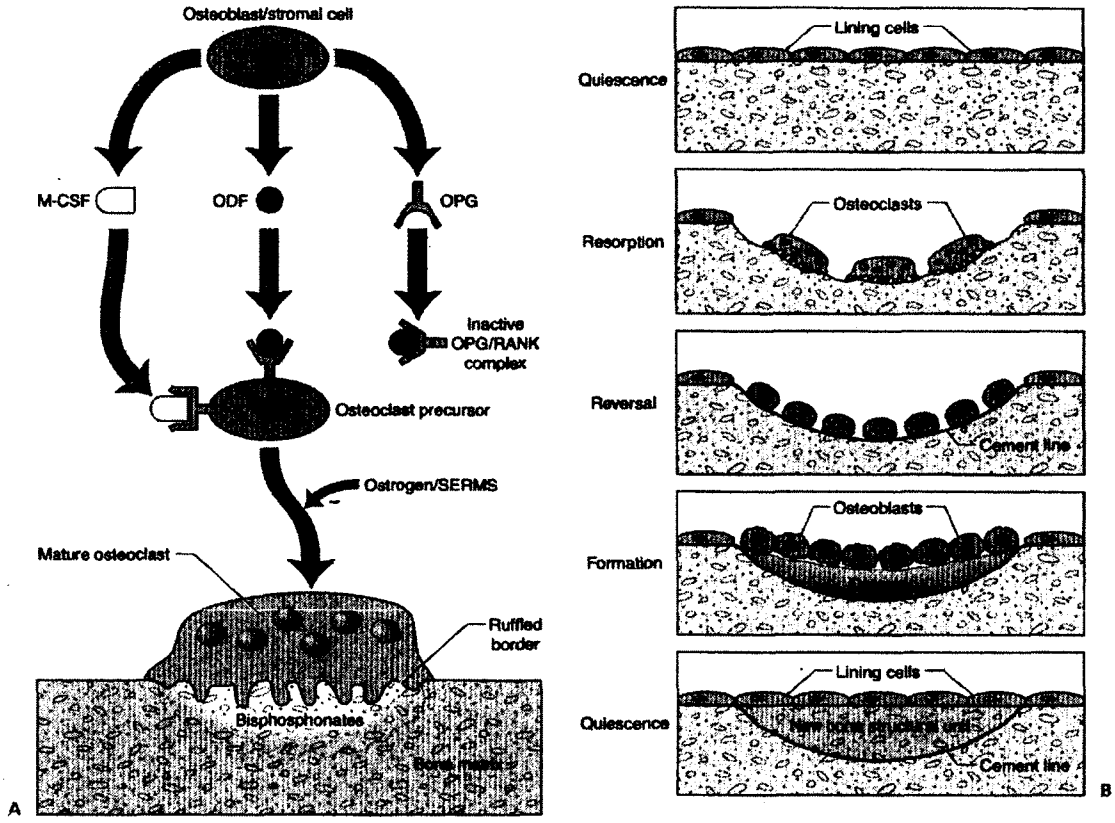

Fig. 4: Osteoblast action. (A) Osteoblasts and stromal cells produce both macrophage colony-stimulating factor (M-CSF) and osteoclast differentiating factor (ODF). In the presence of permissive concentrations of cellular or soluble M-CSF, binding of ODF to its receptor (RANK) on osteoclast precursor cells results in their differentiation and activation. This process is regulated by an inhibitor of ODF, osteoprotegerin (OPG), which competes with RANK for binding of ODF to produce an inactive complex. The sites of action of various agents used for treating osteoporosis are also shown: SERMS =selective oestrogen-receptor modulators. (B) The bone remodeling sequence is initiated by osteoclasts. Subsequently, osteoblasts appear within the resorption bay and synthesize matrix, which is mineralized later (Philip Sambrook, 2001).

The decrease of plasma oestrogen may lead to increased levels of of cytokines, IL-1, IL-6, TNF-α, (Pacifici, 1998) but also can trigger osteocytes apoptosis (Tomkinson et al., 1997). An inflammation - local or systemic – could trigger also the release of cytokines and limphokines, leading to osteolysis.

The osteoclasts are formed as a response to several osteotropic factors like 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], interleukin (IL)-6, PGE_2 (Takahashi et al., 1988) which induce the expression of NFκB ligand (RANKL, a member of the TNF family) in osteoblasts. The binding of RANKL to the RANK receptor found on the surface of the osteoclast progenitors (monocytes) activates the osteoclastogenesis. The PTH induction of RANKL is conditioned by the presence of cyclooxigenase-2 (COX-2) via cAMP production (Okada et al., 2000). The activation of RANKL by the IL-1, IL-6, IL-17 and $1,25(OH)_2D_3$ requires the presence of COX-2 as well. (Li et al., 2006).

The osteoprotegerin (OPG), a soluble "decoy receptor", regulates the RANK effect by competing for the same ligand, thus inhibiting the RANK osteolytic effect, but it is down-regulated by PTH (Lee & Lorenzo, 1999). These are not the only participants involved, different publications mentioning TNF, annexin-II, TGF-β, etc (Mundy, 1996; Boyce et al., 1999).

The damaged area of the bone is signalled by the apoptotic osteocytes to the lining cells. The target is confined to minimise the bone loss, creating a Basic Multicellular Unit (BMU) (Hauge et al., 2001). It is possible that the trigger is the collagen digestion around the mineralised bone and the exposure of the calcified area. The bone lining cells may be responsible for this action as they express collagenase mRNA. (Chambers et al., 1985). More than that, Parathyroid hormone (PTH) stimulates collagenase production and secretion by osteoblastic cells and it appears to be involved in clearing the osteoblast-lining surfaces of the bone to permit access to the osteoclasts (Chiusaroli et al., 2003). The PTH binds to the PTH receptor 1 (PTHR1) found on the osteoblasts, leading to an increase of RANKL, activating the osteoclastogenesis (Fu et al., 2002). Some authors report that PTH acts directly on the osteoclasts receptors, although there are not sufficient studies to accustom this theory yet (Dempster et al., 2005).

It is accepted today that the parathyroid hormone (PTH) has dual effect, anabolic and catabolic, depending on the signalling cascade (Datta, et al., 2009) or depending on the way it is administered. Intermittent administration has an anabolic effect (Horwitz et al., 2003; Pettway et al., 2005; Pettway et al., 2008), whereas continuous administration induces bone resorption (Kaji, 2007).

Calcitonin is a peptide hormone synthesised and secreted by thyroid parafollicular C cells. It is regulated by extracellular calcium levels and by gastrointestinal hormones such as gastrin. Calcitonin receptors are present on osteoclasts, preosteoclasts. The hormone blocks bone resorption probably via mature osteoclasts by enhancement of adenylate cyclase and cAMP or as a mitogen acting on bone cells.

The mechanical load is also a trigger of bone remodelling. The signal is the deformation of the bone, followed by the local release of cytokines and also of AA (arachidonic acid) (Cissel et al., 1996) and consecutively of PGE_2 leading to the bone resorption in this first step (Rodan, 1998). The bone deposition under the release of androgens and BMPs follows.

There are many other proposed mechanisms of acting on the bone turnover including a bidirectional regulation between osteoclasts and osteoblasts through a signalling system (Zhao et al., 2006), and a nervous/neuroendocrine system regulation based on leptin. Leptin is a polypeptide hormone that influences body weight, satiety and lipid metabolism. It plays a role in the central hypothalamic modulation of bone formation, as well as locally within the skeleton, by enhancing differentiation of bone marrow stroma into osteoblasts and by inhibiting its differentiation into osteoclasts and adipocytes (Ducy et al., 2000) (Takeda et al., 2002) thus proving that the bone remodelling is a complex process yet to be discovered.

1.4. Eicosanoids and Prostanoids

Although prostaglandins were the first biologically active eicosanoids to be identified, it is now known that the essential fatty acids are converted into a number of different types of eicosanoids. Eicosanoid is a term meaning a 20 carbon fatty acid derivative.

Arachidonate and some other 20 carbon polyunsaturated fatty acids give rise to eicosanoids, physiologically and pharmacologically active compounds known as prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and lipoxins (LX). Physiologically, they are considered to act as local hormones generated *in situ*, rapidly metabolised, functioning through G-protein-linked receptors to elicit their biochemical effects in the immediate vicinity.

Both von Euler in Sweden and Goldblatt in England discovered marked stimulation of smooth muscle by seminal plasma. Von Euler (1935) then showed that lipid extracts of ram vesicular glands contained the activity and this was associated with a fatty acid fraction. The factor that showed this effect was named prostaglandin and it was thought to possess a variety of physiological and pharmacological properties. The name prostaglandin (and the related prostanoic acid structure) derives from the fact that early researchers believed that the prostate gland was the site of their synthesis.

In 1947, Bergström started to purify these extracts and soon showed that the active principle was associated with a fraction containing unsaturated hydroxy acids. In 1956, with the help of an improved test system (smooth muscle stimulation in the rabbit duodenum), Bergström isolated two prostaglandins in crystalline form (PGE_1 and $PGF_{1\alpha}$). Their structure, as well as that of a number of other prostaglandins, was elucidated by a combination of degradative, mass spectrometric, X-ray crystallographic and NMR studies. The nomenclature is based on the fully saturated 20 carbon acid with C8 to C12 closed to form a 5-membered ring, called prostanoic acid. Thus PGE_1 is designated 9-keto-11 α,15 α-dihydroxyprost-13-enoic acid. The 13,14 double bond has a *trans* configuration; all the other double bonds are *cis*. In the Fig. 6 one can see the difference between the 'E', which have a keto group at position 9 and 'F' series,

which have a hydroxyl group at the same position; 'α' refers to the stereochemistry of the hydroxyl, and the suffix 1, 2 or 3 is related to the number of double bonds contained in the prostaglandin structure (Michael I. Gurr, 2002).

PGA, PGB and PGC are ketones, PGD and PGE are hydroxyl ketones and PGF α is a diol. PGI has a different structure, given the second ring attached to the cyclopentane structure.

1.4.1 Prostaglandin Synthesis

Arachidonate (AA) is usually derived from the *sn*-2 position of glycerophospholipids in the plasma membrane by the action of phospholipase A2 (Fig. 5). It is released from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by phospholipase A2, or from phosphatidylinositol (PI) by phospholipase C (PLC) pathway. The arachidonate can also come from the diet. AA is the substrate for the synthesis of the PG_2, TX_2 series (prostanoids) by the cyclooxygenase pathway (Fig. 6), or the LT_4 and LX_4 series by the lipoxygenase pathway (Fig. 7), with the two pathways competing for the arachidonate substrate (Tang et al., 2006).

The control of the AA release depends mainly of the presence of type IV cytosolic PLA2 which can be translocated to the nuclear envelope, endoplasmic reticulum (ER) and Golgi apparatus.

The metabolism of arachidonate substrate by cyclooxygenase pathway gives cyclic endoperoxides from which the classical prostaglandins or thromboxanes and prostacyclin can be synthesised. A third pathway occurs via cytochrome P450 oxygenation where atomic oxygen is introduced leading to fatty acid hydroxylation or epoxidation of double bonds. Whereas both the lipoxygenase and cyclooxygenase reactions arise from the formation of a fatty acid radical, in P450-oxygenation, activation of atmospheric molecular oxygen is involved. After this, one oxygen is transferred to the fatty acid substrate and one is reduced forming water (Porubsky et al., 2008; Moreno, 2009).

Besides being the substrate for the eicosanoid synthesis,the arachidonic acid has other biological properties, like the modulation of Ca^{2+} influx and the regulation of protein kinase C and phospholipase C. (Lopez-Nicolas et al., 2006; Boittin et al., 2008).

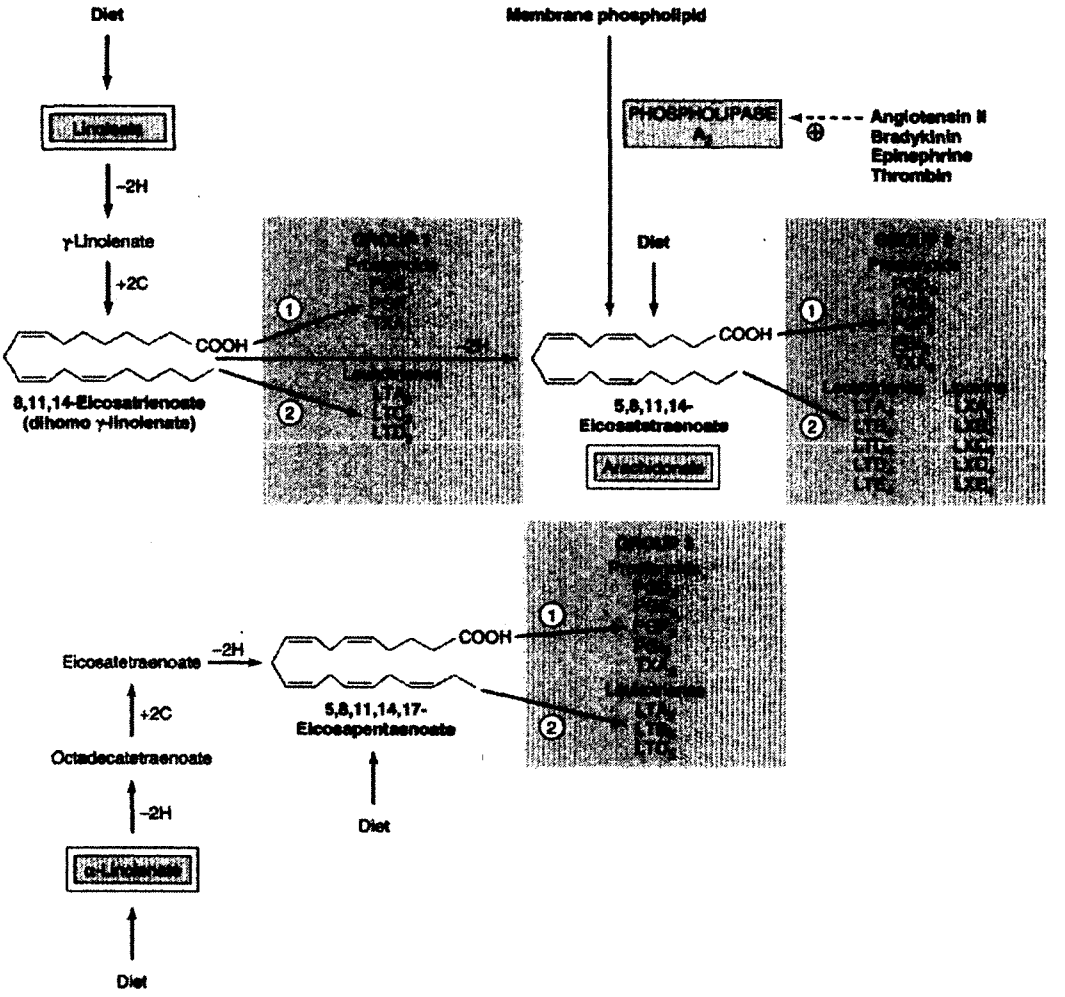

Fig. 5: Biosynthesis of eicosanoids starting from polyunsaturated fatty acids.

I.4.2. Synthesis enzymes: PGHS

The key initial reaction in prostaglandin formation is catalysed by the enzyme Prostaglandin Endoperoxide Synthase (PGHS). For simplicity this is usually known as cyclooxygenase COX (Burdan et al., 2006).

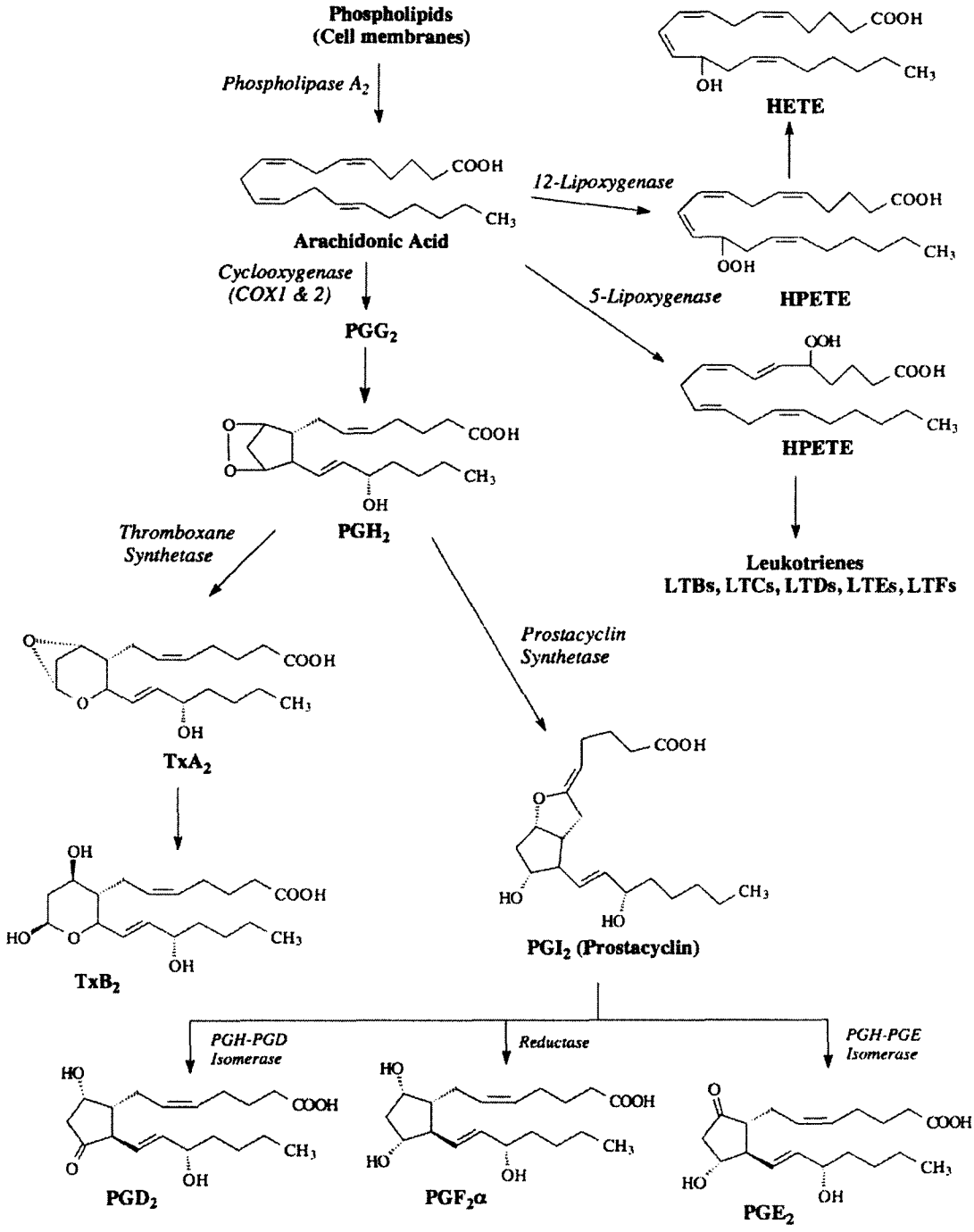

Fig.6: Conversion of free arachidonic acid to prostaglandins and other eicosanoids is initiated by oxidative enzymes of the cyclooxygenase. Adapted after Harper's Illustrated Biochemistry, Twenty-Sixth Edition, 2003.

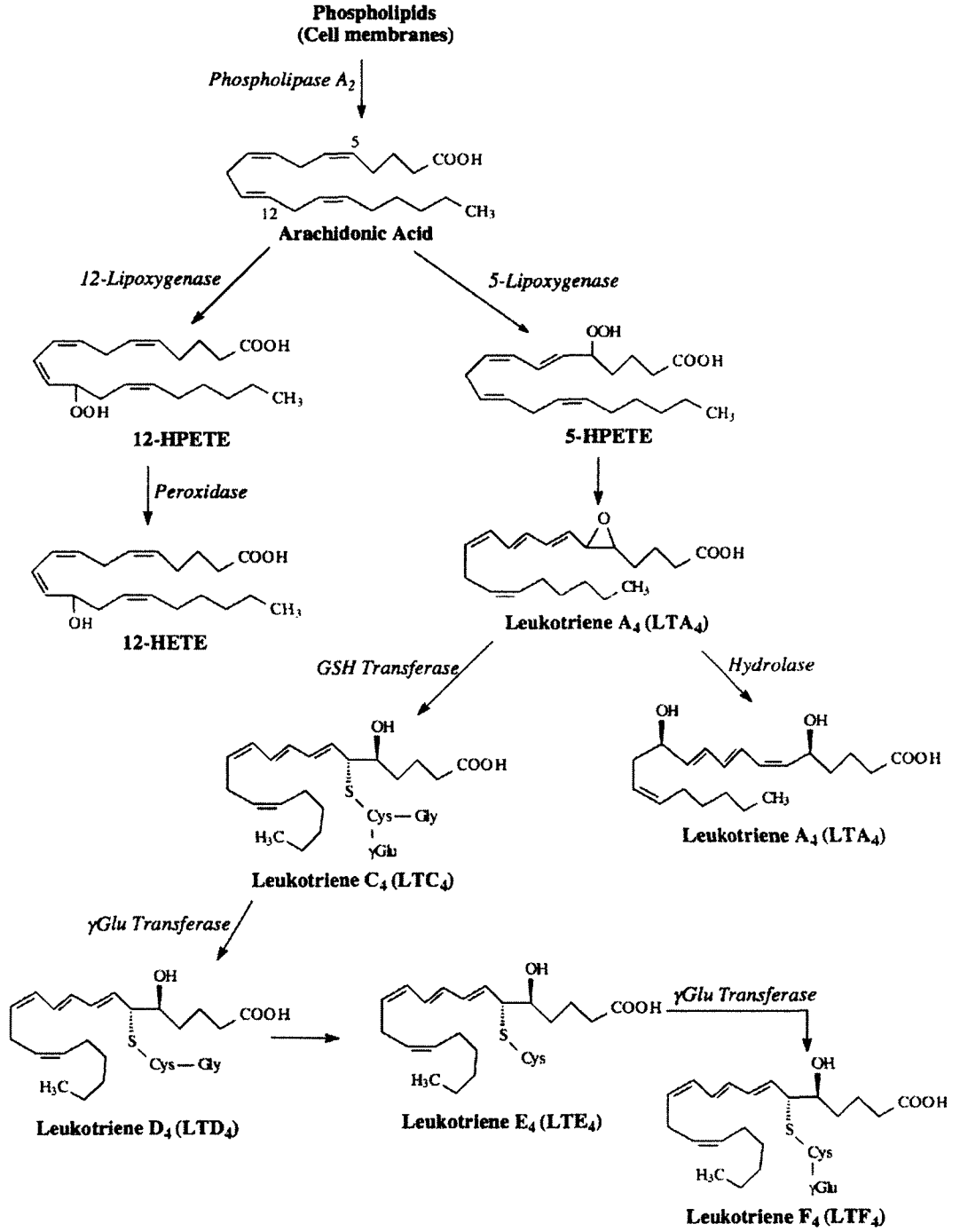

Fig. 7: Lypoxygenase pathway. The lipoxygenase pathway of arachidonic acid metabolism produces a variety of acyclic lipid peroxides (hydroperoxyeicosatetraenoic acids or HPETEs) which can be reduced to the corresponding alcohols (hydroxyeicosatetraenoic acids or HETEs). Adapted after Harper's Illustrated Biochemistry, Twenty-Sixth Edition, 2003.

There are two major isoforms of the enzyme and crystal structures for both have been obtained: PGHS-1 or COX-1 and PGHS-2 or COX-2 (discovered by Daniel L. Simmons in 1991). The cyclooxygenases are haemoproteins and they present both cyclooxygenase and peroxidase activity (Garavito and Mulichak, 2003). Also, they use a variety of substrates like linoleates (α and γ) giving rise to different endoperoxides. There is a 60% aminoacid homology between the two COX isoforms, but there is a difference in localisation, COX-2 acting at the nuclear envelope and COX-1 mainly in the ER and close to the cell membrane (Versteeg et al., 1999).

The product of PGHS is an endoperoxide, subsequently converted to prostaglandins D, E and F, to a thromboxane (TXA_2) and prostacyclin (PGI_2).

Today, we acknowledge the existence of the two isoforms, one with constitutive expression and the other inducible. COX enzymes are integral membrane proteins that sit within one leaflet of the lipid bilayer of intracellular phospholipid membranes of the nuclear envelope and ER. The cyclooxygenase active site is located in a channel formed in the center of enzyme, allowing the hydrophobic fatty acid substrate access without leaving the membrane. The peroxidation function is located on the outside of the enzyme and appears to be similar in both enzymes.

It has been proposed that cyclooxygenase COX-1 and COX-2 serve different physiologic functions largely because of the striking differences in their tissue expression and regulation. COX-1 displays the characteristics of a "housekeeping" gene and is constitutively expressed in almost all tissues. COX-1 appears to be responsible for the production of prostaglandins (PG) that are important for homeostatic functions, such as maintaining the integrity of the gastric mucosa, mediating normal platelet function, and regulating renal blood flow. In contrast, COX-2 is the product of an "immediate-early" gene that is rapidly inducible and tightly regulated. Under basal conditions, COX-2 expression is highly restricted; however, COX-2 is dramatically upregulated during inflammation. For example, synovial tissues in patients with rheumatoid arthritis (RA) express increased levels of COX-2. In vitro experiments on endothelial cells, chondrocytes, osteoblasts, synoviocytes and monocytes/macrophages have revealed increased COX-2 expression after stimulation with proinflammatory cytokines, such as interleukin 1 (IL-1) and tumour necrosis factor-alpha (TNF-alpha).

COX-2 is also increased in some types of human cancers, particularly colon cancer. Mechanisms underlying the association between COX-2 over-expression and tumourigenic potential may include resistance to apoptosis (Garavito et al., 2002).

A PGHS-1 variant, COX-3 (Chandrasekharan et al., 2002) have been under investigation after its presence has been confirmed at the cartilage level (Gosset et al., 2006).

The Prostaglandin Synthase action is the concluding step into the formation of a specific prostaglandin. All known PG's have at least one PGS who generates the specific PG from the PGH2 substrate.

1.4.3. Prostaglandins - general effects and roles.

The prostaglandins are involved in modulations of different systems, from the CNS (central nervous system) to gastrointestinal (GI) and immune systems. Moreover, they are involved in a plethora of pathological states ranging from inflammation to cancer.

They are considered potent pro-inflammatory mediators and they play an important role in nociception and pain, since the COX-2 and mPGES-1 (microsomal prostaglandin E_2 synthase-1) expression are induced in the CNS via the pro-inflammatory cytokines like IL-1β and TNFα (Zeilhofer, 2007). They are involved in the contraction and the relaxation of the smooth muscles, sleep mechanisms, fever induction, renal tubular reabsorption, apoptotic regulation, cell differentiation.

Fig. 8 presents a general cellular structure activated by possible triggering mechanisms like mechanical trauma, inflammation (cytokines). The inducible form of Prostaglandin Synthase (COX-2) can also contribute to the increased expression of PG's. The synthesised prostaglandins are carried by the prostaglandin transporter (PGT) to exert actions on specific receptors. Of course, their action is perhaps extended to some non-specific receptors - like the nuclear hormone receptor PPAR.

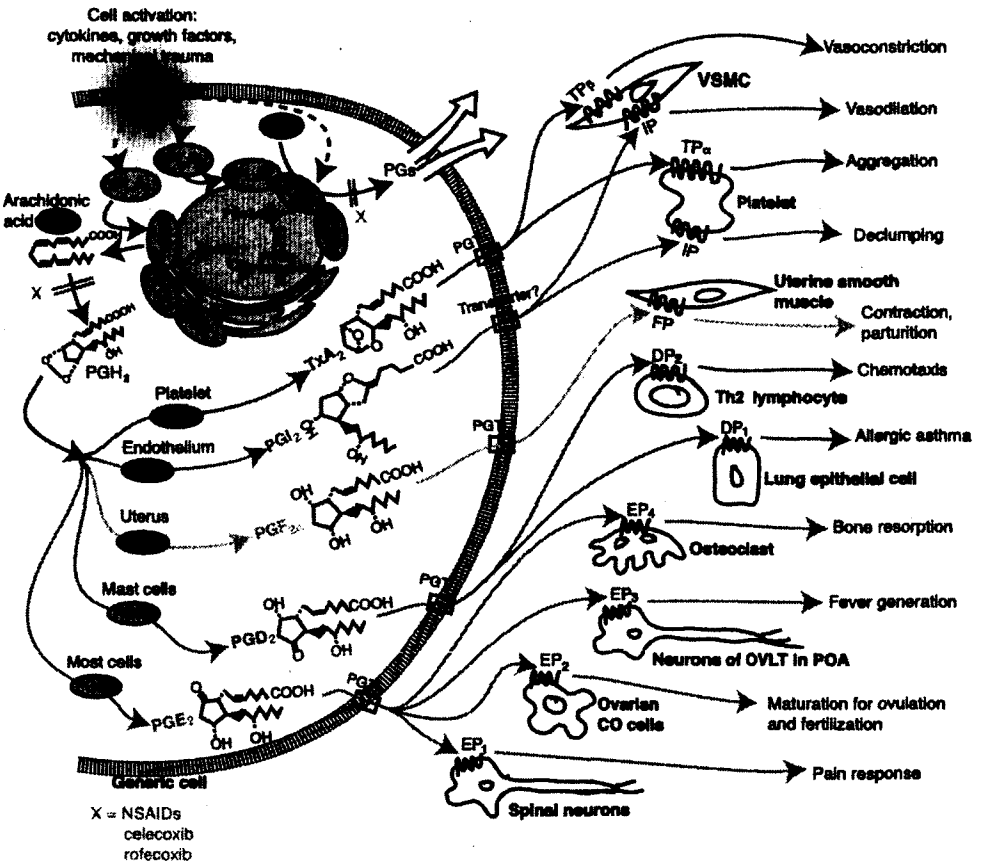

Fig. 8: Prostaglandin synthesis and actions in a generic cell. (Funk, 2001). The "X"s show the inhibition sites of COX-1 and COX-2 with non-specific enzyme blockers (aspirin, ibuprofen), or specific COX-2 inhibitors (celecoxib).

Free radicals are generated in the course of PG synthesis, and inhibitors of PG synthesis are free radical scavengers.

Prostaglandins are autacoids with a quick onset of action over the biochemical targets. They are not stored, and they have immediate release. Because of their ionic structure, PG's do not cross the cell membranes easily, although they are fatty acids derivatives. This is why, once their action yielded the wanted cascade of effects, they need to be quickly inactivated. Most tissues have the necessary equipment to do so, and it is considered that the half-life of the active prostaglandin is less than 5 minutes. PGD_2, PGE_2, $PGF_{2\alpha}$ are eliminated in part through the lungs in a single pass (Ferreira & Vane, 1967).

Concentrations of major active prostaglandin products in blood are less than 0.1 nM and because of their rapid catabolism they can only act as local hormones modifying biological events close to their sites of synthesis.

Degradation occurs via β and ω oxidation, de-methylation or by oxidation of the C-15 hydroxyl group to a ketone by 15-hydroxy-PG dehydrogenase (15-OH-PGDH) present in tissues (peroxizomes) (Gosset et al., 2006) (Tai et al., 2002). On the other hand, TNF-α, IL-β decrease the 15-OH-PGDH mRNA, impeding the keto-transformation of the PG's, thus prolonging their effects.

A prostaglandin transporter (PGT), which plays a primary role in mediating prostanoid transport and metabolic clearance, has been identified (Chi & Schuster, 2010). The PGT plays a role in the uptake of newly released prostanoids, thus acting as a carrier across the plasma membrane before intracellular oxidation (Chi et al., 2006). Moreover, PGT can facilitate intracellular actions of circulating as well as intracellularly produced prostanoids. The PGT preferentially transports PGE_2, PGE_1, $PGF_{2\alpha}$, PGD_2, with high affinity and to a lesser extent, TXB_2 and PGI_2 (Funk, 2001).

1.4.4. Prostaglandin receptors.

There are eight types of the prostanoid receptors (Table 1) conserved in mammals from mouse to human. They are the PGD receptor (DP1), four subtypes of the PGE receptor (EP1, EP2, EP3, EP4), the PGF receptor (FP), PGI receptor (IP) and the TXA receptor (TP). They all are G-protein coupled rhodopsin-type receptors with seven transmembrane domains, and each is encoded by different genes. In addition, there are several splice variants of the EP3, FP, and TP receptors, but they differ only in their C-terminal tails.

For PGD_2 there is a distinct type of receptor, chemoattractant receptor-homologous molecule expressed on T helper type 2 (Th2) cells (CRTH2). This receptor was originally cloned as an orphan receptor expressed in Th2 lymphocytes, and has recently been shown to bind PGD_2 with an affinity as high as that of DP, although the binding profile to other PGD analogs differs from that of DP. The CRTH2 recep-

Class	Subtype	Agonists	Antagonists	Signaling	
TXA_2	TP	U46619, I-BOP, STA_2	SQ29548, S-145, ramatroban	G_q, G_s (a), G_i (h), G_h (a), G_{12}	z IP_3/DAG/Ca^{2+}, z cAMP, A cAMP
PGD_2	DP	BW245C, L-644,698, ZK110841	BWA868C,[a] S-5751	G_s	z cAMP, z Ca^{2+}
	CRTH2	13,15-Dihydro-15-keto-PGD_2, indomethacin, 15-R-methyl-PGD_2, 15d-PGJ_2	Ramatroban	G_i	A cAMP, z Ca^{2+}, PLC, PI3K, MAPK
PGE_2	EP1	ONO-KI-004, iloprost, 17-phenyl-trinor PGE_2, sulprostone	SC51322, SC51089, ONO-8713	?	z Ca^{2+}
	EP2	Butaprost, 11-deoxy PGE_1, AH13205, ONO-AEI-259		G_s	z cAMP, EGFR transactivation, h-catenin
	EP3	Sulprostone, MB28767, misoprostol, SC46275, ONO-AE-249	ONO-AE3-240, L-826266	G_i, G_q, G_s	A cAMP, z IP_3/DAG, z cAMP
	EP4	PGE_1-OH, misoprostol, ONO-AEI-329	AH23848B ONO-AE3-208	G_s	z cAMP, PI3K, ERK1/2, h-catenin
PGI_2	IP	Iloprost, cicaprost, carbacyclin		G_s, G_q, G_i	z cAMP, z IP_3/DAG, A cAMP
PGF_{2a}	FP	Fluprostenol, latanoprost		G_q	z IP_3/DAG, Rho, EGFR transactivation, h-catenin

[a] Partial agonist.

Table 1: Prostaglandin receptors - signaling (Hata and Breyer, 2004).

tor belongs to the family of chemokine receptors, and mediates chemotaxis in Th2 lymphocytes as well as in eosinophils or basophils (Nagata & Hirai, 2003).

The IP, DP, EP2, and EP4 receptors mediate a cyclic adenosine monophosphate (cAMP) rise and have been termed "relaxant" receptors, whereas the TP, FP, and EP1 receptors induce calcium mobilisation and constitute a "contractile" receptor group. The EP3 receptor induces a decline in cAMP levels and has been termed the "inhibitory" receptor. Also, the prostaglandins bind with the highest affinity to their cognate receptor but with lower affinity to other receptors within the family (Breyer et al., 2001).

A. Tromboxane, TP receptors

TxA2 is a potent stimulator of both platelet aggregation and smooth muscle contraction, the latter leading to vasoconstriction. The TxA2 production is implicated in the pathogenesis of atherosclerosis and myocardial infarcts. The human TxA2 re-

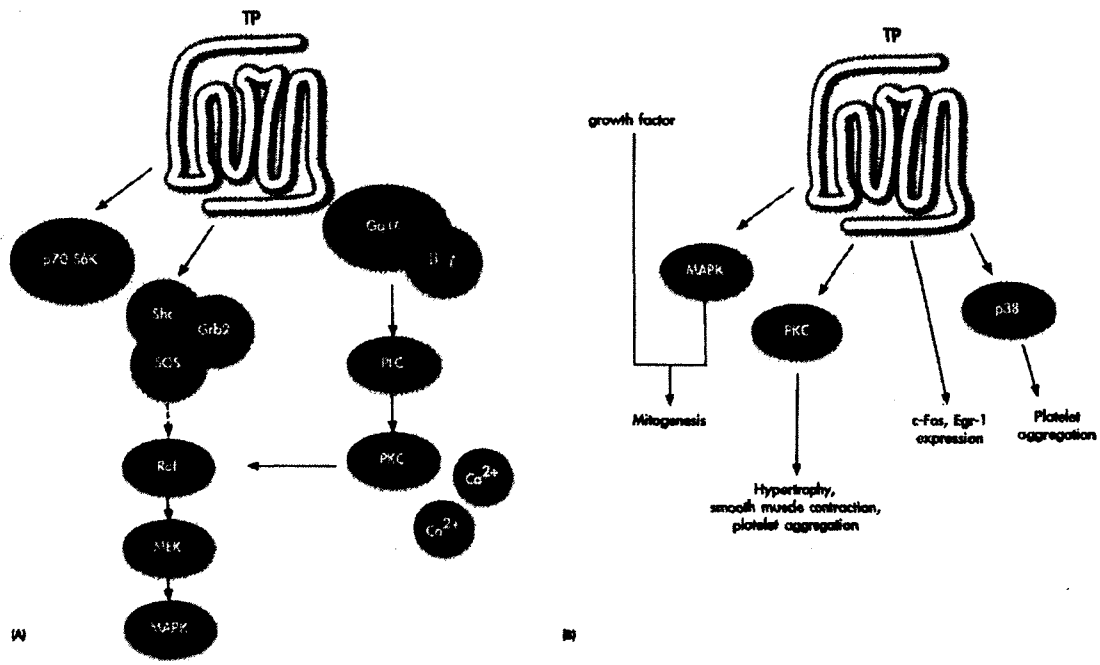

Fig. 9: TxA2-induced signaling via TP. (A) TxA2 treatment leads to activation of MAP kinase via Gq and PKC.(B) Stimulation of TP by TxA2 leads to a set of cellular signals. Adapted after (Bos et al., 2004).

ceptor (Fig. 9), designated "TP", was the first eicosanoid receptor cloned (Hirata et al., 1991).

B. $PGF_{2\alpha}$ and FP receptors

$PGF_{2\alpha}$ is generally associated with physiological processes, such as hypertrophic cell growth, the induction of interleukin synthesis, luteolysis and uterine contraction, plays a critical role in mammalian reproduction, and potentiates mitogenic signals and transcriptional activity (Fig. 10).

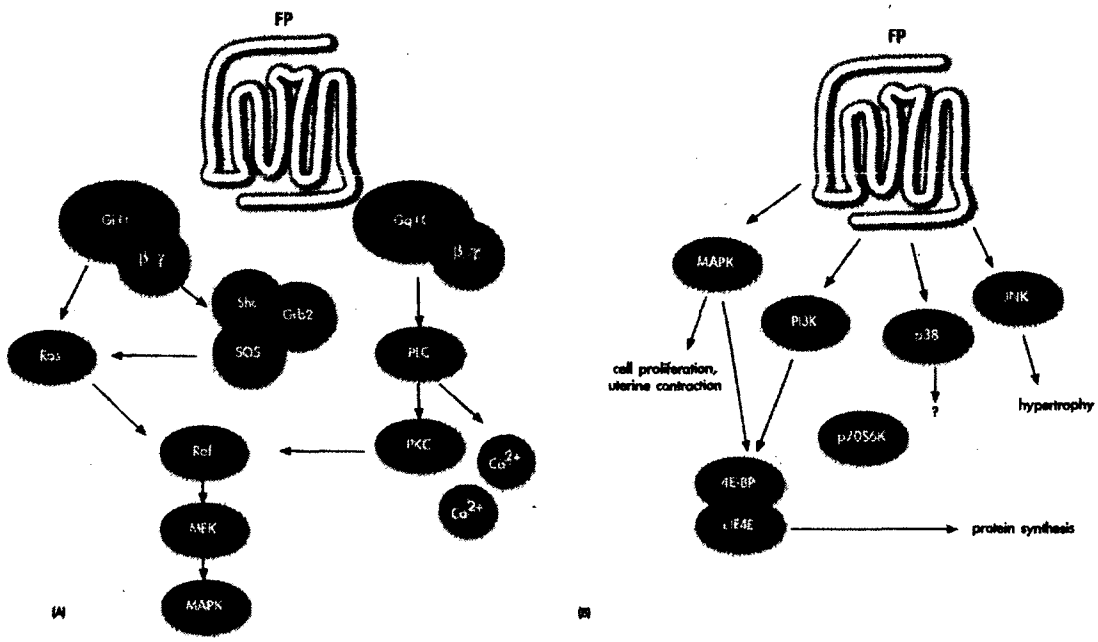

Fig. 10. $PGF_{2\alpha}$-induced signaling via FP. (A) Upon binding of $PGF_{2\alpha}$ to its receptor, MAP kinase activation will generally occur via the activation of the heterotrimeric G-protein Gq, leading to the activation of the PKC pathway. $PGF_{2\alpha}$ might also exert its action via Gi. (B) Next to MAP kinase activation, stimulation of FP leads to activation of several other signaling proteins, such as PI3 kinase (PI3K), p38 MAP kinase, JNK, and p70 S6 kinase. These components have been shown to be essential for $PGF_{2\alpha}$-mediated physiological events. Adapted after (Bos et al., 2004).

C. PGE_2 and EP receptors

PGE_2 induces vasodilatation, is a pro-inflammatory compound, regulates production of a variety of cytokines such as TNFα and IL-6, induces relaxation in arterial smooth muscle beds, plays a protective role in maintaining the integrity of the gastric mucosa, preserves renal blood flow and glomerular filtration rate, controls salt and water transport in the distal tubule, stimulates renin release from the juxtaglomerular apparatus (Breyer et al., 2001). PGE_2 has also been shown to play a role in the maintenance of blood pressure.

The diverse and sometimes opposing effects of PGE_2 may be accounted for in part, by the existence of four receptors, designated EP1, EP2, EP3, and EP4, and to the heterogeneity in coupling of these receptors to intracellular signal transduction pathways (Fig. 11).

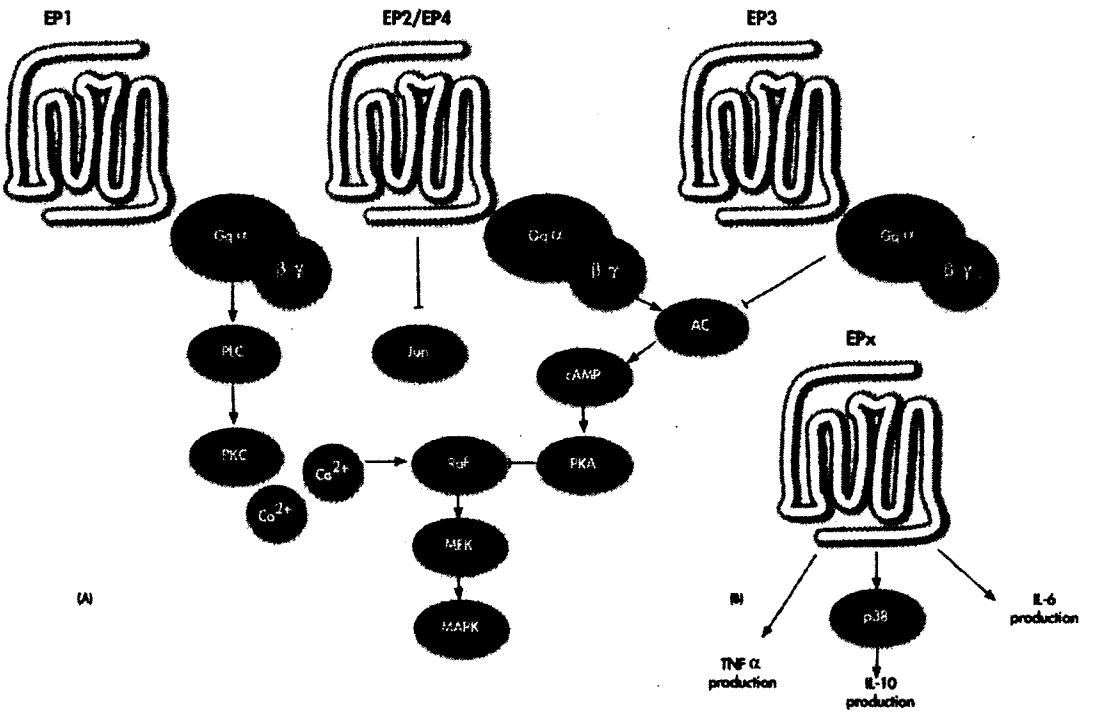

Fig. 11: Activation of distinct EPs by PGE_2 induces several signaling pathways and cytokine release. (A) PGE_2 binds to distinct EPs to induce either activation of the MAP kinase pathway via Gq or Gi, or to induce upregulation of cAMP and subsequently activation of protein kinase A (PKA). The latter pathway leads to decreased MAP kinase activation. (B) Exposing certain cells to PGE2 results in the regulation of cytokine production, such as TNFα, IL-6, and p38 MAP kinase-dependent IL-10 production. Adapted after (Bos et al., 2004).

The EP1 receptor was originally characterised as acting on stimulation of intracellular calcium. Both EP2 and EP4 receptors couple to Gs resulting in stimulation of adenylyl cyclase and increased cAMP levels. The EP3 receptor, which couples to a Gi-type G protein, has been termed inhibitory.(Muallem et al., 1989)

D. PGI_2 receptors

Prostacyclin (PGI_2) is the primary prostaglandin produced by endothelial cells, has vasodilatory and antithrombotic effects, inhibits platelet activation and TXA2-induced vascular proliferation, has cardioprotective effects, and is an important mediator of acute inflammation and inflammatory pain transmission (Crofford, 1997) (Fig. 12).

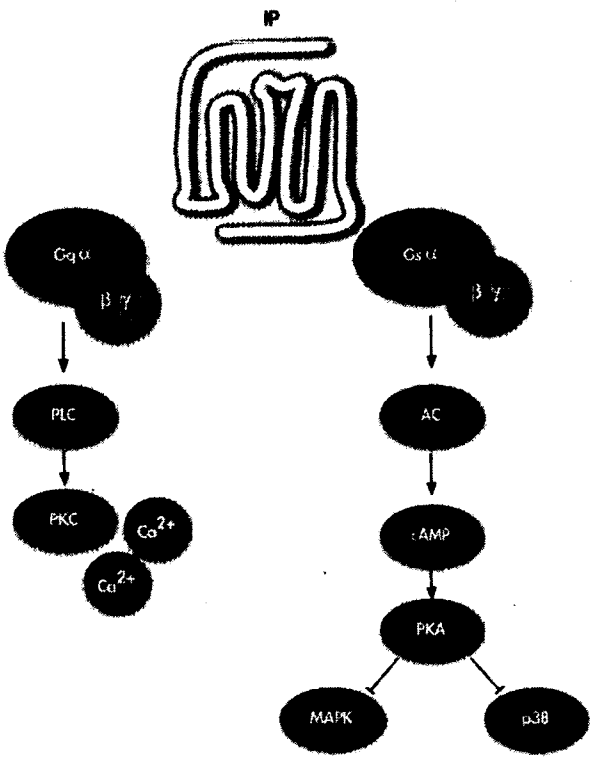

Fig. 12: Binding of PGI_2 to IP induces cell-specific signaling. PGI_2 induces inhibition of both p42/p44 MAP kinase and p38 MAP kinase via the activation of Gs. In some cell types, IP activation triggers the PKC pathway in a Gq-dependent manner. Adapted after (Bos et al., 2004).

E. PGD_2 receptors: DP and CRTH2. PPARγ.

PGD_2 is produced in various tissues, and just like PGI_2, it inhibits platelet aggregation and causes relaxation of both vascular and non-vascular smooth muscle cells. PGD_2 has been shown to bind and activate two G-protein-coupled receptors, DP and CRHT2 (chemoattractant receptor-homologous molecule expressed on T Helper type 2 cells). It is also known as DP2 receptor.

PGD_2-dependent activation of the DP receptor (Fig. 13) mediates various physiological events including sleep induction (Cui et al., 2008), cell survival (Gervais et al., 2001), and allergic responses (Matsuoka & Narumiya, 2007).

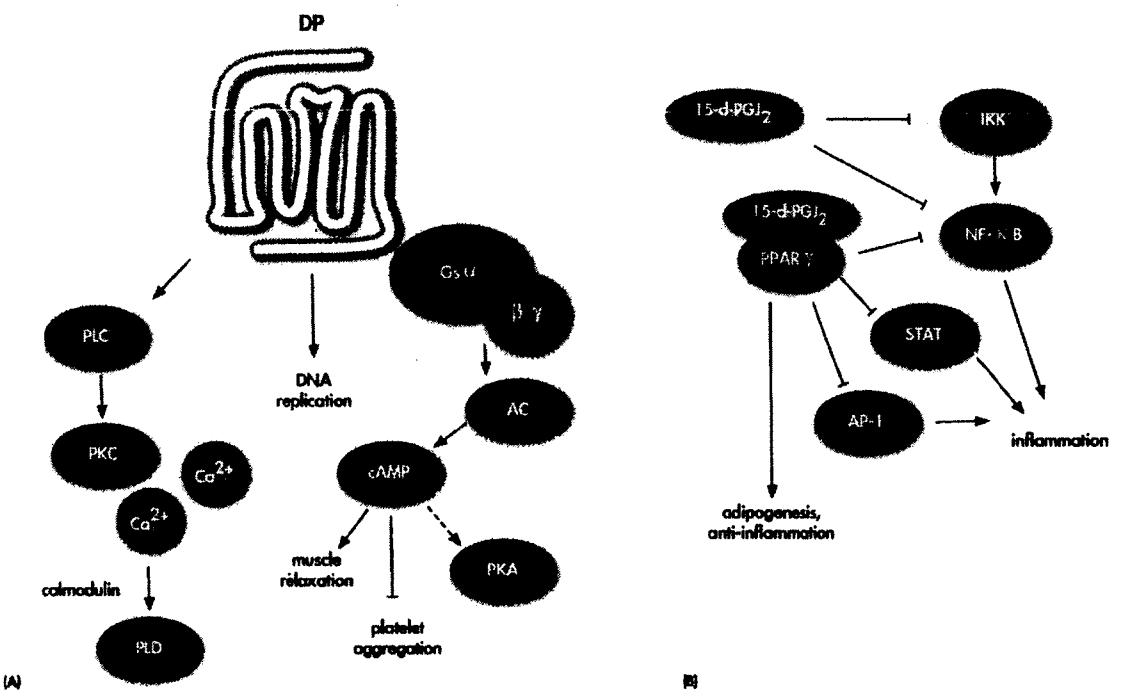

Fig. 13: PGD_2 and PGD_2 metabolite-induced signaling. (A) PGD_2 normally triggers the Gs-dependent PKA pathway leading to inhibition of platelet aggregation and muscle relaxation. It might also lead to activation of PLC, PKC, and PLD. (B) The PGD_2 metabolite 15-d-PGJ_2 antagonizes the proinflammatory actions of prostanoids through the activation of the cytoplasmic receptor PPARγ and independent of PPARγ by inhibition of typical inflammatory intracellular signaling proteins, such as NF-κB and the STATs. Adapted after (Bos et al., 2004).

CRTH2 activation by PGD_2 (Fig. 14) can modulate both eosinophil morphology and degranulation and can also increase eosinophil and T Helper type 2 cell motility. (Gervais et al., 2001; Hirai et al., 2001).

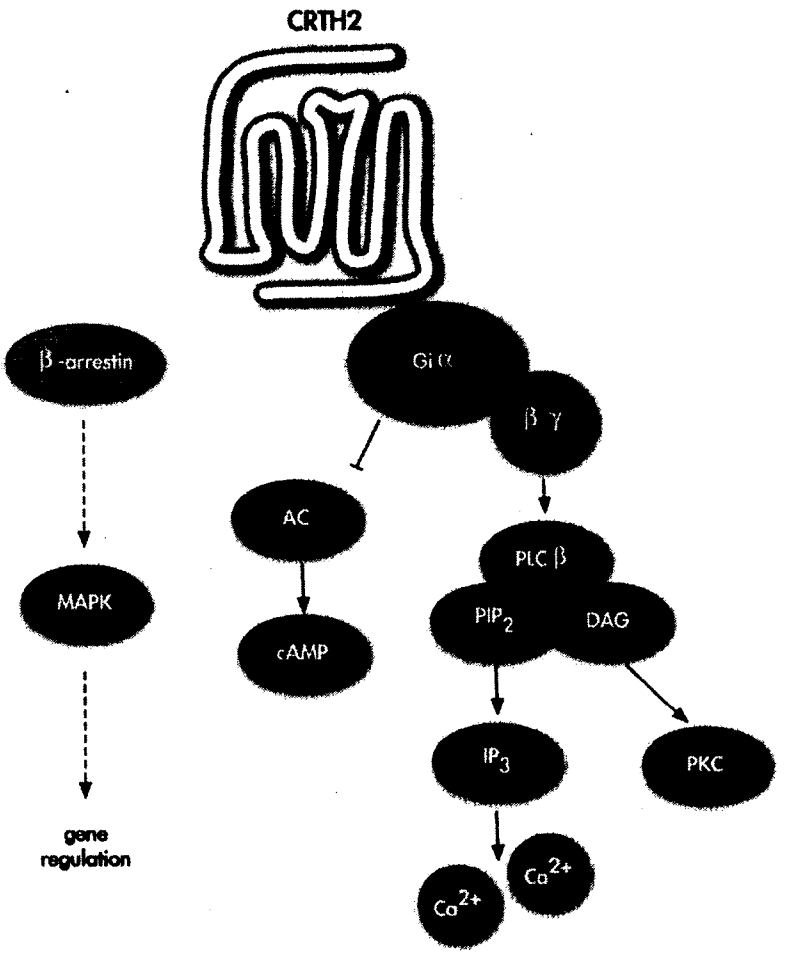

Fig. 14: CRTH2 induced signaling. CRTH2 inhibits adenylyl cyclase (AC) through Gi proteins; this leads to a decrease of intracellular cAMP levels. Gβγ- subunit complexes released upon Gi activation directly stimulate phospholipase Cβ (PLCβ) isoforms, which generate diacylglycerol (DAG) and inositol trisphosphate (IP3) through breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2). IP3 mobilizes Ca^{2+} from endoplasmic-reticulum stores. CRTH2 also signals through arrestin proteins in a G-protein-independent fashion, which is a newly discovered mode of signaling that partly underlies eosinophil shape change after PGD_2 challenge. It is possible but not yet proven that arrestin recruitment might also lead to G-protein-independent MAP-kinase activation. (Ulven and Kostenis, 2006).

F. PGD_2 - Generalities

The prostaglandin D_2 has an important role in the regulation of different body functions like sleep regulation, allergic responses, asthma and pregnancy.

The role of PGD_2 in wake-sleep mechanisms has been studied for a long time, and published works connected the PGD_2 with the non rapid eye movement and sleep (Hayaishi, 2002), with pathological states triggered by systemic or infectious diseases (Jordan et al., 2004), with narcolepsy (Jordan et al., 2005), and perhaps with more complex mechanisms involving melatonine and GABA systems in the brain (Urade & Mohri, 2006).

The PGD_2, being released from basophiles and mast cells (Schleimer et al., 1984), is an important mediator in allergy (Naclerio et al., 1983) having a bronchoconstrictor effect in allergic asthma (Hardy et al., 1984). This effect is probably regulated through the CRTH2 receptor (Boehme et al., 2009a; Boehme et al., 2009b). PGD_2 is a coronary constrictor in anaphylaxis (Weinerowski et al., 1985) and is a mediator in skin dermatitis, probably through CRTH2, (Oiwa et al., 2008).

The transformation of PGH_2 into PGD_2 is realised through the catalytic activity of specific synthetase, called the PGDS, which has two known forms: lipocalin-type (L-PGDS) and the hematopoietic-type (H-PGDS) (Urade & Eguchi, 2002).

Cyclopentenone prostaglandins (PGs), such as 15-deoxy-12,13-didehydro-14,15-didehydro-PGJ_2 (15d-$\Delta^{(12,14)}$-PDJ_2), 12,13-didehydro-PGJ_2 (Δ^{12}-PGJ_2), are the products resulting from the PGD_2 dehydration. The PGJ_2 synthesis was initially related to the presence of serum albumin (Keelan et al., 2003), but studies showed that the conversion could be done through an albumin-independent mechanism (Shibata et al., 2002), and through the intermediaries like PGJ_2 and 15d-PGD_2 to the final compounds 15d-PGJ_2 and Δ^{12}-PGJ_2 .

The biological responses of PGD_2 could be in fact the result of the combined effects at different levels of the PGD_2 and its dehydrated products. The ultimate metabolite of PGD_2, 15deoxy-$\Delta^{12\text{-}14}$-PGJ_2, binds specifically to a nuclear receptor, the gamma isoform of the peroxisome proliferator-activated receptor (PPARγ), thereby

promoting adipogenesis (Negishi & Katoh, 2002) and triggering anti-inflammatory responses. It also binds to the PGD_2 receptors, DP1 and CRTH2 (Scher & Pillinger, 2005). The intranuclear target of the 15d-PGJ_2, the PPAR's, are transcription factors that regulate gene expression of enzymes associated with lipid homeostasis, inflammation, cell proliferation, and malignancy. The 15d-PGJ_2 could intervene in other intracellular cascades in a PPAR-independent manner, through a mechanism of covalent binding to proteins from the NF-κB system (the IκB kinase-IKK). It blocks the activation of NF-κB and the extracellular signal-regulated kinase (Erk) signalling pathway (Chawla et al., 2001) and also binds to H-Ras and increases cell proliferation (Oliva et al., 2003).

1.5. NSAID's

Non-steroidal anti-inflammatory drugs - NSAIDs, are drugs with analgesic, antipyretic and anti-inflammatory effects. The term "non-steroidal" is used to distinguish these drugs from steroids, which have a similar eicosanoid-blocking, anti-inflammatory action. NSAIDs are non-narcotic. The most prominent members of this group of drugs are aspirin, ibuprofen, and naproxen partly because they are available over-the-counter in many areas.

Prostaglandins are potent hyperalgesic mediators which modulate multiple sites along the nociceptive pathway and enhance both transduction (peripheral sensitising effect) and transmission (central sensitising effect) of nociceptive information. Inhibition of the formation of prostaglandins at peripheral and central sites by NSAIDs thus leads to the normalisation of the increased pain threshold associated with inflammation.

The structure of the COX proteins consists of three distinct domains: an N-terminal epidermal growth factor domain, a membrane-binding motif, and a C-terminal catalytic domain that contains the COX and peroxidase active sites. The COX active site lies at the end of a hydrophobic channel that runs from the membrane-binding surface of the enzyme into the interior of the molecule (Dannhardt & Kiefer, 2001).

NSAIDs act at the COX active site in several ways:

- Aspirin irreversibly inactivates both COX-1 and COX-2 by acetylating an active-site serine. This covalent modification interferes with the binding of arachidonic acid at the COX active site.

- By contrast, reversible competitive inhibitors of both COX1 and COX2 isoforms (e.g., mefenamate, ibuprofen) compete with arachidonic acid for the COX active site.

- A third class of NSAIDs (e.g., flurbiprofen, indomethacin) causes a slow, time-dependent reversible inhibition of COX-1 and COX-2 which results from the formation of a salt bridge between the carboxylate of the drug and the arginine 120, followed by conformational changes. (Kalgutkar et al., 2000)

Most of the NSAIDs are non-selective inhibitors acting at the active site of both enzymes, COX-1 and COX-2, but selective COX-2 inhibitors have been available for use in arthritis public use starting from 1998 (under the U.S. Food and Drug Administration approval). Apart from its involvement in inflammatory processes, COX-2 seems to play a role in angiogenesis, colon cancer and Alzheimer's disease. This is based on the fact that it is expressed during these diseases. Lately, acetaminophen is believed to act on the COX-3 izoenzyme (Chandrasekharan et al., 2002) decreasing fever and pain. Of course, the specificity has a certain degree, interactions could occur in a crossover way, the COX-1 inhibitors interacting with the COX-2 sites (Hinz et al., 2006). Studies have shown that the use of NSAIDs is associated with a reduced incidence of colon cancer (COX-2) and a reduced risk of Alzheimer development (COX-2). The NSAIDs have an important role in controlling pain associated with rheumatoid arthritis (Simmons et al., 2004).

I.6. Prostaglandins and bone

Prostaglandins have multiple effects on bone cells, and sometimes opposite effects in different species. Their role is therefore difficult to discern. They are powerful bone and cartilage resorbing agents in certain in vitro studies, yet they are potent anabolic (bone forming) agents when administered in vivo. Experimental investigations proved their influence on inflammatory processes, and, above all, on the bone destruction in the pathogenesis of rheumatoid arthritis and osteomyelitis.

Prostaglandins very probably induce the local intercellular communication and regulate the metabolism of bone and cartilage. They are supposed to be local mediators of mechanical stress, electric phenomena, as well as of hormonal control mechanisms in cytobiologic and cytochemical reactions within the skeletal system.

Prostanoids produced by bone include PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, the metabolite of PGI_2, as well as PGD_2 and thromboxane.

The first thing worth mentioning is the involvement of dietary fatty acids into the bone metabolism. The poly-unsaturated fatty acids (PUFA, n-6 and n-3) seem to modulate hOB metabolism by influencing the OPG/RANKL system responsible for the PGE_2 interactions (Coetzee et al., 2007).

Osteoblasts and osteoclasts, the most important contributors to bone remodelling, produce PGs, which are shown to modulate the cell function in normal bone metabolism and in bone healing (Gajraj, 2003). Several receptors have a proven existence on the hOB (Sarrazin et al., 2001), and the hOB themselves produce these autacoids (Hackett et al., 2006). Moreover, in fluids containing albumin, PGE_2 and PGD_2 are slowly dehydrated within the cyclopentane ring to the cyclopentenone prostaglandins PGA_2 and PGJ_2, respectively. PGJ_2 is metabolised further to yield 15-deoxy-Δ^{12-14} PGJ_2 (15d-PGJ_2). The cyclopentenone PGs, which are not thought to act via the classical PG receptors, are active when given exogenously and can have opposite effects from some of the primary PGs (Negishi & Katoh, 2002).

The PGE_2 is the most abundant PG produced by the OB, producing through the EP2 and EP4 receptors, either anabolic or catabolic effects on the bone (Fig. 15). A

large number of cytokines, hormones and growth factors control the PGE_2 production: IL-1, IL-6 which lead to bone resorbtion (Kwan Tat et al., 2004), IL-4 and IL-3 which suppress the PGE_2 production and inhibit the bone resorbtion (Onoe et al., 1996). Fresh serum is a potent stimulator of COX-2 expression and PGE2 production in cultured osteoblastic MC3T3-E1 cells (Pilbeam et al., 1995). The same effect is obtained with selective agonists for IP, TP, FP and DP receptors (Sakuma et al., 2004).

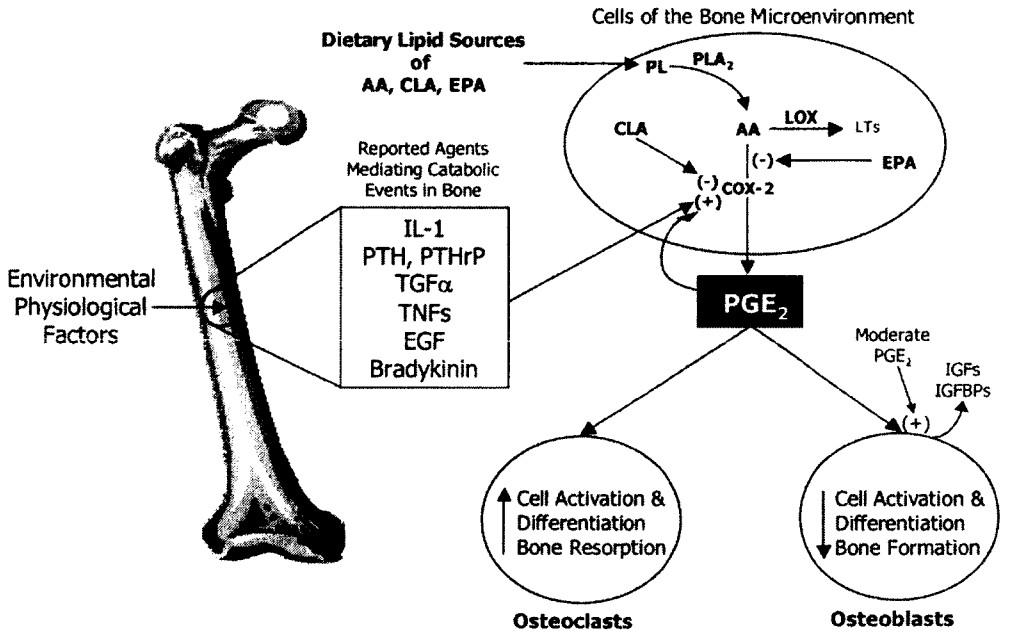

Fig. 15: Possible effects of dietary lipids on the bone. AA: arachidonic acid; CLA: conjugated linoleic acid; EPA: eicosapentaenoic acid; IGF/IGFB:insulin-like growth factors/IGF binding proteins. (Watkins et al., 2001).

Systemic administration of PGE_2 induces bone formation and increases bone mass (Marks & Miller, 1988), it increases the blood flow and callus formation in rabbits by inhibiting osteoclastogenesis (Mano et al., 2000) and by increasing the AlkP expression (Kakita et al., 2004). It can also have a catabolic effect leading to bone resorbtion (Suzawa et al., 2000). As a confirmation of the opposite roles of the PGE_2, we mention that it can be made active by Bone Morphogenetic Protein 2 (BMP-2) which acts as a bone repair factor and normally increases bone formation. It can also induce osteoclastogenesis in mice (Blackwell et al., 2009).

Both PGD_2 receptors, the DP and the CHRT2, are present on the hOB (Gallant et al., 2007). By transduction signals through these receptors, the PGD_2 induces calci-

fication (possible by its metabolite, 15d-PGJ_2) (Koshihara & Kawamura, 1989) and has probable proliferative (Tsushita et al., 1992) or indirect anti-proliferative properties (Haberl et al., 1998). In human primary OB, PGD_2 activates the DP receptor thereby decreasing the osteoprotegerin expression, but in the same cells, through the CRTH2 receptor, it decreases the RANKL production. The production of PGD_2 is stimulated by TNF-α, IL-1, PTH, VEGF (Gallant et al., 2005) and also by mechanical strain (perhaps through the system of Δ^{12} PGJ_2/PPAR) (Siddhivarn et al., 2006).

The PGD_2 has anabolic or catabolic effects on the bone, carrying a controversial role in bone homeostasis. PGD_2 stimulates calcification of hOB (Koshihara & Kawamura, 1989) and has proliferative effects (Tsushita et al., 1992) or anti-proliferative effects through its metabolites (Haberl et al., 1998). PGD_2 stimulates the osteoclastogenesis (via IL-6) (Durand et al., 2008; H. Tokudaa, 1999) probably by inhibiting the OPG production (Samadfam et al., 2006).

The PGI receptor, IP, has been identified also on the cultured hOB (Fortier et al., 2001), (Sarrazin et al., 2001) and its proven role seems to be inducing the expression of COX-2 enzyme (Sakuma et al., 2004).

The TP and FP receptors presence on human osteoblasts have been also proven (Sarrazin et al., 2001) (Samadfam et al., 2006). The FP acts as an inducer of COX-2 on rodent cloned osteoblastic cells, inhibits the collagen synthesis (Pilbeam et al., 1995) and increase the OPG accumulation on MG-63 cells, but not on hOB (Samadfam et al., 2006).

I.7. Bone pathology

The bone pathologies are the diseases resulting from disorders of bone formation and/or resorption, by changing the rates of bone turnover. These include the metabolic bone disease and those related to cancer and inflammatory processes.

1.7.1. Osteoporosis

Osteoporosis is the most common metabolic bone disease. Osteoporosis was defined as "a disease characterised by low bone mass and micro-architectural deterioration of bone tissue, leading to enhanced fragility and a consequent increase in fracture incidence" (1993, Am J Med, 94, 646-50). The disease is diagnosed when the bone mineral density is 2.5 standard deviations below the young adult mean. A bone mineral density between 1 and 2.5 standard deviations below the young adult mean is termed "osteopenia".

In Canada, 1 in 4 women and 1 in 8 men have osteoporosis (Brown & Josse, 2002); 54% of postmenopausal women in the United States have osteopenia and another 30% have osteoporosis.

It has been estimated that bone mass reaches a peak about 10 years after linear growth stops. Peak mineral density is lower in women, especially Caucasian women as compared to black women and men. Women can expect to lose 35% of cortical and 50% of trabecular bone with age. Men can expect to lose about two-thirds of these values. In women, about half the loss can be ascribed to menopause (Serhan, 2004; Giguere & Rousseau, 2000; Brown & Josse, 2002).

	Cellular changes	Probable culprits
Sex steroid deficiency	↑Osteoblastogenesis	Increased IL-6, TNF, IL-1RI/IL-RII
	↑Osteoclastogenesis[a]	MCSF; decreased TGF-β; OPG
	↑Life-span of osteoclasts	Loss of proapoptotic and antiapoptotic
	↓Life-span of osteoblasts	effects of sex steroids, respectively
	↓Life-span of osteocytes	
Senescence	↓Osteoblastogenesis[β]	Increased PPARγ2, pgJ2, noggin;
	↓Osteoclastogenesis	decreased IL-11, IGFs
	↑Adipogenesis	
	↓Life-span of osteocytes	
Glucocorticoid excess	↓Osteoblastogenesis	Decreased Cbfa1 and TGF-β R1;
	↑↓Osteoclastogenesis[c]	and BMP-2 and IGF1 action
	↑Adipogenesis	Increased PPARγ2
	↓Life-span of osteoblasts	Decreased Bcl-2/BAX ratio
	↓Life-span of osteocytes	

[a]Oversupply of osteoclasts relative to the need for remodeling.
[b]Undersupply of osteoblasts relative to the needs for cavity repair.
[c]Osteoclastogenesis may increase transiently at the early stages of steroid therapy, but decreases subsequently.

Table 2: Cellular Changes and Their Culprits in the Three Most Common Types of Osteoporosis. (George C.Tsokos, 2000).

There are many factors that influence the bone mass loss leading to osteoporosis: genetic background (Albagha & Ralston, 2006), the lifestyle (Lock et al., 2006), low estrogen levels (Amin et al., 2006), calcium and vitamin D deficiency (Lips, 2001) (Table 2.).

The accumulation of adipocytes in bone marrow is often observed in patients who manifest bone diseases such as osteoporosis, showing a lack of balance between the osteoblastogenesis and adipogenesis (Nishimura et al., 2007) (Fig. 16).

Fig. 16: "Fatty bone" - Femoral head presenting rough (destroyed) articular surface and large, spongiform trabeculae filled with fat. 54 years old male patient.

1.7.2. Rickets and Osteomalacia

Rickets and osteomalacia are characterised by a defect of mineralisation due to calcium and phosphate deficiencies. This could be due to inadequate absorption by the gastrointestinal tract, lack of sunlight exposure or disorders of vitamin D metabo-

lism, increased renal excretion, increased catabolism, or drug induced (e.g., fluoride, etidronate). The lack of vitamin D results in a fall of serum calcium concentration and bone mineralisation becomes defective. Rickets is a disease of the growing skeleton and affects the epiphyseal plate and bones of children. Osteomalacia is the adult counterpart.

The characteristic histological feature of rickets and osteomalacia is deficiency or lack of mineralisation of the organic matrix of bone accompanied by low values for serum calcium and phosphorus. Osteomalacia is defined as excess osteoid (hyperosteoidosis) and a defective bone matrix mineralisation and this is why the biochemical markers associated with increased osteoid production, such as bone-specific alkaline phosphatase and osteocalcin, will be elevated in states of rickets and osteomalacia (Cole et al., 1985).

The disease has proven hereditary causes (Nguyen et al., 2006), but can be triggered by a osteosarcoma or by mesenchymal tumours (Folpe et al., 2004), posing problems for the differential diagnostics.

1.7.3. Osteopetrosis

Osteopetrosis results from a reduction in bone resorption relative to bone formation, leading to an accumulation of excessive amounts of bone. The relative decrease in resorption is a consequence of inadequate osteoclastic bone resorption. Although the osteoclasts are unable to resorb the bone being incapable of acidifying their ruffled border, they continue to stimulate the osteoblasts, leading to an increase in bone mass (Karsdal et al., 2007). There are three major categories of the disease (malignant, intermediate, and mild) with a variety of subtypes.

1.7.4. Hypophosphatasia

Hypophosphatasia (HPP) has been reported throughout the world and seems to affect all races (Whyte et al., 1986). However, it is especially prevalent in Mennonites in Manitoba, Canada, where about 1 in 25 individuals is a carrier, and 1 in 2500 newborns manifests severe disease (Greenberg et al., 1993). In HPP, circulating levels

of calcium and inorganic phosphate are usually normal or elevated, yet the skeleton does not mineralise properly, thus showing the role of AlkP in biomineralisation (Whyte et al., 1995; Anderson et al., 1997; Whyte et al., 2000).

1.7.5. Paget's Disease

Paget's disease or *Osteitis Deformans*, named after James Paget, (1814-1899) is a localised disorder of bone, with increased bone remodelling, bone hypertrophy, and abnormal structure. It is seen in patients living above their fourth decade, but has a slight male predominance. It is common among the white population of England, France, Austria, Germany, Australia, New Zealand, and the United States (with an estimated incidence of 3%). It is rare in Scandinavia, China, Japan, and Africa (Gomez et al., 2001).

The majority of patients are without symptoms. When symptoms are present, skeletal deformity and pain are most common. Deformities are usually most apparent in the skull, face, and lower extremities. Bone fragility and disorganisation of the matrix result from the accelerated bone turnover yielding increased levels of bone markers including serum alkaline phosphatase, osteocalcin, and the propeptide of type I collagen. Bone is brittle in spite of the increase in bone density and size. Secondary malignancies have been described in long-standing Paget disease, the commonest of which is osteosarcoma (Singer, 2009).

1.7.6. Rheumatoid arthritis

RA is considered to be an autoimmune disease that may involve multiple organ systems. Joint involvement predominates in most patients. The disease occurs more often in women than men in a ratio of about 3:1. It may appear at any time during life but has its peak incidence between 35 and 50 years of age (David M. Reid, 2008).

RA is an illness that affects much more than just the joints, but the joint structure, including the synovium and the bone and periarticular muscles. Systemic symptoms are common (Abramson & Yazici, 2006). Symptoms often start gradually, over months. Patients often experience difficulty in pinning down exactly when their ill-

ness started. The first symptoms are often vague and include fatigue, lack of energy, feeling depressed or washed out, and stiffness in the morning that lasts longer than 30 minutes.

A variety of predispositions are recognised for individuals with RA, including genetical and environmental factors, age, gender, trauma and exercise (Yoshitomi & Sakaguchi, 2005).

1.7.7. Cancers

The bone marrow is one of the more common organs to be involved by tumours that metastasise via the bloodstream. In adults the tumours most often seen are carcinomas of the prostate gland, breast and lung, although any tumour that causes blood-borne metastases may infiltrate the marrow (Ingle et al., 1978).

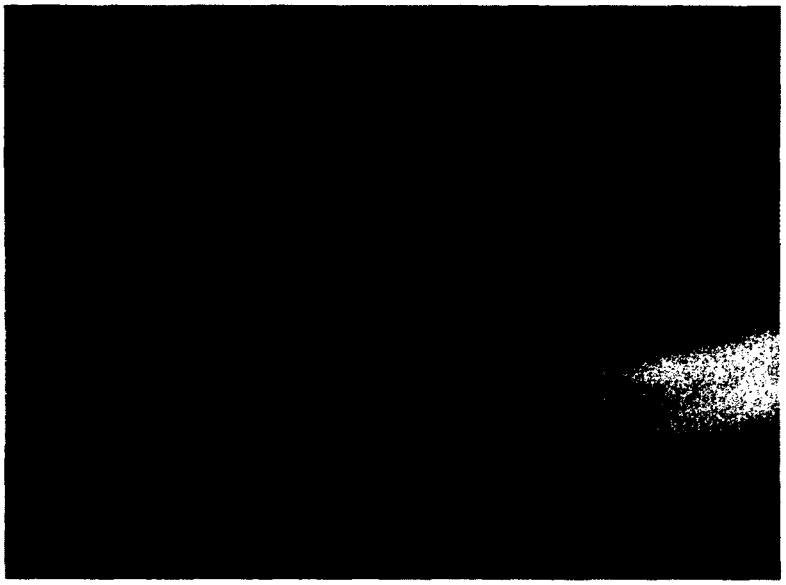

Fig. 17: Fracture on pathological bone. Bone osteolitic metastasis following a diagnosed breast cancer (25 years before). Woman, aged 51. By courtesy of M.D. E. Nedelcescu.

Infiltration of the marrow may be suspected on the basis of the following: (i) bone pain; (ii) pathological fractures, lytic lesions (Fig. 17) or sclerotic lesions demonstrated radiologically; (iii) unexplained 'hot spots' on isotopic bone scans or positron emission tomography (PET) scanning; (iv) an abnormal magnetic resonance imaging scan; (v) hypercalcaemia or elevated serum alkaline phosphatase activity; or (vi) unex-

plained haematological abnormalities (Anner & Drewinko, 1977; Syed et al., 2007). Osteosarcomas (also known as osteogenic sarcoma) are malignant neoplasms of bone that are composed of proliferating cells that produce osteoid. Osteosarcomas appear in Paget's disease, but with an occurrence of less than 1% (Singer, 2009).

Rationale

A survey of the literature has shown that stimulation of human OB by PGD_2 induces an increase in collagen type I synthesis and matrix mineralisation but the receptor subtypes responsible for these actions were not described (Tasaki et al., 1991).

Studies performed in our laboratory showed that the major prostaglandin receptors exist at human osteoblasts level (Sarrazin et al., 2001). The osteoblasts produce the PGD_2 through the lipocalin-type PGD_2 synthase (L-PGDS) and both PGD_2 receptors, the DP and CRTH2, are present on cultured cells (Gallant et al., 2005).

These results support our working hypothesis that PGD_2 may act as an autocrine or paracrine agent in bone and have a positive impact on bone anabolism. Other reasons that make PGD_2 an interesting possible pharmacological target is the existence of two receptors mediating its actions, thus allowing more specific and less side effect-prone pharmacological targeting.

Objectives

In the present study we complete and extend the initial observations from our laboratory and test our hypothesis using human bone cells in culture. Our specific objective is to determine the roles of DP and CRTH2 on the control of osteoblast metabolism, by using the PGD_2 and specific DP and CRTH2 agonists and antagonists, and evaluate the following parameters: proliferation by incorporation of [^{3}H]thymidine, cell differentiation (expression of differentiation markers) and mineralisation.

II. Materials and methods

II.1. Materials

The fetal bovine serum (FBS) were purchased from Gibco BRL (Rockville, MD, USA). Dulbecco's Modified Eagle's Medium (DMEM) came from Sigma-Aldrich Ltd. Compounds BW 245C, BW A868C, PGD_2 and 13,14-dihydro-15-keto PGD_2 (DK-PGD_2), T0070907, CAY 10471 were from Cayman Chemical Co. Compounds were dissolved in dimethyl sulfoxide or ethanol and the solvents were kept at less than 0.1% in the final solutions. Penicillin/streptomycin, L-ascorbate, amphotericin, trypsin, and 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] were purchased from Sigma (St. Louis, MO, USA). [^{3}H]Thymidine, and the Alkaline Phosphatase Substrate Kit were purchased from Bio-Rad Laboratories (2000 Alfred Nobel Drive, Hercules, CA 94553). The calcium QuantiChrom assay kit was purchased from BioAssay Systems (Hayward, CA, USA). All other reagents used were of high-grade quality.

II.2. Cell culture

Specimens of human trabecular bone were obtained from patients undergoing total hip arthroplasty. In conformity with Québec's civil law, each patient signed an informed consent. hOBs were cultured from these tissues following a previously reported method with minor modifications (Sarrazin et al., 2001). Briefly, thin slices of trabecular bone were treated with 2.5 mg/ml of trypsin in phosphate-buffered saline (PBS) for 1 h at 37°C, washed extensively in PBS, and cut into fragments of approximately 2 mm^2. Fragments were seeded in 100-mm culture dishes containing 10 ml DMEM supplemented with 10% FBS, 50 U/ml of penicillin, 150 µg/ml of streptomycin, 2.5 µg/ml of amphotericin, 2.2 mg/ml of sodium bicarbonate, and 50 µg/ml of L-ascorbate. The plates were incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was replaced at 3-to 4-day intervals. After reaching confluence (4-5 weeks), cells were trypsinized (0.1%trypsin in PBS) and subcultured at a ratio of 20,000 cells/

cm^2 in 100-mm culture dishes. First-passage cells were grown to confluence, incubated 24 h in DMEM containing 0.2% FBS, and used in the experiments. These cells have been shown previously to exhibit phenotypic characteristics of osteoblasts such as response to parathyroid hormone (PTH) and production of alkaline phosphatase and osteocalcin.

II.3. Cell proliferation

Osteoblast proliferation was studied by measuring [^{3}H]thymidine (2 μCi/ml) incorporation into trichloroacetic acid (TCA)-insoluble material. Briefly, cells were plated at a density of 30,000 cells/well (15,000/cm^2) in 24-well plates and cultured for 24h in DMEM complete medium, in the presence of [^{3}H]thymidine. 24 h later, the plates were washed with cold PBS two times followed by washing with a 10% TCA cold solution. After fixation with 50μl of EtOH/Ether (3:1) they were left to dry at room temperature for at least 2 hours.

Incorporated [^{3}H]thymidine was released through washing with 600μl of 0.2N of NaOH after 30 minutes wait time followed by trituration. A 500μl collected volume for each well was supplemented with 50μl of acetic acid (glacial) and 5 ml of scintillation liquid. The radioactivity was measured using a β-scintillation counter. Results are expressed as counts per minute of triplicates.

II.4. Measurement of alkaline phosphatase activity

Alkaline phosphatase was used as a marker of osteoblast differentiation and was measured in disrupted cells using *p*-nitrophenyl phosphate as substrate. Briefly, cells were seeded at a density of 120,000 cells/well (60,000/cm^2) in 24-well plates left to adhere for 24h and then cultured in the presence of 10 nM of $1,25(OH)_2D_3$ as a positive control with varying treatments. The timeframe has been set to 7 days, stimulating the cells every second day. A baseline control for this alkaline phosphatase production consisted of cells cultured in the absence of vitamin D_3. After the incubation pe-

riod, cells were harvested in 0.4 M of Tris buffer and sonicated, and the alkaline phosphatase activity in the supernatant was measured spectrophotometrically at 405 nm. Values obtained were compared with a standard curve using *p*-nitrophenol as standard. Data were expressed as micro-moles of alkaline phosphatase activity per milligram of protein. Protein concentration in the supernatants was assessed using the BioRad method (BioRad, Mississauga, Ontario, Canada).

II.5. Calcium measurements

Calcium measurements have been performed in two different ways, by using the Von Kossa method and using a calcium assay kit, commercially available.

The cells were seeded at 200,000 cells/well (100,000/cm^2) in 24 wells plate and conditioned for 35 days, every second day.

The mineralising buffer medium used contains the complete culture medium supplemented with 5 mM β-glycerophosphate, 50μM L-ascorbate (vitamin C). The Dexamethasone was added as a positive control for mineralisation at 10^{-7}M.

Before analysis, the wells are washed and incubated for 24 hours at room temperature with 0.5ml solution HCl 0.6N.

For the Von Kossa method the cells have been washed with 1 ml PBS, fixed with 1 ml EtOH 100% and then left for 15 minutes. The EtOH residues were removed by washing the wells with distilled water several times.

In each well we added 3 ml of 5% silver nitrate solution (Sigma) end exposed the plate in bright light for 60 minutes. The wells have been washed 5 times with distilled water to remove the traces of silver nitrate and then left with 3 ml of sodium thiosulphate solution (5%) for 2 minutes. After washing again the wells with distilled water, they have been finally dehydrated and fixed with EtOH 95% for 2 minutes.

Pictures of each well were taken using a Nikon camera attached to the microscope and the following settings:

- blue filter;

- intensity: 3;

- smallest objective (Zeiss 2.5/0.08);

- camera balance-AUTO

- camera gain +12dB, FIX;

- camera detail: on/gamma: 1/D-SUB OUT: RGB.

The Ca^{2+} quantification has been also done using an analysis commercial kit - QuantiChrom™ colorimetric assay - designed to measure calcium directly in biological samples without any pretreatment. A phenolsulphonephthalein dye in the kit forms a very stable blue colored complex specifically with free calcium. The intensity of the colour measured at 612 nm, is directly proportional to the calcium concentration in the sample (mg/dl or 250 μM Ca^{2+}).

II.6. Data analysis

Data were analysed with Mann-Whitney two-tailed non-parametric test using Prism 4 software (GraphPad Software, San Diego, CA, USA) and $p < 0.05$ was considered significant. Mean ±S.E.M are represented on the graph. The numbers of donors (different cell lines) is marked as "N". The statistical significance in the graphs has been marked with a "*" sign for the statistical significance of $p< 0.05$, or "**" for $p< 0.01$. The "•" represents the condition they have been compared to.

III. Results

In this chapter we present the results of the experimental assays on the human osteoblastic cells. We have tried to assess the proliferation, differentiation and matrix mineralisation on primary cultures of hOB by treating human osteoblasts in culture with different concentrations of PGD_2 or the corresponding agonists/antagonist of PGD_2 receptors. In non-stimulated conditions, the hOB do not produce detectable quantities of PGs.

III.1. Proliferation of the cultured human osteoblastic cells.

The osteoblast proliferation was determined by incorporation of [^{3}H]thymidine. The [^{3}H]thymidine is incorporated only into newly forming DNA, thus being an indicator of the cell advancement through the osteoblast lineage (Aubin, 1998). It is known that mature osteoblasts and osteocytes do not divide, therefore the radiolabelled thymidine appears in the pre-osteoblasts (Timothy R. Arnett, 1998).

As described in the Methods chapter, we analysed the incorporation of [^{3}H]thymidine (2 μCi/ml) for lines of cells stimulated with different concentrations of PGD_2. We used the same method for the agonists/antagonists of the PGD_2 receptors.

III.1.1. Time-course proliferation assay

The first assay was in fact a time-course assessment of the incorporated radiolabelled thymidine at different timeframes.

When the cells have reached the confluence, they have been trypsinized, seeded in 24-well plates at 30000 cells/well and allowed to adhere for 24 hours before starting the stimulation. The cells have been stimulated with PGD_2, BW245C (DP specific receptor agonist) and DK-PGD_2 (CRTH2 specific agonist), respectively. The concentrations used were of 1 μM.

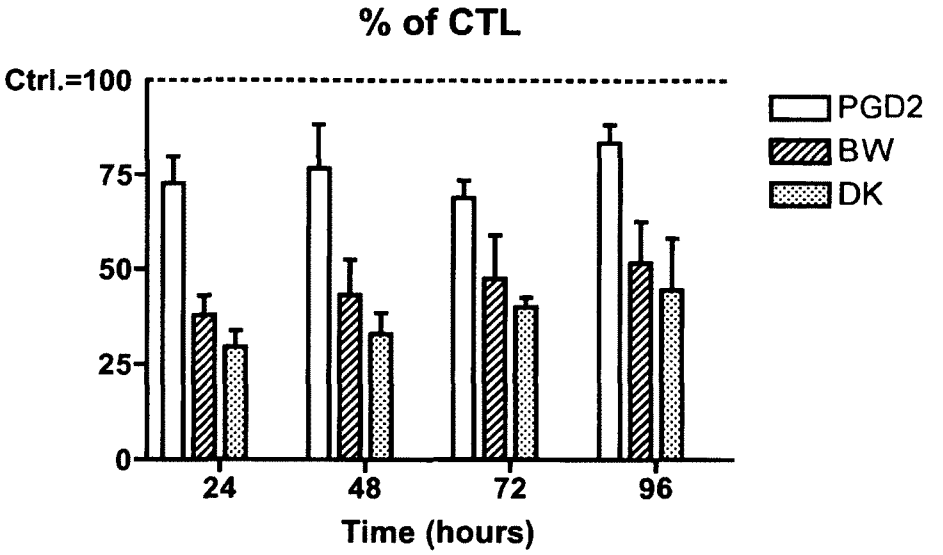

Fig.18: Effect of the PGD_2, BW245C (DP specific receptor agonist) and DK-PGD_2 (CRTH2 specific agonist), all at 10^{-6} M, on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml) - time course. The control (vehicle) considered as the baseline has been removed from graph, but is marked as 100%. The added PG's represented as percentage of control. The parameter measured: number of radioactive counts/well. N=3. Mean ±S.E.M. One-way ANOVA.

Three lines of hOB have been stimulated for different periods of time with afore mentioned compounds. No COX inhibitors have been added. The determinations were represented as a percentage compared to the control (Fig. 18).

Exogenous PGD_2 decreased the newly formed DNA at around 75%, compared to control. The effect was even more visible for the BW245C and DK-PGD_2. The have decreased the effect to 30% and 40%, respectively. However, no significant change has been detected for the same compound trough different timeframes using the one-way ANOVA, hence allowing us to establish the 24h timeframe for proliferation as suitable.

III.1.2. Overall effect of exogenous PGD_2 / agonists on proliferation

To assess the effects of the exogenous PGD-like compounds over the cultured hOB, we tested the effects of PGD_2, BW245C (DP specific receptor agonist) and DK-

PGD_2 (CRTH2 specific agonist) on the proliferation of the cells in a comparative test (Fig. 19).

The confluent cells have been detached with trypsin, seeded in 24-well plates at 30000 cells/well and allowed to adhere for 24 hours before starting the stimulation. Subsequently, the cells have been stimulated with PGD_2 (1 µM), BW245C (1 µM) and DK-PGD_2 (1 µM) in presence of Diclofenac, a nonspecific COX inhibitor (1 µM), and Naproxen (10 µM) alone for 24 hours. The "Normal" condition represents the non-stimulated wells, containing the solvent vehicles. The Naproxen has been added for all conditions to insure that the endogenous production of prostaglandins is being seized.

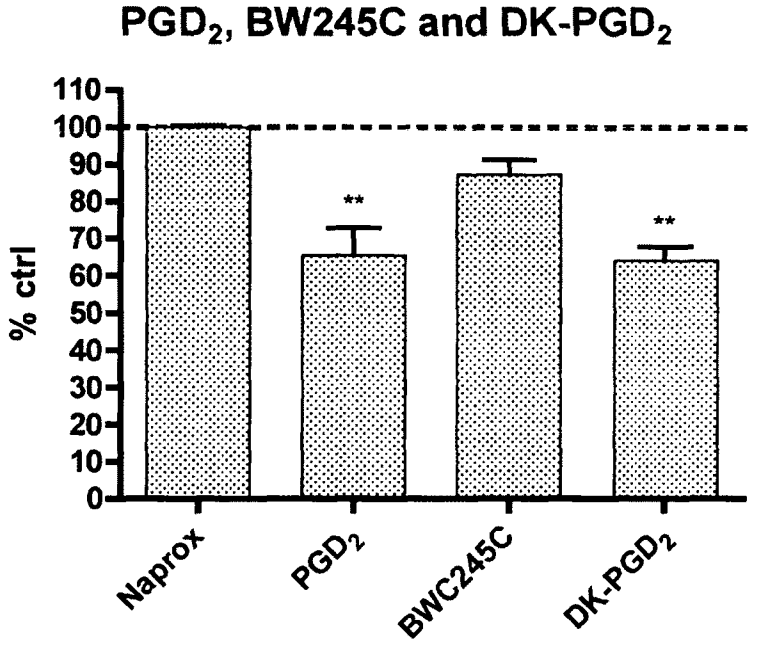

*Fig. 19: Effect of the PGD_2, BW245C (DP specific receptor agonist) and DK-PGD_2 (CRTH2 specific agonist) on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 µCi/ml) - in the presence of Naproxen. The Naproxen condition is considered as the baseline and marked as 100%. The added PG's represented as percentage of Naproxen. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test. ** $p < 0.01$.*

On a 24 stimulation in the presence on nonspecific COX inhibitors, we can observe a decrease of the measured parameter for proliferation for PGD_2 and DK-PGD_2 to 65% when compared to Naproxen (N=5. Mean ±S.E.M. Two-tailed non-

parametric test. ** p< 0.01). However, no effect is seen for the BW245C condition, although it decreased the measured effect to 90%.

III.1.2. The effect of different concentrations of exogenous PGD_2 on hOB proliferation

In the next series of experiments we tested the effect of PGD_2 at different concentrations on 6 different lines of hOB. We used two different nonspecific COX inhibitors to block the endogenous PG production: Naproxen was used at a concentration of 10 μM and Diclofenac at 1 μM, the latter having a negative impact on hOB proliferation at higher concentrations (Matziolis et al., 2002; Kaspar et al., 2005).

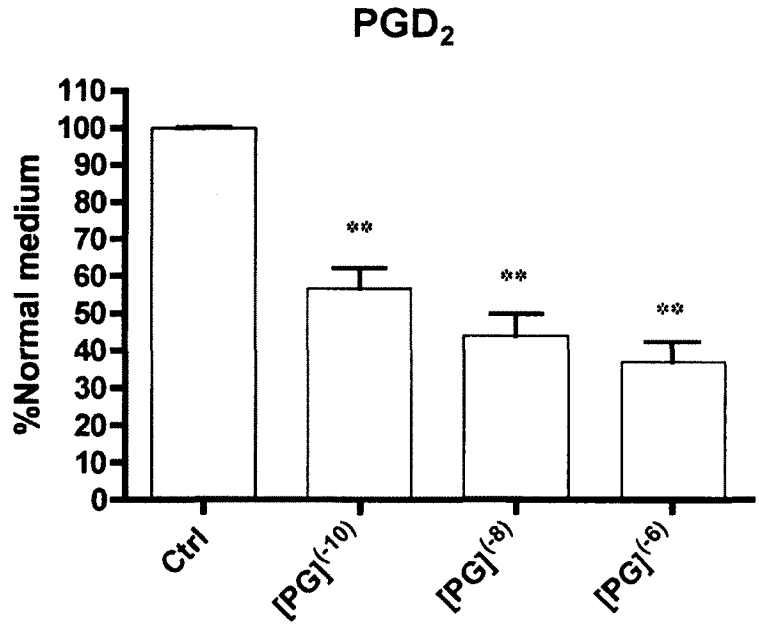

*Fig. 20: The effect of different concentrations of exogenous PGD_2 on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml). The control condition (Vehicle) is considered as the baseline and marked as 100%. The added PG's and NSAID represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=6. Mean ±S.E.M. Two-tailed non-parametric test. ** p< 0.01.*

In the first test (Fig. 20), we have added the PGD_2 at different concentrations, varying from 10^{-10} M to 10^{-6} M. We have not inhibited the local production of endoge-

nous prostaglandins with any of the mentioned COX blockers. We observed a significant change between the control and the conditioned wells. The added PGD_2 decreased significantly the newly formed DNA (N=6. Mean ±S.E.M. Two-tailed non-parametric test. ** $p< 0.01$). The PGD_2 at 10^{-10} decreased the effect at 55%, PGD_2 at 10^{-8} decreased the effect at 45% and PGD_2 at 10^{-6} decreased the effect at 35% compared to Control (vehicles).

In the second test (Fig. 21), we added Naproxen in all conditioned wells (10 μM) except for the control. The same protocol was used for the stimulation. The parameter measured was the number of radioactive counts/well after the 24 hour stimulation in presence of [^{3}H]thymidine (2 μCi/ml). The PGD_2 was used at different concentrations, varying from 10^{-10} M to 10^{-6} M.

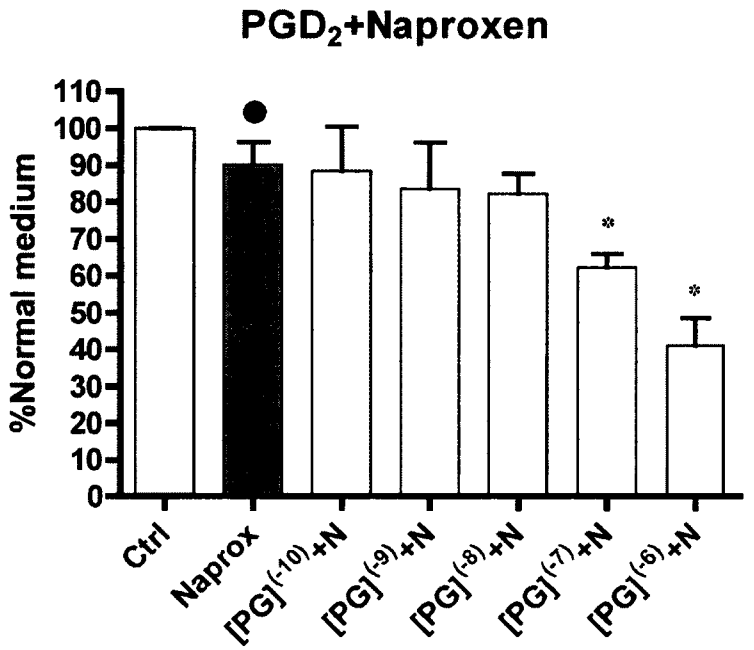

*Fig. 21: The effect of different concentrations of exogenous PGD_2 on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml) in the presence of Naproxen. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added PG's and NSAID represented as a percentage of control. The parameter measured: number of radioactive counts/well. Naproxen added to every condition, except control. N=6. Mean ±S.E.M. Two-tailed non-parametric test. * $p< 0.05$.*

Both the Naproxen and the PGD_2 at 10^{-10} M decreased the effect at 90% of Control (vehicles). The PGD_2 at 10^{-9} M and PGD_2 at 10^{-8} M decreased the effect at around 80% of Control. The PGD_2 at 10^{-7} decreased the effect at 60% and the PGD_2 at 10^{-6} at 40%. By comparing the Naproxen conditioned wells to PGD_2 stimulated wells, we observed a significant inhibiting effect on the hOB proliferation, but only for PGD_2 at 10^{-7}M and 10^{-6}M. Although not statistically significant, the PGD_2 at 10^{-9}M and 10^{-8}M could have also an effect suggesting a dose-response effect (N=6. Mean ±S.E.M. Two-tailed non-parametric test. * $p< 0.05$).

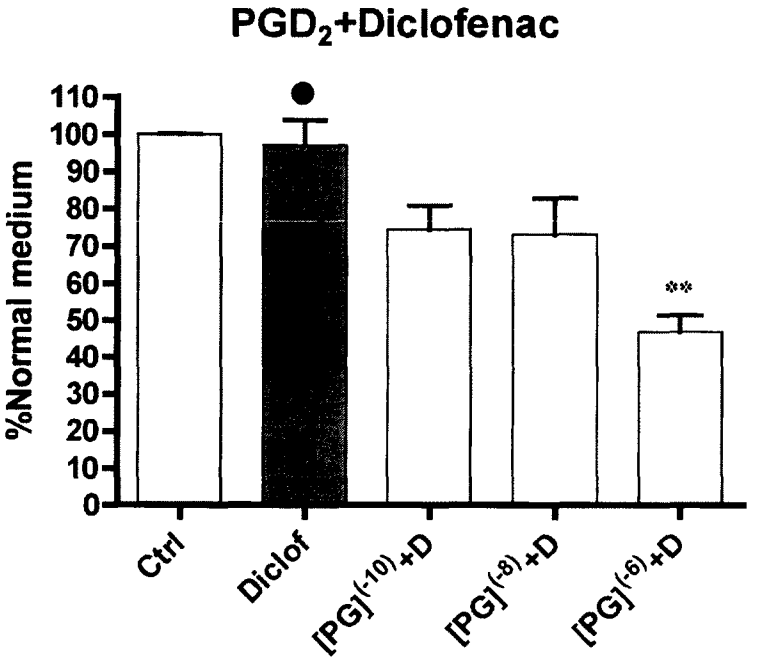

*Fig. 22: The effect of different concentrations of exogenous PGD_2 on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml) in the presence of Diclofenac. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added PG's and NSAID represented as a percentage of control. The parameter measured: number of radioactive counts/well. Diclofenac added to every condition, except control. N=6. Mean ±S.E.M. Two-tailed non-parametric test. ** $p< 0.01$.*

In the third test (Fig. 22) we inhibited the COX enzyme with Diclofenac at 1 μM added in every conditioned well, except the control. The PGD_2 was used at different concentrations, varying from 10^{-10} M to 10^{-6} M.

The Diclofenac alone decreased the measured effect at 95% of the Control (vehicles). The PGD_2 at 10^{-10} and the PGD_2 at 10^{-10} both yielded the same efect, decreasing the proliferation at around 73% of the Control. We saw a significant decrease of the synthesised DNA only for a concentration of 10^{-6}M PGD_2, although the trend might suggest a dose-response effect (N=6. Mean ±S.E.M. Two-tailed non-parametric test. ** $p< 0.01$).

III.1.3. The effect of different concentrations of BW245C (DP specific receptor agonist) on hOB proliferation

We performed a series of experiments using the BW245C (DP specific receptor agonist). The used protocol was the same as the one used for the PGD_2 series.

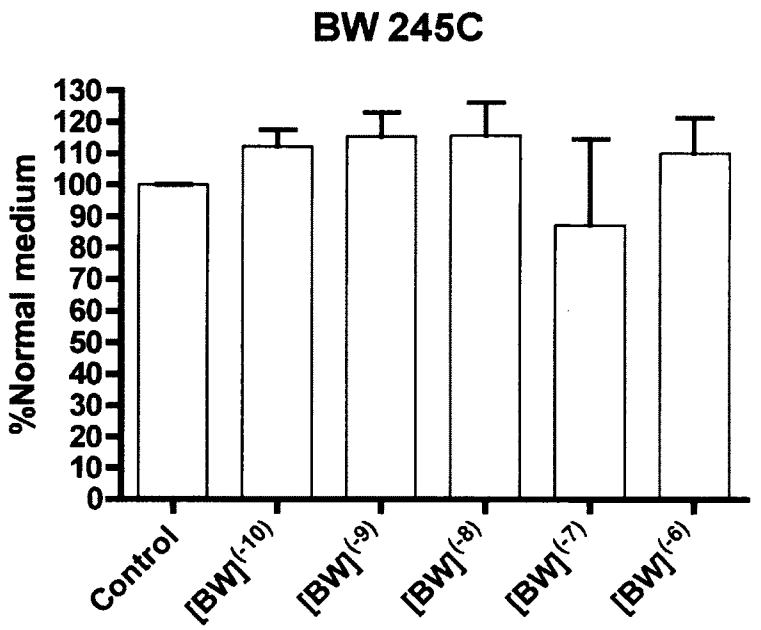

Fig. 23: Effect of the BW245C on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml) at different concentrations. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added BW245C is represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

In the first assay (Fig. 23), we have compared the stimulated conditions to the control. No NSAIDs have been used. We have used increasing concentrations of BW245C from 10^{-10}M up to 10^{-6}M.

We observed an increase of the positive effect over the proliferation (up to 25%) with the increasing concentrations of BW245C up to 10^{-8}M. For the wells conditioned with BW245C at 10^{-7}M, we observed a drop of 85% compared to the Control, but we think that the effect cannot be taken into consideration because of the wide errors. The last sample (BW245C at 10^{-6}M) seem to be continuing the trend. No statistically significant effect could be assessed (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

In the second test (Fig. 24), by using the same protocol as described, we added Naproxen to every conditioned well, simply to remove any endogenous PG's through the nonspecific inhibition of COX enzyme.

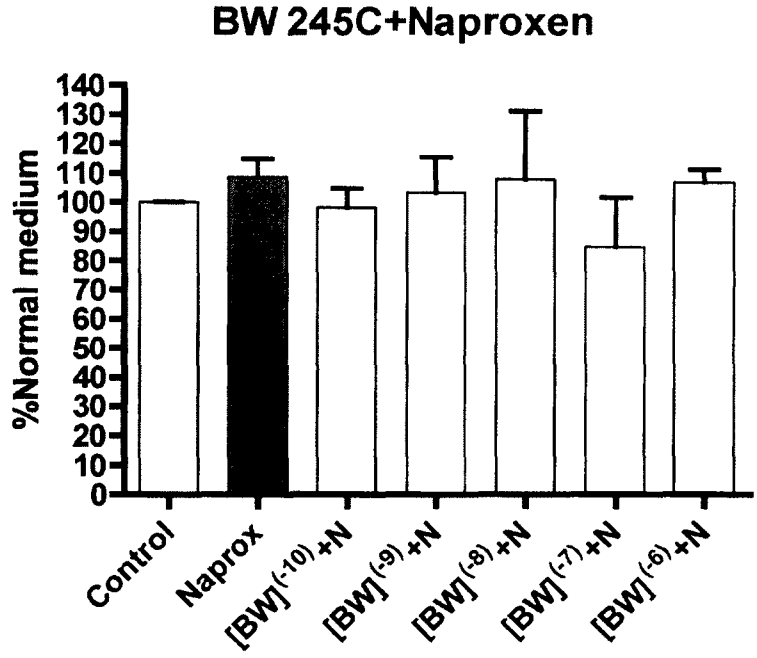

Fig. 24: Effect of the BW245C on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml) at different concentrations in presence of Naproxen. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added BW245C and the Naproxen are represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

The stimulated conditions have been compared to the Naproxen, this one being present in all the wells. Naproxen, BW245C at 10^{-8} and BW245C at 10^{-6} M gave an increase of the measured effect of 5-8% when compared to Control. BW245C at 10^{-10} and BW245C at 10^{-9} responses were comparable to the Control, while The BW245C at 10^{-7} M lowered the effect at around 85%.

Compared to Naproxen, there is no statistically significant correlation to BW245C at any concentration of BW245C (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

The third test was similar (Fig. 25), but we replaced the Naproxen with Diclofenac (nonspecific COX inhibitor). The Diclofenac has been added to all wells, except the control. Again, the Diclofenac was used at a concentration of 1 μM to avoid the deleterious effects. Diclofenac had a positive effect on proliferation of around 10%,

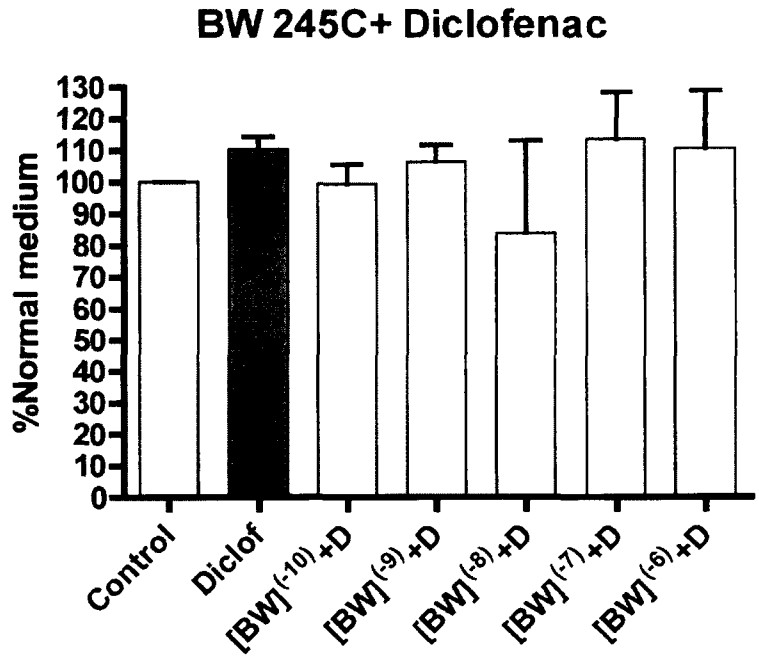

Fig. 25: Effect of the BW245C on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 μCi/ml) at different concentrations in presence of Diclofenac. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added BW245C and the Diclofenac are represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

when compared to Control (vehicles). BW245C at 10^{-10} M response was comparable to the Control. BW245C at 10^{-9} response was 5% higher than the Control, while BW245C at 10^{-8} decreased the effect at around 80%. The BW245C at 10^{-7} M increased the effect at over 10% compared to the Control and the BW245C at 10^{-6} at around 9%.

We have not seen an important effect or a significant variance comparing the Diclofenac to the stimulated conditions (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

III.1.4. The effect of different concentrations of DK-PGD_2 (CRTH2 specific agonist) on hOB proliferation

We performed a series of three tests similar to the former ones. The protocol used is the same as the one described. The parameter measured was the number of

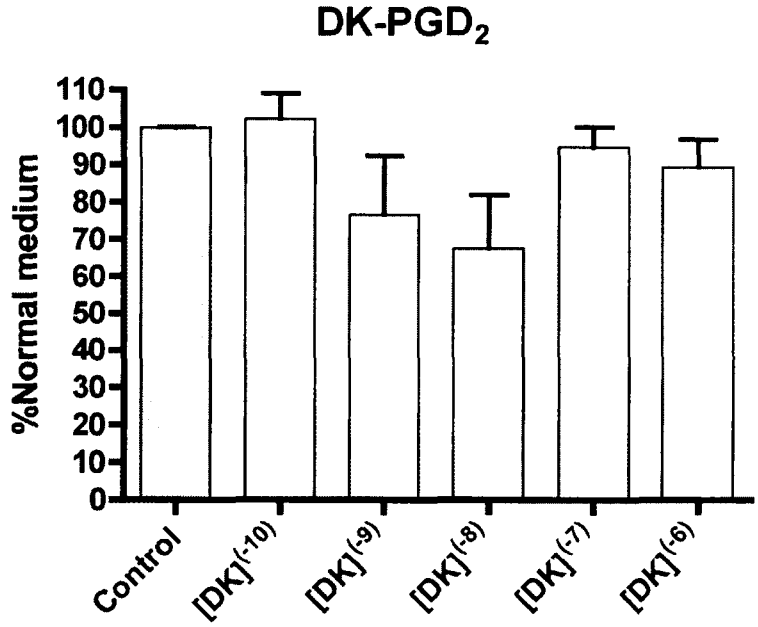

Fig. 26: Effect of different concentrations of DK-PGD_2 on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 µCi/ml). The control condition (Vehicle) is considered as the baseline and marked as 100%. The added DK-PGD_2 is represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

radioactive counts/well after the 24 hour stimulation in presence of [^{3}H]thymidine (2 µCi/ml). In the first assay (Fig. 26) we have compared the stimulated conditions to the control (vehicles) using the DK-PGD_2 in a range of concentrations from 10^{-10} M to 10^{-6} M. No NSAIDs have been used. While the DK-PGD_2 at 10^{-10} M had an effect comparable to the Control (vehicles), the DK-PGD_2 at 10^{-9} M decreased the effect at 75% of Control, and the DK-PGD_2 at 10^{-8} M lowered that effect at 65%. Surprisingly, DK-PGD_2 at 10^{-7} M and DK-PGD_2 at 10^{-6} M restored the effect at 95% and 90%, respectively.

One can observe a tendency of proliferation decrease towards the higher concentrations of DK-PGD_2, but this stops suddenly at 10^{-8}M - not a statistically relevant value (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

The second test (Fig. 27) of this series is performed using the Naproxen to block the endogenous PG's production (10 µM). We have compared the stimulated

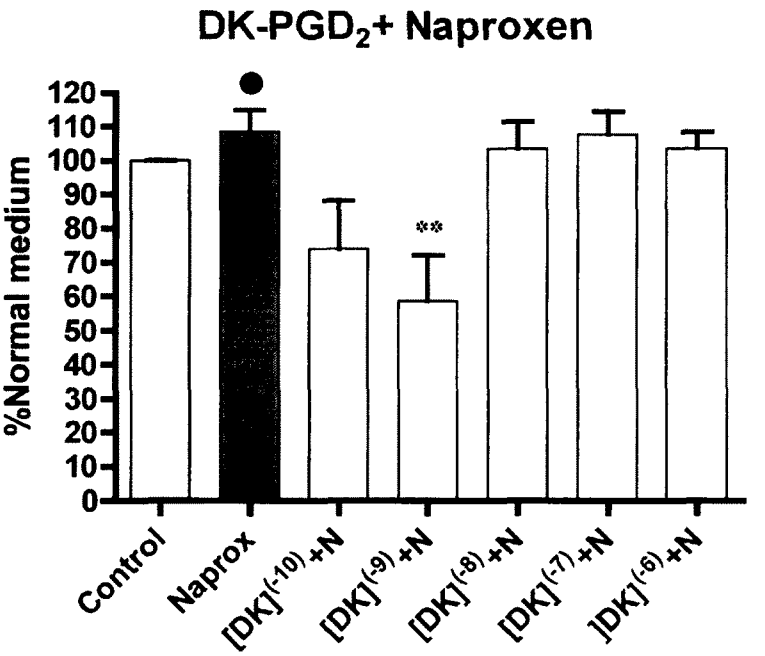

*Fig. 27: Effect of different concentrations of DK-PGD_2 on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 µCi/ml) in presence of Naproxen. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added DK-PGD_2 and the Naproxen are represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test. ** $p< 0.01$.*

conditions to Naproxen (present for every condition) using the DK-PGD_2 in a range of concentrations from 10^{-10} M to 10^{-6} M. The Naproxen increased the effect with around 7% when compared to the Control (vehicles). DK-PGD_2 at 10^{-10} M and at 10^{-9} M decreased the proliferation to 70% and 55%, respectively. DK-PGD_2 at 10^{-8} M, at 10^{-9} M and DK-PGD_2 at 10^{-6} yielded effects going over 100%.

We observed a dose-dependent effect which stops suddenly when the DK-PGD_2 reaches a concentration of 10^{-9}M, the only significant value. Surprisingly, for the following conditions the measured effect level is similar to that of Naproxen (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

The third assay (Fig. 28) using the DK-PGD_2 concentrations ranging from 10^{-10}M to 10^{-6} M has been performed using Diclofenac as an inhibitor of the local PG's production - added to all conditioned wells. Diclofenac alone had a positive effect on

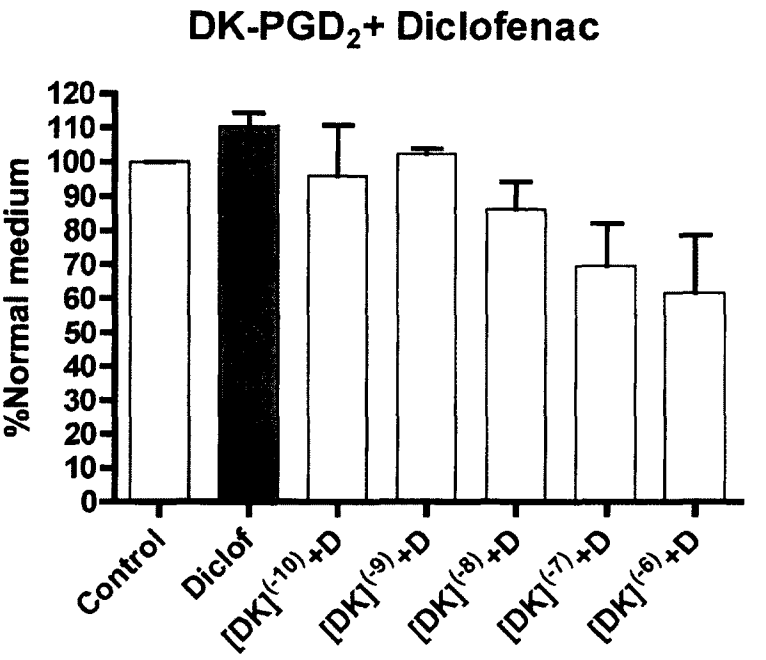

Fig. 28: Effect of different concentrations of DK-PGD_2 on human osteoblast cell proliferation determined by incorporation of [3H]thymidine (2 µCi/ml) in presence of Diclofenac. The control condition (Vehicle) is considered as the baseline and marked as 100%. The added DK-PGD_2 and the Diclofenac are represented as a percentage of control. The parameter measured: number of radioactive counts/well. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

the proliferation of 110% when compared to Control (vehicles). DK-PGD_2 at 10^{-10} M and at 10^{-9} M had an effect comparable to Control. DK-PGD_2 at 10^{-8} M lowered the effect to 85%. DK-PGD_2 at 10^{-7} M and at 10^{-6} M continued this trend, going to 70% and 60%, respectively.

No significant effect is seen, however the proliferation seems diminished compared to control, suggesting a dose-response effect for the increasing concentrations of DK-PGD_2 compared to the Diclofenac (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

III.2. Differentiation of the cultured human osteoblastic cells

Skeletal alkaline phosphatase (ALP) is an enzyme localised in the membrane of osteoblasts which is released into the circulation by an unclear mechanism. The enzyme is considered as a marker for the mature osteoblast and we must mention that along with its secretion, the mineralisation takes place. (Marc C. Hochberg, 2003)

The method used has been described in the "Methods" chapter. Briefly, the confluent cells have been detached with trypsin, seeded in 24-well plates at 120,000 cells/well and allowed to adhere for 24 hours before starting the stimulation. The positive control is the Vitamin D used at 10^{-9}M.

Alkaline phosphatase has been quantised as ALK/proteins[μmol/mg] for each conditioned well. The timeframe has beed set to 7 day, stimulating the cells every second day (Maciel et al., 1997; Morisset et al., 1998; da Rocha & de Brum-Fernandes, 2002).

III.2.1. Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts

In the first experiment (Fig. 29) we have used the PGD_2 at three different concentrations (10^{-10}M, 10^{-8}M and 10^{-6}M) in order to measure the effect on the two receptors, DP and CRTH2. The endogenous production of PG's has not been blocked with NSAID. Vitamin D stands as positive control.

The vitamin D alone yielded a 400% increase of differentiation compared to the Normal, non-stimulated condition. The PGD_2 at three different concentrations (10^{-10}M, 10^{-8}M and 10^{-6}M) had almost the same effect as the Normal condition, with just a few percents below the marked 100% line.

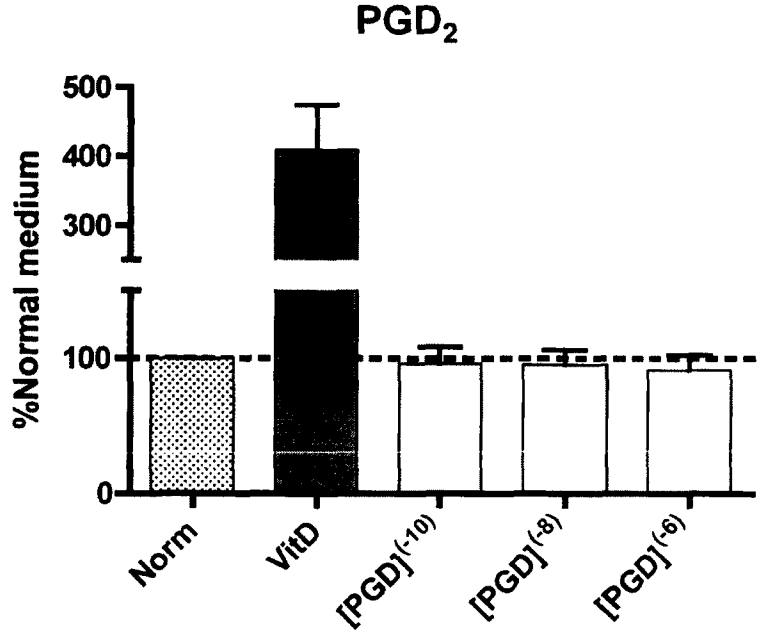

Fig. 29: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts. The control condition (Normal or Vehicle) is considered as the baseline and marked as 100%. The Vitamin D is represented as positive control. The parameter measured: µmol AlkP/mg protein. N=6. Mean ±S.E.M. Two-tailed non-parametric test.

No statistical significant variation has been observed (N=6. Mean ±S.E.M. Two-tailed non-parametric test).

The second assay (Fig. 30) reproduces the previous one, but using the Naproxen (10^{-5}M) as a COX inhibitor in all conditioned wells and the PGD_2 at three different concentrations (10^{-10}M, 10^{-8}M and 10^{-6}M). Vitamin D stands as positive control.

For this test, the Naproxen condition has been considered as 100%, this COX inhibitor being added to all stimulated wells. When comparing the Normal condition with the Naproxen, we can say that the latter impaired the differentiation.

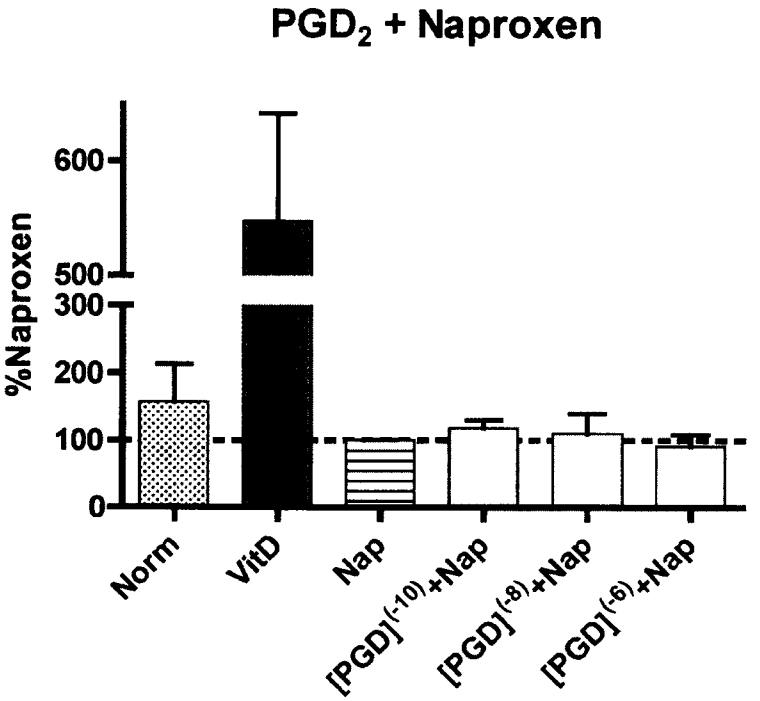

Fig. 30: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblast in presence of Naproxen. The Naproxen is considered as the baseline and marked as 100%. The Vitamin D is represented as positive control. The parameter measured: µmol AlkP/mg protein. Normal stands for Vehicle. N=6. Mean ±S.E.M. Two-tailed non-parametric test.

Vitamin D had a positive effect of 540%. The PGD_2 at 10^{-10}M yielded a positive effect of roughly 110% when compared to Naproxen. For the two other concentrations used - 10^{-8}M and 10^{-6}M - we consider that the effect is similar with the one given by Naproxen.

No correlation could be established between the dose of the added PG's and the production of AlkP when comparing the conditioned wells to the Naproxen which was present in all conditions (N=6. Mean ±S.E.M. Two-tailed non-parametric test).

The third assay is similar (Fig. 31), but we used Diclofenac as an endogenous PG inhibitor at 10^{-6}M for the conditioned wells and the PGD_2 at three different concentrations (10^{-10}M, 10^{-8}M and 10^{-6}M). Vitamin D stands as positive control and had a positive effect of around 250% compared to the Diclofenac standard. Diclofenac alone had a positive impact on differentiation, compared to the Normal (vehicles) condition.

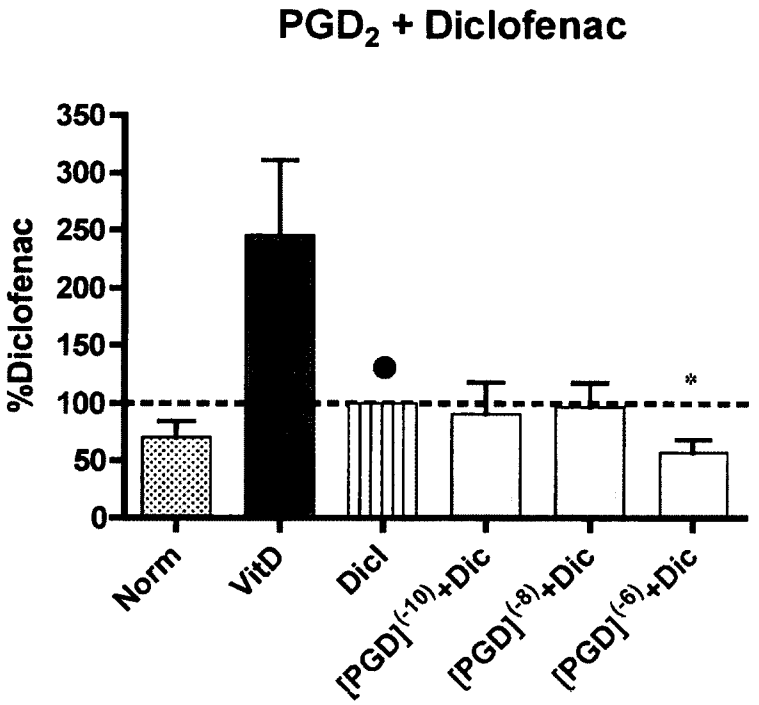

*Fig. 31: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblast in presence of Diclofenac. The Diclofenac is considered as the baseline and marked as 100%. The Vitamin D is represented as positive control. The parameter measured: µmol AlkP/mg protein. Normal stands for Vehicle. N=6. Mean ±S.E.M. Two-tailed non-parametric test. * p< 0.05.*

We did not see a significant modification for the production of AlkP, except for the PGD_2 at 10^{-6}M compared to the Diclofenac (N=6. Mean ±S.E.M. Two-tailed non-parametric test. * p< 0.05). The two other conditions - PGD_2 at 10^{-10}M and 10^{-8}M - had a response comparable to the one given by Diclofenac.

III.2.2. Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in presence of DP-receptor and CRTH2-receptor antagonists

In the next test (Fig. 32) we used a DP antagonist - BWA868C and a CRTH2 antagonist - CAY10471, both at 10^{-6} M (Ulven & Kostenis, 2005; Royer et al., 2007). The PGD_2 has been used at 10^{-10}M, 10^{-8}M, and 10^{-6}M, in competition with each one of

the afore mentioned antagonists. No COX blockers have been added; the vitamin D stands as a positive control. The vitamin D yielded a positive effect of 200%.

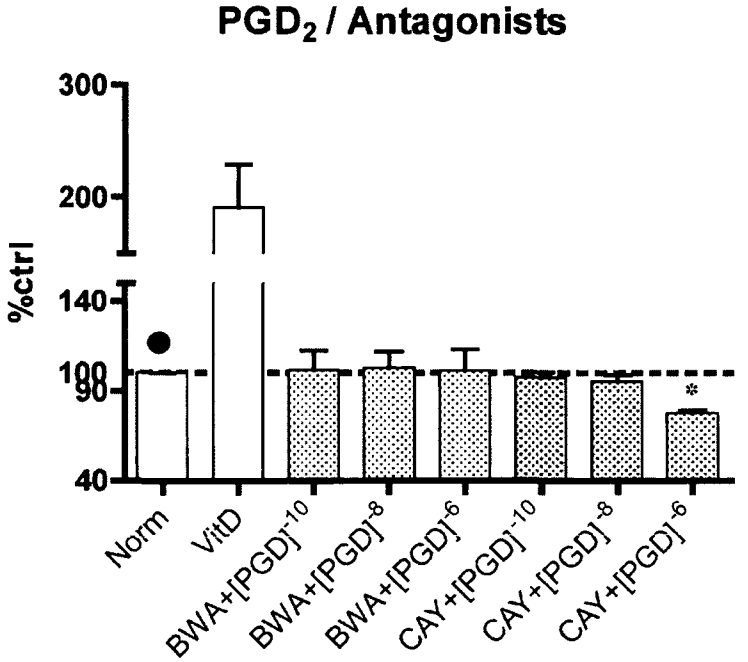

*Fig. 32: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in presence of DP and CRTH2 antagonists. The Normal (vehicles) is considered as the baseline and marked as 100%. The added PGD_2 and the antagonists ($10^{-6}M$) are represented as a percentage of control. The parameter measured: µmol AlkP/mg protein. N=5. Notation: BWA=DP antagonist; CAY=CRTH2 antagonist. Normal stands for Vehicle. Mean ±S.E.M. Two-tailed non-parametric test. * p< 0.05.*

Except for the CAY10471 (CRTH2 antagonist)/PGD_2 $10^{-6}M$ condition no significant effect is observed compared to the control. The CAY10471/PGD_2 $10^{-6}M$ is at 95% for the measured effect, this could suggest a possible decreasing effect with concentration (N=5. Mean ±S.E.M. Two-tailed non-parametric test. * p< 0.05).

III.2.3. Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in presence of T0070907, a selective antagonist of the human PPARγ nuclear receptor

The next assay (Fig. 33) was meant to provide some information over the action of the PGD_2 on the PPARγ receptor (Dussault & Forman, 2000b; Khan & Abu-Amer, 2003; Bell-Parikh et al., 2003; Lecka-Czernik & Suva, 2006).

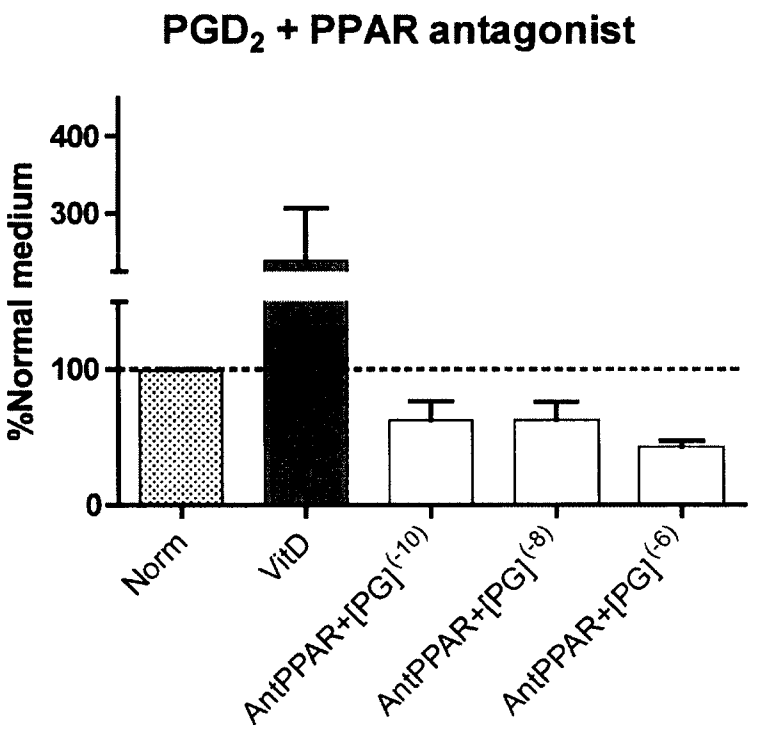

Fig. 33: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in presence of T0070907, a selective antagonist of the human PPARγ nuclear receptor. The Normal (Vehicle) is considered as the baseline and marked as 100%. The added PGD_2 and the PPARγ antagonist (10^{-6}M) are represented as a percentage of control (PPAR and the PGD_2 at molar concentration). The parameter measured: µmol AlkP/mg protein. N=4. Notation: AntPPAR = PPAR antagonist. Normal stands for Vehicle. Mean ±S.E.M. Two-tailed non-parametric test.

We have used the same protocol described for the determination of AlkP. The PPARγ receptor antagonist chosen was T0070907, a highly selective compound active on human PPARγ receptor (Lee et al., 2002) at 10^{-6}M. No NSAID's have been used. The PGD_2 has been used at 10^{-10}M, 10^{-8}M, and 10^{-6}M, alone and in conjunction with T0070907. The vitamin D has been added as a positive control and yielded a positive effect of over 200% compared to the Normal (vehicle condition). The PGD_2 at 10^{-10}M/ PPAR Ant and PGD_2 at 10^{-8}M/PPAR Ant decreased the measured effect to around 60%, while PGD_2 at 10^{-6}M/PPAR Ant went to 40% when compared to Normal condition.

The combination PGD_2/PPARγ antagonist decreased the alkaline phosphatase production, but without statistical significance (N=4. Mean ±S.E.M. Two-tailed non-parametric test). No COX blockers have been added; the vitamin D stands as a positive control.

III.2.4. Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in the presence of Vitamin D

In order to assess the influence of vitamin D on the AlkP production, we have performed a series of tests by adding vitamin D (10^{-9} M) in every conditioned well.

The first assay (Fig. 34) have used the PGD_2 at three different concentrations: 10^{-10}M, 10^{-8}M and 10^{-6}M. No COX inhibitor has been added.

Vitamin D had more than a double the effect of the non-stimulated condition (Normal) and being present in all conditions has been chosen as reference. The PGD_2 at 10^{-10}M had almost the same effect as the vitamin D condition. PGD_2 at 10^{-8}M decreased the measured effect to 80% and PGD_2 at 10^{-6}M decreased the effect at 65% when compared to the vitamin D reference.

We observed a dose-response trend line comparing the VitD control with the increasing concentrations of PGD_2, although not statistically proven for the entire series (N=5. Mean ±S.E.M. Two-tailed non-parametric test. * $p< 0.05$; ** $p< 0.01$).

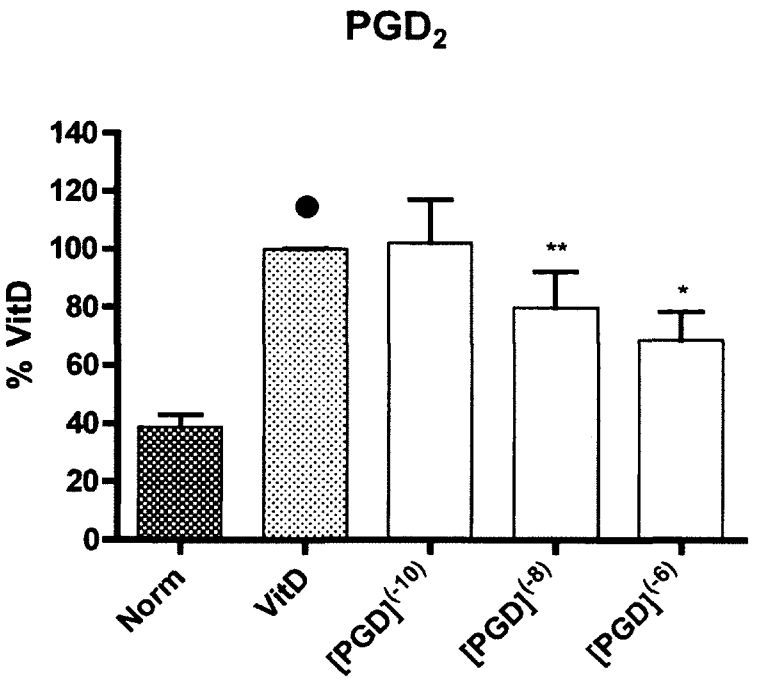

*Fig. 34: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in the presence of VitD. The vitamin D is considered as the baseline and marked as 100%. The added PGD_2 are represented as a percentage of control. The parameter measured: µmol AlkP/mg protein. Normal stands for Vehicle. N=5. Mean ±S.E.M. Two-tailed non-parametric test. * $p< 0.05$; ** $p< 0.01$.*

In the second experiment of these series (Fig. 35), we used Naproxen (10^{-5}M) to block the local PG production and PGD_2 at three different concentrations: 10^{-10}M, 10^{-8}M and 10^{-6}M. Vitamin D is present (10^{-9} M) in every conditioned well.

Vitamin D had more than a double the effect of the non-stimulated condition (Normal) and being present in all conditions has been chosen as reference. Naproxen yielded an increased effect of 110% compared to the vitamin D reference. Naproxen is present in all the samples containing PGD_2. The PGD_2 at 10^{-10}M/Naproxen and PGD_2 at 10^{-8}M/Naproxen had an 85% effect when compared to vitamin D standard. The PGD_2 at 10^{-6}M/Naproxen had the same effect as the vitamin D alone.

The PGD_2 conditions did not show a dose-response trend-line compared to the VitD control (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

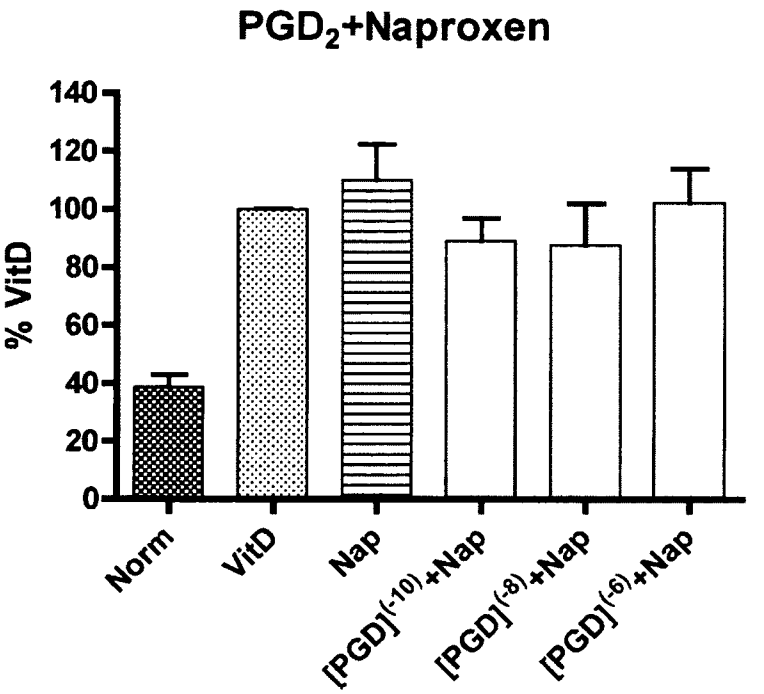

Fig. 35: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in the presence of VitD and Naproxen. The vitamin D is considered as the baseline and marked as 100%. The added PGD_2 and the Naproxen (10^{-5}M) are represented as a percentage of control. The parameter measured: µmol AlkP/mg protein. Normal stands for Vehicle. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

In the third experiment of these series (Fig. 36), we used Diclofenac (10^{-6}M) to block the local PG production and PGD_2 at three different concentrations: 10^{-10}M, 10^{-8}M and 10^{-6}M. Vitamin D is present (10^{-9} M) in every conditioned well. The Diclofenac is present in all the samples containing PGD_2.

Vitamin D had more than a double the effect of the non-stimulated condition (Normal) and being present in all conditions has been chosen as reference. PGD_2 at 10^{-10}M, 10^{-8}M and 10^{-6}M yielded decreased effects when compared to vitamin D (standard) of 90%, 80% and 70%, in this order.

No significant effect is seen for the any of the conditions, although the decrease of the measured parameter with the increasing concentrations of PGD_2 could suggest a dose-response (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

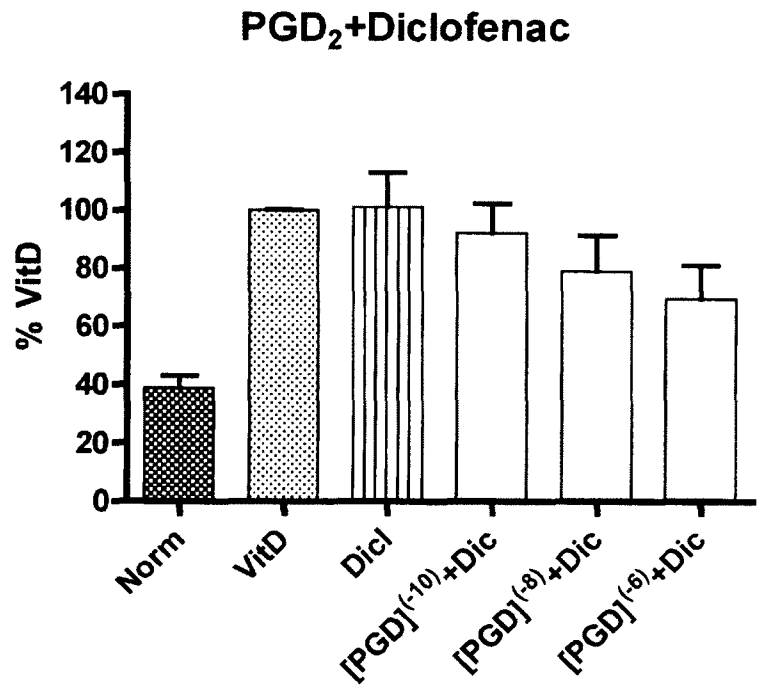

Fig. 36: Effect of different concentrations of exogenous PGD_2 on the differentiation of human osteoblasts in the presence of VitD and Diclofenac. The vitamin D is considered as the baseline and marked as 100%. The added PGD_2 and the Diclofenac ($10^{-6}M$) are represented as a percentage of control. The parameter measured: µmol AlkP/mg protein. Normal stands for Vehicle. N=5. Mean ±S.E.M. Two-tailed non-parametric test.

III.3. Matrix mineralisation (calcification) from the cultured human osteoblastic cells

The mature osteoblast shows all the functions necessary in bone synthesis. The mineralisations is the following step after the organic matrix has been created. The active osteoblasts surround themselves by this matrix and this is the point where the crystals of hydroxyapatite begin to form.

To measure the hOB capacity of generating mineral bone tissue, we determined the secreted Ca^{2+} in different conditions after a period of 35 days. Briefly, cells are seeded at the ratio of 200,000 cells/well in 24 well plates and cultured in media containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. Dexamethasone has been used as a positive control.(Cheng et al., 1996; Mikami et al., 2007). The cells are washed and incubated 24 h at room temperature with 0.5 ml 0.6 N HCl.

The Ca^{2+} quantification has been also done using a colorimetric assay designed to measure calcium directly in biological samples without any pretreatment. A phenolsulphonephthalein dye in the kit forms a very stable blue colored complex specifically with free calcium. The intensity of the color measured at 612 nm, is directly proportional to the calcium concentration in the sample (mg/dl or 250 μM Ca^{2+}).

III.3.1. Effect of different concentrations of exogenous PGD_2 on the calcification of human osteoblasts. Silver staining von Kossa method.

The cells have been stimulated with different concentrations of PGD_2 (10^{-10}M to 10^{-6}M) in presence of Naproxen. After 35 days in stimulated conditions, an adapted von Kossa method has been used (Fig. 37).

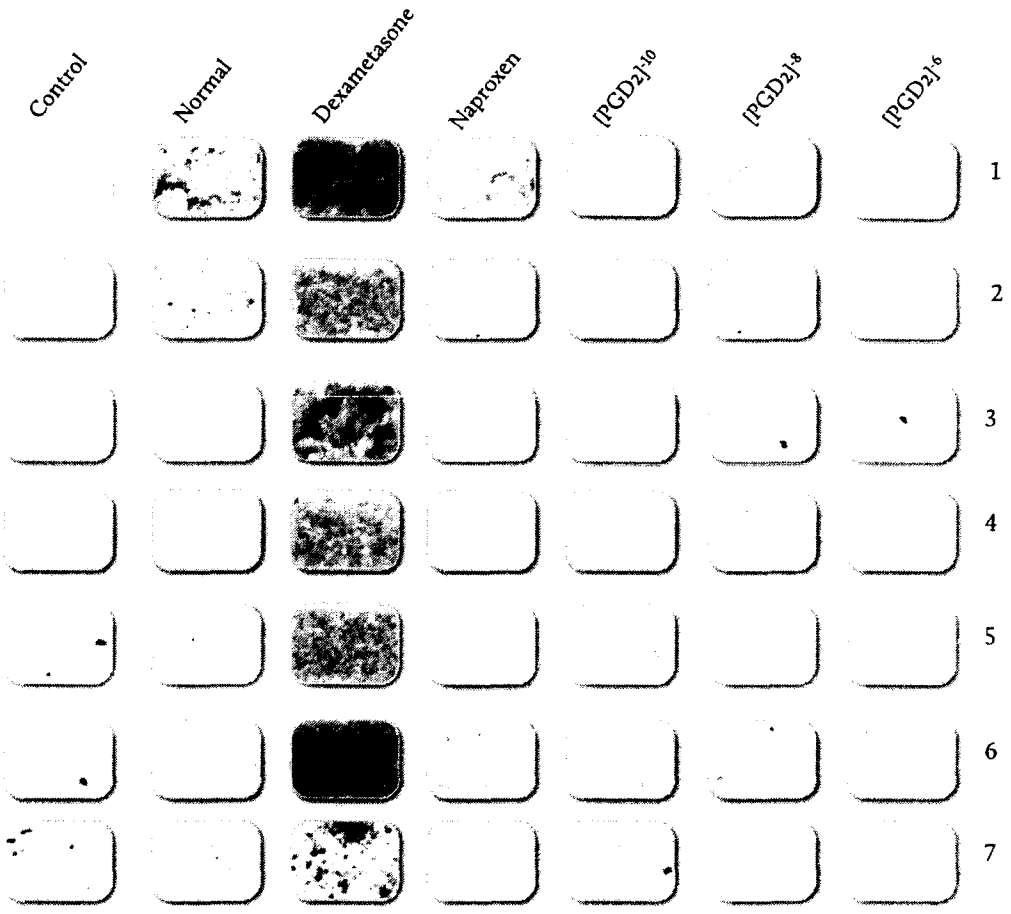

Fig. 37: Effect of different concentrations of PGD_2 on human osteoblast cell calcification in presence of Naproxen. Dexamethasone used as positive control at 10^{-7}M. Vitamin C added at in all samples except Control (10^{-4}M). Naproxen added to all PG samples at 10^{-5}M. β-glycerophosphate added in all wells except Control. N=7. Normal stands for Vehicle.

A certain effect for the wells containing PGD_2 is sometimes noticeable. However, there is no dose-response relationship as we increased PGD_2 concentration further away. A slightly positive effect on calcification can be seen for the Naproxen conditions for a few cell lines.

III.3.2. Effect of exogenous PGD_2 in presence of DP-receptor and CRTH2-receptor antagonists on the calcification of human osteoblasts. Silver staining von Kossa method.

In this assay we used the DP antagonist - BWA868C (10^{-10} M to 10^{-6} M) and the CRTH2 antagonist - CAY10471 (10^{-10} M to 10^{-6} M) in competition with PGD_2 used at 10^{-9}M. The only noticeable effect for calcification was seen for BWA868C at 10^{-6} M and the CAY10471 at 10^{-6} M (Fig. 38).

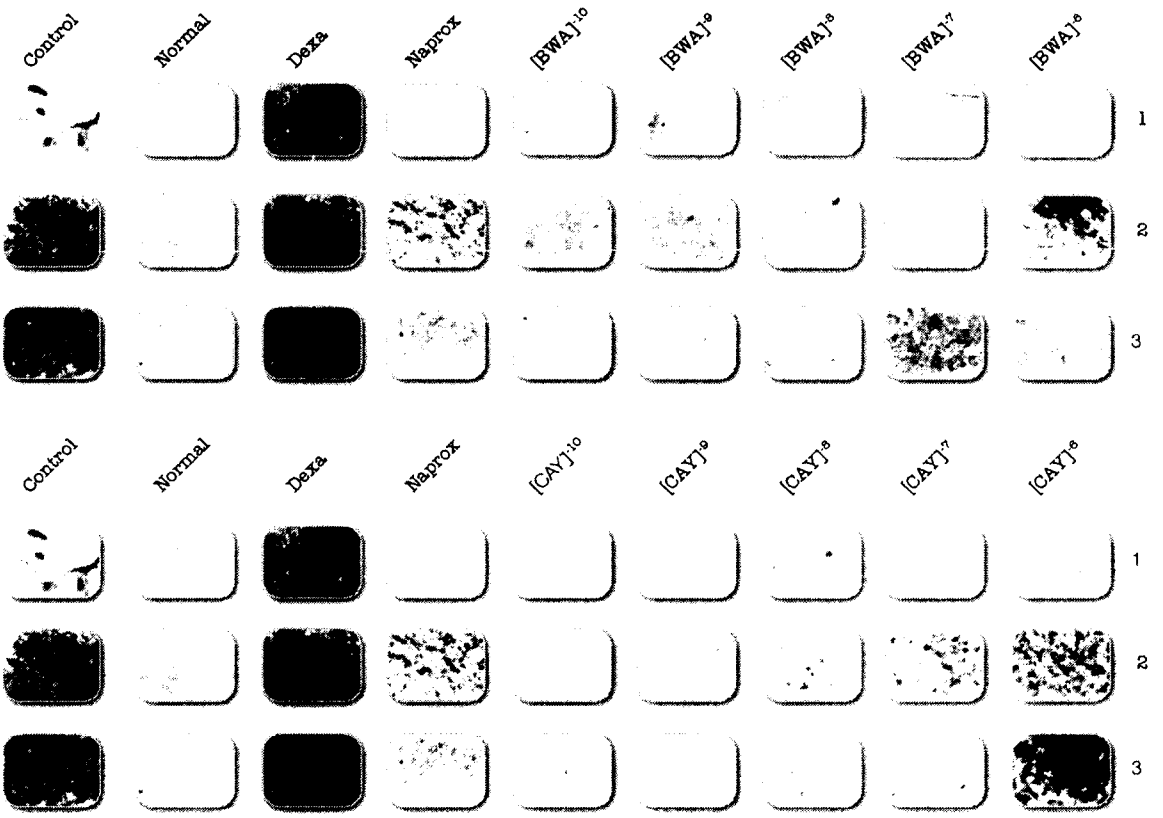

Fig. 38: Effect of DP/CRTH2 receptor antagonists (in the presence of PGD_2 10^{-9}M) on human osteoblast calcification in presence of Naproxen. Dexamethasone used as positive control at 10^{-7}M. Vitamin C added at in all samples except Control (10^{-4}M). Naproxen added to all agonist samples at 10^{-5}M. β-glycerophosphate added in all wells except Control. N=3. Notation: BWA/CAY = BWA868C/ CAY10471. *Normal stands for Vehicle*

III.3.3. Effect of different concentrations of exogenous PGD_2 on the calcification of human osteoblasts. QuantiChrom™ colorimetric assay.

Considering the von Kossa method less accurate, this time the Ca^{2+} quantification has been done using an analysis commercial kit.

For this test (Fig. 39) we used three different concentrations of PGD_2: 10^{-10}M, 10^{-8}M and 10^{-6}M. The Naproxen is present in all conditioned wells and has been chosen as reference. Dexamethasone was used as positive control at 10^{-7}M. Vitamin C was added in all samples (10^{-4}M).

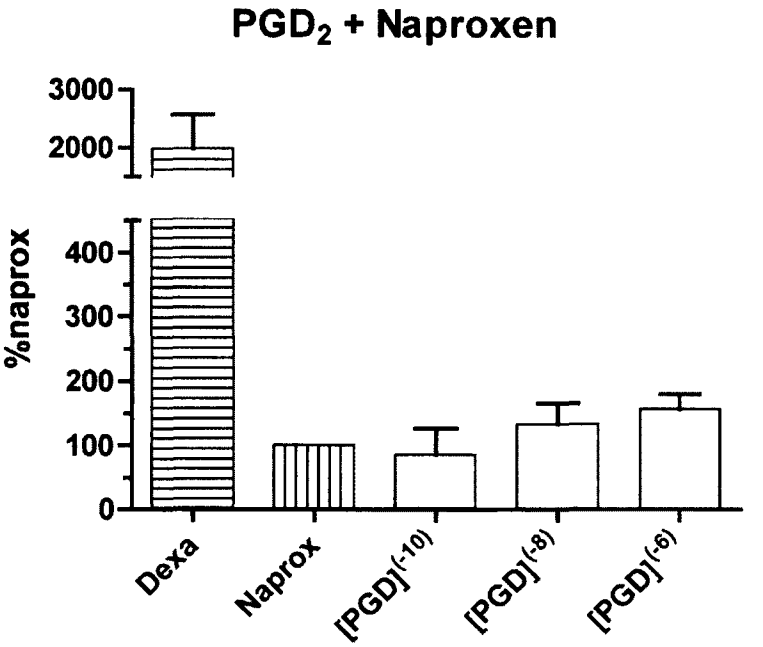

Fig. 39: Effect of different concentrations of PGD_2 on human osteoblast calcification in presence of Naproxen - colorimetric. Dexamethasone used as positive control at 10^{-7}M. Vitamin C added at in all samples (10^{-4}M). Naproxen added to all PG samples (at 10^{-5}M) is represented as 100%. β-glycerophosphate added in all wells. Parameter measured Ca^{2+} concentration (mg/dl). N=6. Mean ±S.E.M. Two-tailed non-parametric test.

The results obtained using this analysis are more accurate. The Dexamethasone alone had a 2000 times the effect of the Naproxen standard. The PGD_2 at 10^{-10}M showed a decreased effect of 80%. The PGD_2 at 10^{-8}M and PGD_2 at 10^{-6}M showed an increased effect of 130% and 150%, respectively.

Although one could suspect a dose-response effect when comparing the PGD_2 samples against Naproxen, no statistically relevant response is seen (N=6. Mean ±S.E.M. Two-tailed non-parametric test).

III.3.4. Effect of exogenous PGD_2 in presence of DP-receptor and CRTH2-receptor antagonists on the calcification of human osteoblasts. QuantiChrom™ colorimetric assay.

In Fig. 40 we show the results of a competition test with PGD_2 at 10^{-9}M and two antagonists, for the DP and CRTH2 receptor, respectively. The antagonists have been used at different concentrations: 10^{-10}M, 10^{-8}M and 10^{-6}M. Dexamethasone was used as positive control at 10^{-7}M. Vitamin C was added in all samples (10^{-4}M).

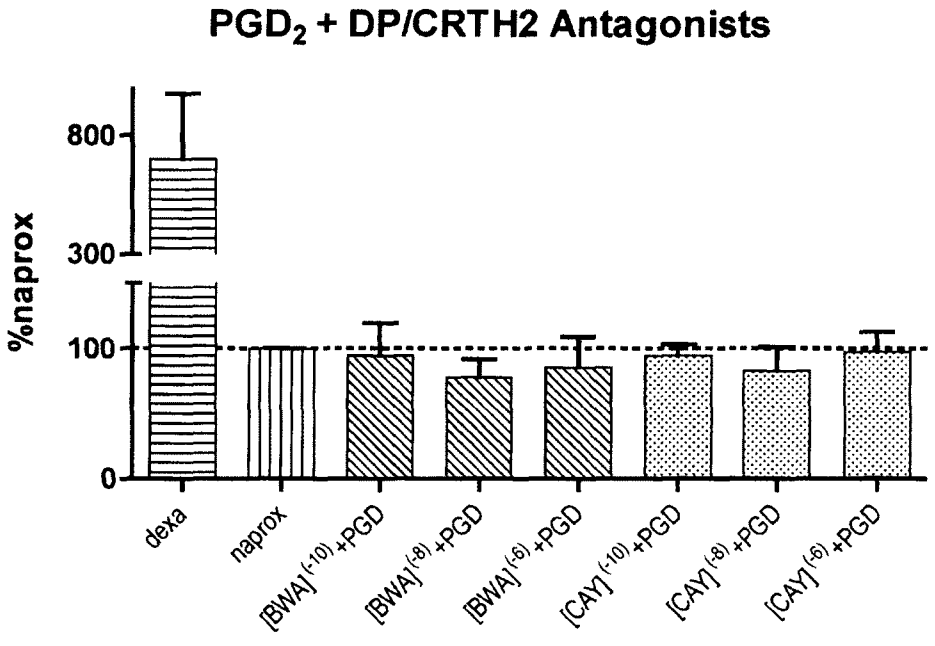

Fig. 40: Effect of DP/CRTH2 antagonists on human osteoblast calcification in presence of PGD_2 (10^{-9}M). Dexamethasone used as positive control at 10^{-7}M. Vitamin C added at in all samples (10^{-4}M). Naproxen added to all PG samples at 10^{-5}M is represented as 100%. β-glycerophosphate added in all wells. Parameter measured Ca^{2+} concentration (mg/dl). N=5. Notation: BWA/CAY = BWA868C/CAY10471. Mean ±S.E.M. Two-tailed non-parametric test.

Dexamethasone had a positive effect of 700% roughly when compared to the Naproxen standard. The BWA868C at 10^{-10}M/Naproxen yielded the same effect as Naproxen alone. The BWA868C at 10^{-8}M/Naproxen decreased the effect to around 80%, and the BWA868C at 10^{-6}M/Naproxen decreased the effect at around 85% compared to the Naproxen reference.

The CAY10471 at 10^{-10}M/Naproxen and the CAY10471 at 10^{-6}M/Naproxen had almost the same effect as Naproxen alone, while CAY10471 at 10^{-8}M/Naproxen showed a decreased effect of around 80%.

There is no significant change between the control (Naproxen) and the stimulated conditions for either of the two antagonists used (N=5. Mean ±S.E.M. Two-tailed non-parametric test).

III.3.5. Effect of different concentrations of exogenous PGD_2 on the calcium production of human osteoblasts in presence of T0070907, a selective antagonist of the human PPARγ nuclear receptor. Quanti-Chrom™ colorimetric assay.

The PPARγ receptor antagonist used was T0070907, a highly specific antagonist of the human PPARγ receptor at a concentration of 10^{-6}M with PGD_2 at 10^{-10}M, 10^{-8}M, and 10^{-6}M. Naproxen at 10^{-5}M has been added in all conditioned wells (Fig. 41).

Dexamethasone used as positive control at 10^{-7}M. Vitamin C added at in all samples (10^{-4}M).

Dexamethasone, when compared to the Naproxen standard, yielded a 200% effect.

The PGD_2 at 10^{-10}M/T0070907 condition showed only a slight increase in the measured effect when compared to Naproxen standard. The PGD_2 at 10^{-8}M/T0070907 lowered the measured effect to about 50%, while PGD_2 at 10^{-6}M/T0070907 decreased it even more, to around 30%.

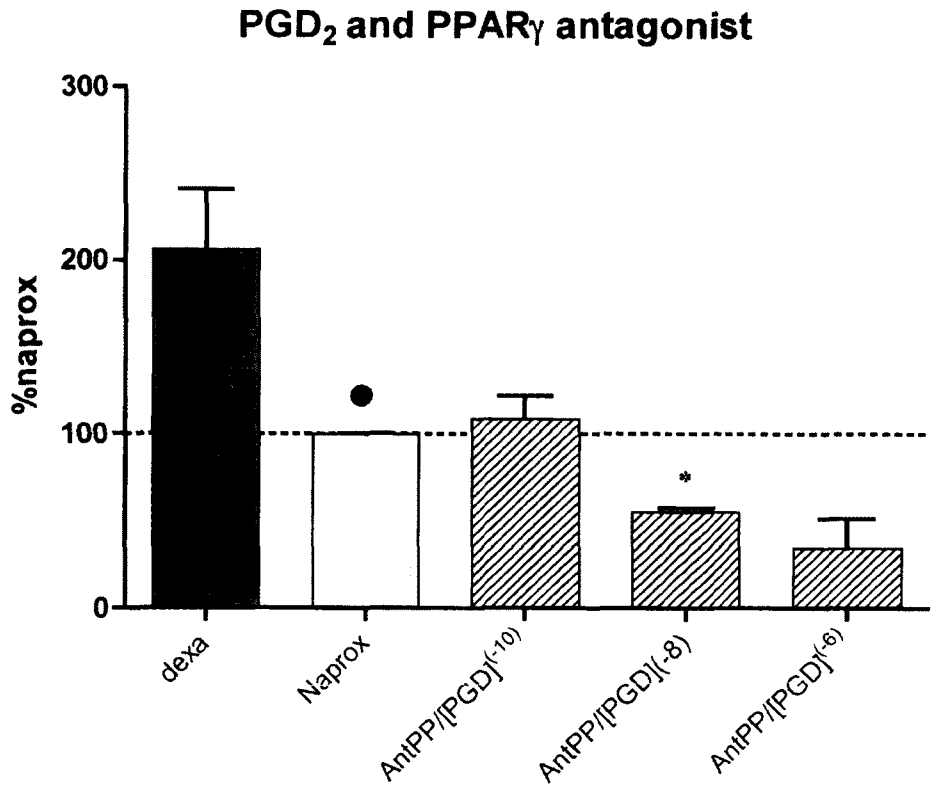

*Fig. 41: Effect of PGD_2 in conjunction with T0070907, PPARγ antagonist on human osteoblast calcification . Dexamethasone used as positive control at $10^{-7}M$. Vitamin C added at in all samples($10^{-4}M$). Naproxen added to all samples (except Dexa) at $10^{-5}M$ is represented as 100%. β-glycerophosphate added in all wells. Parameter measured Ca^{2+} concentration (mg/dl). N=4. Notation: AntPP= PPAR antagonist. Mean ±S.E.M. Two-tailed non-parametric test. * p< 0.05.*

We observe significant changes for the PGD_2 at $10^{-8}M$. The treatment seems to diminish the calcification, even if the last value AntPP/$[PGD_2]^{-6}$ is not accounted as significant (N=4. Mean ±S.E.M. Two-tailed non-parametric test. * p< 0.05).

IV. Discussion

Prostaglandins (PGs) are active biologic substances that are involved in a wide range of physiologic processes; when their production is out of balance, they are factors in the pathogenesis of illness. Modulation of PGs by inhibition or stimulation is promising for the management of various conditions.

Prostaglandin synthesis is up-regulated in virtually all forms of inflammation including RA. They are produced on demand and are not stored in cells, acting locally. The key regulatory step in the production of prostaglandins is the conversion of membrane phospholipids to arachidonic acid by the phospholipase A2 (PLA2) family of enzymes in response to a variety of stimuli, including pro-inflammatory cytokines, lipopolysaccharide, oxidised low-density lipoproteins, and small peptide growth factors.

Red meat and certain vegetable oils (corn, sunflower, and safflower) contain omega 6 fatty acids, which are transformed into arachidonic acid, the precursor for prostaglandins and leukotrienes. Reducing the amount of omega 6 while substituting omega 3 oils may help reduce pain and inflammation (Maggio et al., 2009). Omega 3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), fatty acids that compete with omega 6 fatty acids to form arachidonic acid. In fact, diets high in omega 3 fatty acids appear to have a protective effect against rheumatoid arthritis onset (Watkins et al., 2003).

The biologic effects of prostaglandins are numerous. Their role in regulating inflammatory responses includes the induction of fever, pain, swelling, and the regulation of leukocyte function. Fever is induced, in part, by local production of PGE_2 in the brain, through interaction with neurones in the hypothalamus. (Ushikubi et al., 1998) Prostaglandins do not directly stimulate pain responses, but they contribute to pain responses by inducing a state of hyperalgesia. PGE_2 and PGI_2 cause sensitisation of peripheral nerve terminals and modulate pain processing at a central level in the spinal cord (Wise, 2006; Burian & Geisslinger, 2005)

In addition to the classical prostanoid receptors, new evidence is emerging that suggests nuclear receptors for eicosanoids (Negishi & Katoh, 2002). The candidate receptors identified to date are classified as peroxisome proliferator-activated receptors (PPARs) (Giaginis et al., 2007). These nuclear receptors are transcription factors that regulate gene expression of enzymes associated with lipid homeostasis. PPARs complex with the nuclear receptor for 9-cis-retinoic acid (RXR) and bind a specific DNA motif termed the PPAR-response element (PPRE). Several eicosanoids, including PGJ_2 and LTB_4, bind to PPARs and affect transcription. PPREs are present in genes involved in the oxidation pathway for metabolism of fatty acids. At least one potentially important mechanism underlying PPAR-mediated eicosanoid action is auto-regulation of eicosanoid levels through increased synthesis of enzyme-mediating degradation. In addition, PPAR agonists, including PGJ_2, suppress cytokine production (Xu and Drew, 2007).

Non-steroidal anti-inflammatory drugs have been shown to delay fracture healing in both clinical series (Giannoudis et al., 2000) and animal models (Altman et al., 1995). The mechanism of action is thought to be related to inhibition of prostaglandins resulting in delayed ossification by diminishing regional blood flow or hindering primitive osteoblasts. More recently, selective COX-2 non-steroidal anti-inflammatory drugs have been shown to impair fracture healing (Seidenberg & An, 2004; Vuolteenaho et al., 2008).

IV.1. PGD_2

PGD_2 is implicated in the control of osteoblast function and bone anabolism. In human primary osteoblasts, PGD_2 activates the Gs-coupled PGD_2 receptor (DP) and decreases osteoprotegerin production (Gallant et al., 2005). In the same cells, PGD_2 decreases RANKL production upon binding to another PGD_2 receptor, chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2), which couples to Gi/o. Thus, PGD_2 appears to have both bone-resorptive and -forming activities through two different receptors DP and CRTH2. The latter activity may be responsible for the anabolic effects of PGD_2 on bone in vivo.

Two inflammatory cytokines (TNF-α and IL-1) and three regulators of bone formation (PTH, VEGF, and insulin-like growth factor-1) strongly stimulate the production of PGD_2 in human primary osteoblasts. Mechanical loading by strain application increases the mRNA expression of PGD synthase and PPARγ-1 in MC3T3-E1 cells(Siddhivarn et al., 2006). The mechanical loading increases the production of PGD_2, and its metabolite Δ^{12} PGJ_2, the natural ligand for PPARγ-1 (Siddhivarn et al., 2007). In addition, stretching of osteoblasts induces bone nodule formation and the activation of PGD synthase. Thus, the Δ^{12} PGJ_2 -PPARγ-1 pathway may have a significant influence on bone formation upon mechanical loading.

IV.2. Primary culture of osteoblasts

The choice of using the primary osteoblasts culture came from the supposition that the human osteoblasts in culture will maintain the overall characteristics of human bone cells. In this method, the osteoblasts are derived from trabecular bone explants from patient undergoing hip arthroplasty (removed femoral head); the cells are allowed 4 to 6 weeks to reach confluence, and they are used at first passage.

The utility of this method is somehow limited, the access to bone specimens not being continuous, the outgrowth time being rather long, and because of the high cost associated to the full usage of the cells at first passage.

The limitations arise from the heterogeneity of the cultured cells (Matsuyama et al., 1990) and their age-dependent response (Sutherland et al., 1995) - osteoblastic cultures derived from older donors are likely to contain fewer osteoprogenitor cells, and represent more differentiated osteoblasts compared with those derived from younger donors (Zhou et al., 2008).

We believe that the heterogeneity of the cultured cells has been overcome with the use of dexamethasone (as a glucocorticoid) and vitamin D ($1,25(OH)_2D_3$), both having a "levelling" effect in respect to the cell phenotype, acting as "osteoblastic inducers" (Jorgensen et al., 2004). Moreover, the osteoblast response to specific stimuli

is present: the cells are capable of producing alkaline phosphatase induced by vitamin D, and mineralised nodules followed a Dexamethasone stimulation.

The major prostaglandin receptors have been already proven to exist at human osteoblasts level (Sarrazin et al., 2001). The local production of the PGD_2 through the lipocalin-type PGD_2 synthase (L-PGDS) as well as the presence of both PGD_2 receptors, the DP and CRTH2, has been also established (Gallant et al., 2005) however it is considered that the endogenous production is low or absent in unstimulated conditions.

VI.3. Osteoblast proliferation

A preliminary test (time-course) was necessary to estimate the differences between different timeframes of stimulation. We can confirm that we did not see any significant differences throughout the 24h to 96h stimulation with the PGD_2 or DP/CRTH2 agonists. Exogenous PGD_2 and the agonist for CRTH2 receptor (DK-PGD_2) decreased the proliferation compared to the unstimulated condition, but the effect seem to be restored at higher concentration. The effect however was more prominent for the PGD_2 than for the specific DP/CRTH2 agonists, suggesting a third possible signalling mechanism. PGD_2 appears to have the potential to signal via the cell surface receptors - DP, other PG receptor family members, or CRTH2 - via the nuclear receptor PPARγ, or to modify signalling by direct interaction with intracellular proteins such as NF-κB (NF-κB is a regulator of genes that control cell proliferation and cell survival) (Dussault & Forman, 2000a).

On a 24h stimulation with the PGD_2 or DP/CRTH2 agonists the proliferation decreased significantly for PGD_2 and the CRTH2 agonist, suggesting that the effect could work through the CRTH2 receptor.

We have confirmed that the PGD_2 decreases the osteoblast proliferation in a dose-dependent manner using the PGD_2 alone or in conjunction with the COX inhibitors.

For the DP agonist BW245C we did not see any significant change of proliferation either for the agonist alone, or when used with the COX inhibitors, suggesting that the DP receptor is not involved in signalling at this level.

For the CRTH2 agonist used alone we see a decreasing trend-line up to 10^{-8}M, but the effect seems to be restored when further increasing the concentration. When this agonist is used with Naproxen, this effect could be replicated but for a concentration of 10^{-9} (statistically significant) after which the control level is restored. When Diclofenac is used, we observe the same decreasing trend-line, more consistent as the concentration increases, but with no significant values.

We have to mention that at micro-molar concentrations of 15d-PGJ_2, Δ^{12}-PGJ_2, PGJ_2, and PGD_2 have been shown to activate the nuclear receptor PPARγ (Forman et al., 1995); it seems plausible that PPARγ may represent another mean for micro-molar concentrations of PGD_2 metabolites to inhibit the proliferation. In contrast, a pro-inflammatory (chemotactic) role for 15d-PGJ_2 at much lower ligand concentrations has been observed recently in eosinophils (Kobayashi et al., 2005). That could be explained through the binding of 15d-PGJ_2 to the CRTH2 receptor who is responsible for this effect (Sugimoto et al., 2005), but not through the binding to the DP receptor.

The activation of PPARγ by 15dPGJ_2 converts rodent osteoblastic cells to terminally differentiated adipocytes and simultaneously suppresses the osteoblast phenotype and Cbfal expression and can inhibit proliferation (Maurin et al., 2005) proving that PPARγ is implicated in gene regulation (Kliewer et al., 1997). However, 15d-PGJ_2 levels in vivo are reported to be several orders of magnitude too low to be considered as the endogenous mediator of PPARγ activation (Powell, 2003).

In the first phase, active proliferation is reflected by mitotic activity with expression of genes associated with cell cycling (e.g., histone) and growth (e.g., proto-oncogenes c-myc, c-fos, and c-jun) (McCabe et al., 1995). Several other genes associated with formation of the extracellular matrix (type 1 collagen, fibronectin, and TGFβ) are also actively expressed (Aronow et al., 1990) and then gradually down-

regulated with collagen mRNA being maintained at a low basal level during subsequent stages of osteoblast differentiation.

There is a difference to be underlined in the nature of the non-specific COX inhibitors. Naproxen is known to be also a ligand (agonist) for the PPARγ (Jaradat et al., 2001). We note that Diclofenac is also a ligand for the PPARγ, but having an antagonistic role (Bishop-Bailey & Wray, 2003).

Another phenomenon to account for is that the the PG and the PG agonists induce COX-2 expression in this system in parallel with the PG stimulation of cAMP production and is mediated probably via the phospholipase C pathway - a mechanism of feedback in regulating the COX-2 expression (Pilbeam et al., 1995). The auto-amplification effect may be particularly important in cell cultures, where PGs are not rapidly degraded, and may account for the biphasic or recurrent induction of COX-2 seen in some long-duration cultures.

By using the PGD_2 alone at different concentrations, we confirm the anti-proliferative effect given by the growth inhibition (Fukushima, 1992).

The transformation in plasma of PGD_2 to 15d-PGJ_2 leads consequently to the activation of the PPARγ responsible for the anti-proliferative effect, and possibly increased its expression (Maurin et al., 2005). The known *in vitro* concentrations for the 15d-PGJ_2 are in the micro-molar range (5-10 μM) (Khan & Abu-Amer, 2003), while the endogenous concentrations are 0.1-1nM (Bishop-Bailey & Wray, 2003).

By adding Naproxen to all conditions, and accounting for the errors, the measured effect for the proliferation in the presence of increasing concentrations of PGD_2 was slightly decreasing with PGD_2 at 10^{-7} M and 10^{-6} M having significant values. The same test using Diclofenac had a similar effect on proliferation, but the only significant concentration of PGD_2 was 10^{-6} M. Diclofenac is responsible for decreasing the proliferation for SAOS cells, at concentrations of 15×10^{-6} M (Kaspar et al., 2005), if this may be the case, that could explain the same effect for both COX inhibitors, in conjunction with the added PGD_2 although the concentration of Diclofenac used in experiments was fixed at 10^{-6} M.

The controversial effects observed could be the result of multiple interactions, some related to the PGD_2 receptors, and some related to signalling mechanisms involving the PPARγ. Besides the well-known anti-proliferative effects of the PPARγ, some studies show that this nuclear receptor could be implicated in the development of tumours by PGJ_2 and 15d-PGJ_2 induced proliferation of COX-2-depleted colorectal cancer cells at a nano-molar concentration, but reduced by these PGs at micro-molar concentrations (Chinery et al., 1999). Therefore, the proliferative/anti-proliferative effects of PPARγ ligands are likely to be dependent on their intracellular concentrations. It should be noted that PGs formed endogenously at submicromolar concentrations are biologically more relevant in terms of regulating cell proliferation. Some authors (Yamazaki et al., 2002) showed that the effects of NSAIDs on rheumatoid synovial cells were not related to the inhibition of COX isozymes, but to the stimulation of PPARγ which can point to the fact that the inhibition of COX does not have necessarily anti-proliferative effects.

The effect observed in activating the CRTH2 receptor (decreasing proliferation) may be due the indirect activation of arrestins (non-GPCR signaling) that might lead to MAP-kinase activation (Ulven & Kostenis, 2006). Also, the 15d-PGJ_2 can activate the MAP-kinase through an independent PPARγ mechanism (Lennon et al., 2002).

We can conclude that effect of the PGD_2 over the osteoblast proliferation has an overall inhibitory role probably acting through the CRTH2 receptor, however other mechanisms cannot be excluded.

IV.4. Differentiation

Immediately after the down-regulation of proliferation, proteins associated with the osteoblast phenotype are detected such as the alkaline phosphatase. With progression into the mineralisation stage, all cells become positive for alkaline phosphatase.

Osteoblasts synthesise and secrete type I collagen, glycoproteins such as osteopontin (rich in sialic acid; N- and O-linked oligosaccharides) and osteocalcin (contains glutamic and aspartic acid residues), cytokines and growth factors into a region of unmineralised matrix (osteoid) between the cell body and the mineralised matrix. In addition, osteoblasts produce a specific membrane-bound molecule known as receptor activator of NF-κB ligand (Anderson et al., 1997). This factor is responsible for programming osteoclast differentiation and also functions as a dendritic cell survival element (Yasuda et al., 1998).

The exogenous PGD_2 at different concentrations alone or in the presence of Naproxen did not produce a significant effect on the differentiation. When Diclofenac was used, we can say that probably the only significant value (decreased differentiation at 10^{-6}M) is due to the high concentration of the PGD_2 or due to some other mechanisms.

In the series that contained the vitamin D, the PGD_2 alone decreases the differentiation in a dose-dependent manner, and the same effect is partially replicated by using Diclofenac (not significant), but not when Naproxen was used.

For the test with DP/CRTH2 antagonists, without vitamin D, we did not see any effect given by the activation of CRTH2. A possible response was obtained for the activation of the DP receptor with the significant value of 10^{-6} for the PGD_2 when we see a decreased level of alkaline phosphatase. Even if the trend-line could suggest a dose-response effect it is difficult to assess this because of the low number of donors.

Considering the transformation of PGD_2 into 15d-PGJ_2 in plasma, we can mention again the parallel signalling of this prostaglandin in connection to the PPARγ, but contrary to the literature, we did not see any inhibitory effect as mentioned by (Khan & Abu-Amer, 2003). The mechanism is similar to the one explaining the PPARγ inhibiting effect for the proliferation, however in our case no significant modification has been seen, although the cells responded well when stimulated with vitamin D. Interestingly, some studies in vitro suggest that human osteoblasts respond to exogenous vitamin D by decreasing their rate of proliferation, as well as increasing their expression of mRNA species encoding osteocalcin, bone sialoprotein-1, and RANKL (At-

kins et al., 2007) probably preparing the osteoblast for the mineralisation stage. It is probably the transition that was detected when performed the tests in the presence of vitamin D, the stimulation with high concentration of PGD_2 potentiating this effect by unknown mechanisms.

PPARγ is an important nuclear receptor family for adipocyte differentiation balancing the transformation between the osteoblasts and adipocytes since osteoblasts share the same origin with adipocytes in bone marrow cavity (Lecka-Czernik & Suva, 2006) that could explain the fatty marrow, accumulation of adipocytes in bone marrow, observed in the patients who manifest bone diseases such as osteoporosis.

By stimulating the cells with PGD_2 in presence of T0070907 (PPARγ antagonist) we did noticed a drop in alkaline phosphatase expression, but with no significant values, suggesting that the inhibition of the differentiation might use the DP pathway or other signalling systems.

We have to mention studies related to the origin of the harvested cells, indicating that primary osteoarthritis osteoblast-like cells (coming from patients with osteoarthritis) express higher alkaline phosphatase activity and osteocalcin release, both under basal conditions and with 1,25-dihydroxyvitamin D stimulation. They show decreased cAMP synthesis in response to human parathyroid hormone and prostaglandin E, in contrast to normal osteoblast cells, a result that could not be attributed to altered adenylate cyclase activity (George Hilal et al., 1998). Regarding the age of the patients, the literature suggests that is an age-dependent correlation to the decreased proliferation, osteoblastogenesis, differentiation and increased apoptosis for a test involving human mesenchymal cells (Zhou et al., 2008).

We can conclude that from our experiments without vitamin D, PGD_2 has no effect over the differentiation except for PGD_2 at 10^{-6} M used with Diclofenac. The same effect is seen when we activate the DP receptor only, suggesting a possible mechanism by activating this receptor, however the data is not conclusive. By blocking the PPARγ receptor and stimulating the cells with PGD_2 the measured alkaline phosphatase drops below the control level, implying that this receptor is not involved directly. The response is similar to the one observed when we stimulated the cells

with PGD_2 in presence of Diclofenac, a possible antagonist of PPARγ (Bishop-Bailey & Wray, 2003), but not in Naproxen's presence, suggesting that in fact the PPARγ might have a positive effect over the differentiation of the osteoblasts.

For the experiments performed in the presence of vitamin D, PGD_2 decreased the differentiation dose-dependently. By using the Naproxen, we did not see any effect. When Diclofenac was used, we obtained a decreasing trend-line as the PGD_2 concentration increased, suggesting again the involvement of the PPARγ in stimulating the differentiation.

The PGD_2 have an inhibitory effect over the human osteoblast differentiation, probably through the DP receptor activation.

VI.5. Matrix mineralisation

Performing the first measurement of calcium for the PGD_2 stimulated osteoblasts we observed that the prostaglandin had a stimulative effect over the matrix mineralisation, suggesting a dose-response effect, however without any significant values.

In competitive tests of PGD_2 at 10^{-9} M and the specific DP/CRTH2 antagonists BWA868C and CAY10471 respectively, no effect is seen, leading to the conclusion that the positive effect that PGD_2 might have on the calcification is obtained through other mechanisms.

Some publications relate to an altered phenotype of cultured osteoblasts who have elevated alkaline phosphatase and osteocalcin levels coming from patients with osteoartritis, but who have an impaired mineralisation as a consequence of an imbalance in collagen 1A1/collagen 1A2 triggered by TGFβ (Couchourel et al., 2009). The possible change of the phenotype towards the adipocytes by activating the PPARγ is not to be neglected, Naproxen being added to all conditions and 15d-PGJ_2 being present in plasma as previously discussed. The absence of PGE_2 by blocking non-specifically the COX enzymes could also account for the absence of calcification

(Marks & Miller, 1988) although some suggest that the PGD_2 has a bigger role in matrix mineralisation than PGE_2 (Asano et al., 2009) by having a stimulating action on Na inorganic phosphate transport through a MAP-kinase pathway (Asano et al., 2009).

Suspecting a PPARγ activation, we used T0070907, a highly specific antagonist of the human PPARγ receptor at a concentration of 10^{-6}M in conjunction with PGD_2. The treatment seems to diminish the calcification, with T0070907/PGD_2 at 10^{-8}M having a significant value, suggesting that probably the PPARγ is involved in the positive effects on mineralisation.

The products of PGD_2 metabolism can activate separately the DP/CRTH2 receptors, 15-deoxy-^{12,14}-PGD_2 and 12-PGD_2 having for instance a higher affinity for the CRTH2, but the former being an agonist for the PPARγ too, showing that DP and CRTH2 can be regarded as antagonistic receptors, CRTH2 mediating pro-inflammatory and pro-stimulatory effects and DP limiting CRTH2 activation upon exposure to PGD_2 on mast cells (Kostenis & Ulven, 2006). Moreover, some competitive antagonistic effects could exist between the PGD_2 receptors and the PPARγ, as a result of a direct action of PGD_2 or indirectly, by its metabolites. The difference between the rate of recycling of the two receptors on the cell surface could also play a role in the signalling (Gallant et al., 2007), the multiple interactions present changing the internalization rates by acting on arrestins.

As a conclusion, we observed that the PGD_2 might have a positive effect on human osteoblasts matrix mineralisation by acting on the PPARγ directly or through the plasma degradation products of PGD_2.

V. Conclusions

The study confirmed some of the data present in the literature like the effect of PGD_2 on the proliferation and the differentiation, but brought contradictory information in regards to the calcification of the human cultured osteoblasts. Of course, the literature confirms that too, proving once more the controversial effects of the prostaglandins for the *in vitro* studies.

It is becoming clear that we cannot separate the multiple interactions that might appear in the proposed system even though we can measure some of the effects.

The PGD_2 decreases the osteoblasts proliferation proliferation in a dose-dependent manner alone or in conjunction with the COX inhibitors. We can conclude that effect of the PGD_2 over the osteoblast proliferation has an overall inhibitory role probably acting through the CRTH2 receptor, however other mechanisms cannot be excluded.

Exogenous PGD_2 at different concentrations alone or in the presence of Naproxen did not produce a significant effect on the differentiation. By stimulating the cells with PGD_2 in presence of PPARγ antagonist we did not notice a significant response. PGD2 dose-dependently decreases the differentiation of osteoblasts in the presence of vitamin D. The lack of a clear response on the differentiation of the osteoblasts and the inhibitory effect obtained when vitamin D was used implied the existence of other mechanisms.

The PGD_2 has no significant effect over the matrix mineralisation through DP/CRTH2 activation. The mineralisation is decreased when blocking the PPARγ receptor. This receptor is therefore involved in augmenting the calcification in this particular case.

Perhaps the role of the PPARγ becomes more clear on the calcification, but we cannot ignore many other mechanisms given by degradation of the main compound,

secondary signalling through different messengers or even delays in receptor recycling.

The added chemicals can also trigger different messaging systems and some of them could activate partially the DP/CRTH2 receptors.

The human osteoblasts in culture, although presenting a certain heterogeneity are a valid replica of *in vivo* processes. The fact that the origin of the cultured cells come from the patients with osteoarthritis or from old patients with a certain pathology of their bone apparatus may somehow impede over the consistency of the results, but this fact can be taken into account and judged accordingly.

Perspectives

The fact that the PGD_2 could have an anabolic effect on different stages in the evolution of human osteoblasts is not to be neglected.

More studies have to be performed in different conditions, with PPARγ antagonists to eliminate possible contrary effects on assessing the effects of the PGD_2. The number of experiments could also lead to interesting results, having several lines of cultured cells giving a more homogenous answer. The downfall of this is the cell culture timeframe and the supply with the bone explants.

It would be maybe worthwhile to test the line of cells coming from the same patient for all the three assays, proliferation, differentiation and mineralisation, this indicating a more consistent evolution of the cell line. Also, it would be interesting to correlate the cell line with the age of the patient, sex, general habits (alimentation, sports, smoking) and with the disease of the patient.

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