CHAPTER 4

Effects of Arachidonic Acid, Oestrogen and Parathyroid Hormone on Prostaglandin E_2 (PGE_2) Production in MG-63 and MC3T3-E1 Osteoblast-like Cells

4.1 Introduction

Polyunsaturated fatty acids (PUFAs) as well as the bone modulating hormones, oestrogen (E2) and parathyroid hormone (PTH) have positive effects on bone homeostasis. Hormones may affect bone cell activity by stimulating or suppressing the release of locally acting factors such as the prostaglandins in the bone cell environment.[243]

PUFAs are converted via a series of desaturation and elongation steps to various longer chain PUFAs specifically arachidonic acid (AA) and eicosapentaenoic acid (EPA), which in turn can act as precursors for prostaglandins (PGs).[171] (Figure 2.15). Some studies suggested that PGs may mediate the anabolic effects of oestrogen in rats and mice,[311,312] while both PGs and oestrogen seem to target early osteoblast precursors in the bone marrow.[109] PTH induces PG synthesis by osteoblasts,[313,314] and PGs can induce bone resorption, an effect of PTH at physiological concentrations.[313] PTH may therefore exert some of its effects via induction of PG synthesis. Exposure of cultured mouse parietal bones to AA and PTH simultaneously amplified PG production compared to PTH exposure only.[315]

Two iso-enzymes, COX-1 (constitutive) and COX-2 (inducible) regulate PG synthesis (Figure 4.1). Two isoforms of the PGE synthase for PGE_2 biosynthesis have been cloned and characterized. Membrane-associated PGE synthase (mPGES) is thought to be an inducible enzyme whilst cytosolic PGE synthase (cPGES) is constitutively expressed.[316-318] cPGES is functionally coupled with COX-1 and mPGES is functionally coupled with COX-2.[149,233,317] PTH induces COX-2 expression with little

or no effects on COX-1 or $cPLA_2$.[240] PTH and PTH-related peptide are potent stimulators of PGE_2 secretion in cultured neonatal rat calvari.[237]

Following their intracellular synthesis, prostaglandins exit the cell, act on the parent cell and/or neighbouring cells in an autocrine and/or paracrine fashion through specific prostaglandin receptors thereby affecting changes in the levels of second messengers.[170] PGE receptors belong to the G protein-coupled seven transmembrane domain family of receptors. There are at least four distinct receptors for PGE_2 with differential signaling pathways: EP_1 with Ca^{2+} mobilization; EP_2 and EP_4 with stimulation of cAMP production; and EP_3 mainly with the inhibition of cAMP production.[235,236] Recently, Sakuma *et al* (2004), using primary cultures of murine osteoblasts, demonstrated that PGE_2 is an effective stimulator of cAMP production and an inducer of COX-2.[246] This effect was attributed to cAMP-dependent activation of protein kinase A and to be the result of the combined effects of activation of the EP_2 and EP_4 receptors.[246]

Nonsteroidal, antiinflammatory drugs (NSAIDs) inhibit synthesis of prostaglandins. A well known example of this class, indomethacin, reversibly inhibits prostaglandin synthesis by competing with the substrate AA for the active site of the enzyme, thereby blocking both COX-1 and COX-2 activity.[230,319] NS-398 on the other hand, is known to selectively block only COX-2 mediated prostaglandin production.[252,253] It is possible that modulation of PG synthesis in osteoblasts using selective NSAIDs may affect bone formation or resorption. Figure 4.1 illustrates the action of indomethacin and NS-398 on COX-1 and COX-2.

The purpose of the study was to determine whether AA alone or in combination with oestrogen and PTH modulates PGE_2 synthesis in osteoblast-like cells and to determine to what extent COX-1 and COX-2 contribute to the observed effects by employing specific blockers. DHA is not a substrate for prostaglandin synthesis[172] but could interfere with PGE_2 production by displacing AA in the cell membrane thereby limiting the amount of AA available for PGE_2 synthesis.[176] It has also been shown that DHA competes with AA for binding to COX-1 and therefore may be regarded as a strong competitive inhibitor for PGE_2 synthesis.[122] However, as PGE_2 synthesis is hardly detectable in basal (unstimulated) control conditions, the effect of DHA on PGE_2 synthesis was not investigated in our study.

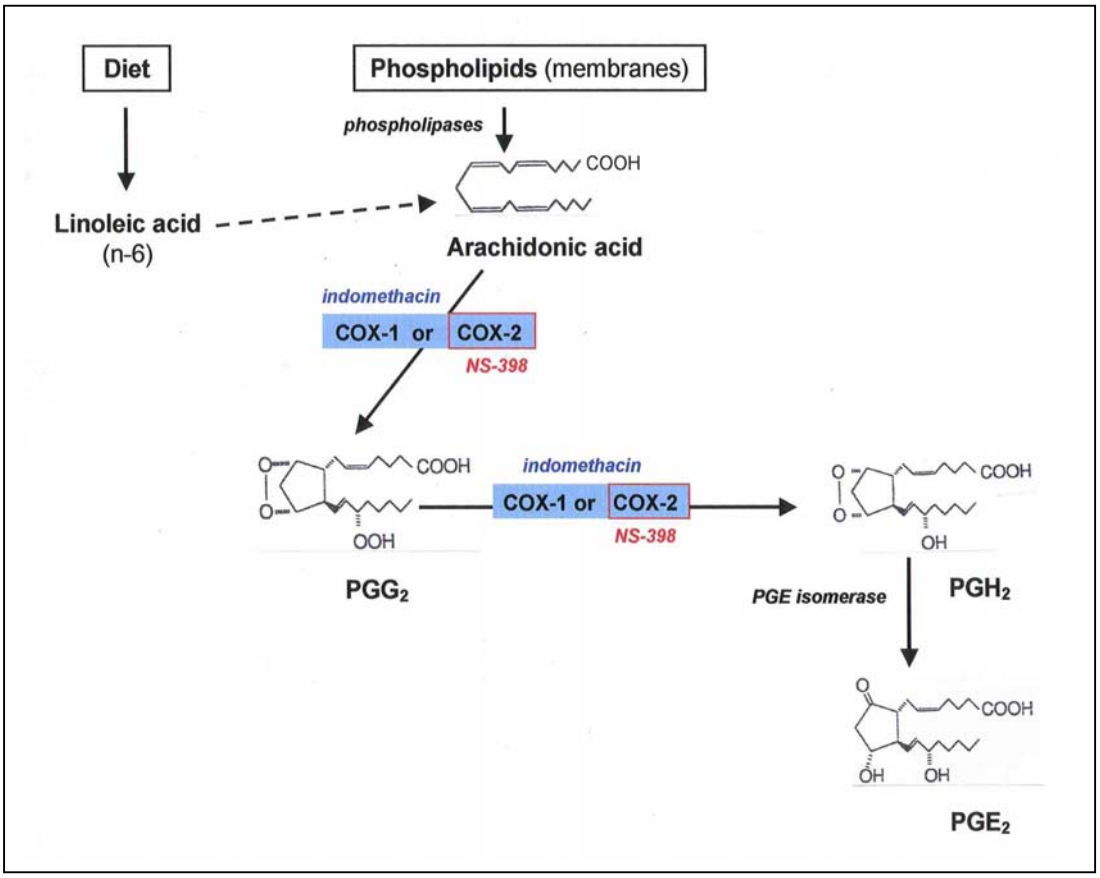

Figure 4.1. The prostaglandin pathway indicating the enzymes involved in the synthesis of prostaglandin E_2 from its substrate AA. Indomethacin is an unselective blocker of both COX-1 and COX 2, while NS-398 blocks only COX-2.

4.2 Materials and Methods

Reagents

Sigma Chemical Co (St. Louis, MO, USA) supplied DMEM, L-glutamine, crystal violet, trypan blue, arachidonic acid, oestrogen, parathyroid hormone fragment 1-34, indomethacin, gamma globulin, dextran-coated charcoal and PGE_2. Heat inactivated fetal calf serum (FCS) was obtained from Highveld Biological (Pty) Ltd. (Sandringham, SA). Gentamycin was supplied by Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). NS-398 was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were of analytical grade and purchased from Sigma Chemical Co (St. Louis, MO, USA).

Cell cultures

MG-63 (human osteoblast-like, osteosarcoma-derived) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Nontransformed MC3T3-E1 mouse calvaria fibroblasts (established from the calvaria of an embryo/fetus C57BL/6 mouse) described to differentiate to osteoblasts,[285] were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Exposure of cell cultures to arachidonic acid, parathyroid hormone and oestrogen

Following trypan blue exclusion, MG-63 and MC3T3-E1 cells were seeded at densities of 40 000 cells per well in 24-well cluster plates. Cells were cultured in DMEM with 10% heat-inactivated FCS, as measurable PGE_2 is not produced by cells in serum-free media.[248] Regulation of PGE_2 biosynthesis in MG-63 cells is cell density-dependent. Subconfluent cultures displayed a greater response to cytokine stimulation than confluent cultures.[249] It therefore seems that signaling pathways resulting in PGE_2 biosynthesis are subject to complex regulation by cell-cell or cell-extracellular matrix interactions, therefore only subconfluent cell cultures were used in these experiments. After 24 hours, medium was changed and subconfluent cells exposed to vehicle (0.2% ethanol), PTH (10^{-7} M), oestrogen (E2) (10^{-6} M) or AA (20μg/ml) for four hours. Concentrations of the agents used, were determined from results of earlier dose-response trials performed in our laboratory. To determine the role of the PGHS enzymes COX-1 and COX-2 on PGE_2 synthesis, cells were incubated with the unselective COX-blocker indomethacin (1μM) or the COX-2 specific blocker NS-398 (0.1μM) which were added to the growth medium 45 minutes prior to the addition of AA and hormones.[248,253] Cell cultures were maintained at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. All cell cultures were supplemented with 2 mM L-glutamine and gentamycin (25 μg/ml). After 4 hours conditioned media were aspirated from cultured cells and stored at -70ºC until analysed. Following removal of medium, cell numbers were determined by crystal violet staining.[290,298] In short, cultures were fixed with 1% glutaraldehyde, stained with 1% crystal violet, and the dye extracted with 0.2% Triton X-100.

Absorbance (OD) was read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA) at a wavelength of 570nm; 0.2% Triton X-100 in water was used as a blank. Crystal violet is a basic dye, which stains cell nuclei.[298] Spectrophotometer readings of colour intensity are therefore an indication of DNA content and therefore cell numbers. Results were analysed using a linear standard curve established from known cell numbers (Refer to Figure 3.3). Three independent experiments were conducted in quadruplicate.

Radioimmunoassay (RIA) of prostaglandin E_2 in cell culture media

The amount of PGE_2 produced by the cells and released into the media was assessed using a competitive binding radioimmunoassay, adapted from a method described by Raisz and Simmons (1985).[237] In this assay, unlabeled PGE_2 samples or standards diluted in working buffer (0.01 M Tris HCl in 0.14 M NaCl, pH 7.6, containing 2 mg gamma globulin/ml) were incubated overnight at 4ºC with radiolabeled PGE_2 ([^{3}H]-PGE_2, New England Nuclear™ Life Science Products, Inc., Boston, MA, USA) and unlabeled PGE_2 antibody (provided by Dr Laurence Levine, Brandeis University, Waltham, Massachusetts, USA). The samples were put on ice and subsequently dextran-coated charcoal was added to each polystyrene tube. Free PGE_2 was removed by centrifugation for 15 minutes at 3000 rpm at 4ºC with a Rotixa 120R, Hettich sentrifuge (Tuttlingen, Germany). Thereafter, the supernatant was transferred to a counting vial and Liquiscent (NEN) scintillation cocktail (Packard Bioscience B.V., Groningen, The Netherlands) added. Samples were counted for 10 minutes each on the ^{3}H channel using a Beckman L55000CE Liquid Scintilation counter (Beckman Instruments, Nuclear Systems Operations, Fullerton, CA, USA). The percentage of bound versus unbound [^{3}H]-PGE_2 was compared to a standard curve to calculate the amount of PGE_2 in the original samples. (Refer to Figure 3.3). The intra and interassay coefficients of variation were 3% and 4.4% respectively.

Indirect immunofluorescence for detection of COX-1 and COX-2 in MG-63 cells

Following trypan blue exclusion, cells were seeded at a density of 200 000 cells per heat-sterilised coverslip in 6-well cluster plates and left to adhere for 24 hours. Fresh growth medium containing vehicle only (0.2% ethanol)(control) or 20 µg/ml AA was added to near confluent layers. Cells were exposed for four hours at 37ºC. At the end of the experiment, after discarding the medium, cells were fixed in 10% formaldehyde (v/v) in (2 mM EGTA in PBS) for 10 minutes at room temperature. The presence of COX-1 and COX-2 after exposure to AA were visualised by indirect immunofluorescence as follows:

The coverslips were transferred to a staining dish and rinsed three times with PBS at room temperature. Cells were then permeabilised in ice cold 97% methanol containing 2 mM EGTA in PBS at –20°C for 4 minutes whereafter they were rinsed thrice with PBS. Primary antibody (25 µl mouse monoclonal antibody against human COX-1 or COX-2 (1:1000 in PBS))(Cayman Chemical Company, Ann Arbor, MI, USA), was added to each coverslip and incubated for 60 minutes at 37ºC in a damp environment. After rinsing the cells with PBS, the cells were incubated with the secondary antibody (25 µl 1:100 biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent))(Diagnostic and Technical Services, Johannesburg, SA.), for 60 minutes at 37ºC in a damp environment. After rinsing, cells were finally incubated with ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent) (Sigma Chemical Co**,** St. Louis, MO, USA) for 60 minutes. The coverslips were then mounted with a glycerol-based mounting fluid after the final wash step. Photographs were taken with 400 ASA film on a fluorescent microscope (Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 nm for FITC).

Statistics

Data presented are the results of three separate experiments (n=4). Statistical analysis was performed using Statistics for Windows software (version 2, Tallahassee, Florida, USA). Student's paired *t*-test was used to evaluate differences between the sample of interest and its respective control. A *P* value of less than 0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Effects of cyclooxygenase blockers and arachidonic acid on prostaglandin E_2 production in MG-63 cells

The effects of the cyclooxygenase blockers indomethacin and NS-398 and AA on PGE_2 production in MG-63 cells are shown in Figure 4.2. Compared to control, incubation of MG-63 cells with either indomethacin or NS-398 resulted in almost 90% inhibition of PGE_2 production. Inhibition was similar for both treatments.

AA increased PGE_2 synthesis six- to seven-fold compared to control. Pre-incubation with COX-blockers prior to AA-treatment inhibited PGE_2 secretion. Indomethacin-mediated inhibition however, was greater than NS-398-mediated inhibition (60% compared to 20%) as indicated in Figure 4.2. Application of blockers did not result in the very low levels of PGE_2 synthesis observed when AA-unstimulated cells (controls) were exposed to these blockers.

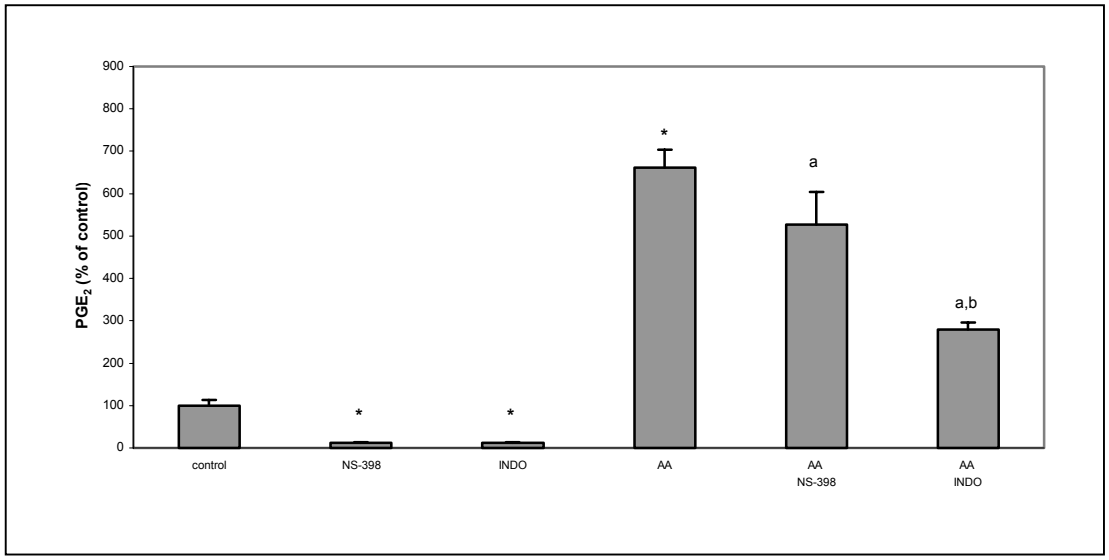

Figure 4.2 Effects of cyclooxygenase blockers and arachidonic acid on prostaglandin E_2 production in MG-63 cells.

MG-63 cells were treated with vehicle (0.2% ethanol) or AA (20 μg/ml) for four hours before sampling of the media. Indomethacin (INDO) (1 μM) and NS-398 (0.1μM) were added 45 minutes prior to AA- treatment. PGE_2 radioimmunoassay was performed as described in Materials and Methods and expressed as percentage of control. *Significant difference from control; **a** significant difference from AA; **b** significant difference from (AA + NS-398), $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

4.3.2 Indirect immunofluorescence staining for COX-1 and COX-2 in control and arachidonic acid-activated MG-63 cells

Figure 4.3 depicts the presence of COX-1 and COX-2 enzymes in control and AA-activated MG-63 cells as shown by indirect immunofluorescence staining. Exposing MG-63 cells to either vehicle (0.2% ethanol) or AA (20 μg/ml) in the presence of FCS, resulted in detection of both COX-1 (Figure 4.3 A and B) and COX-2 (Figure 4.3 C and D) enzymes. Morita *et al* (1995) reported that COX-1 and COX-2 are both located in the endoplasmic reticulum as well as the nuclear envelope.[234] COX-2 however, is more highly concentrated on the nuclear membrane than COX-1.[234] This phenomenon is confirmed in Figure 4.3. No difference in the densities of either COX-1 (Figure 4.3 A and B) or COX-2 (Figure 4.3 C and D) in control or AA-treated cells was found.

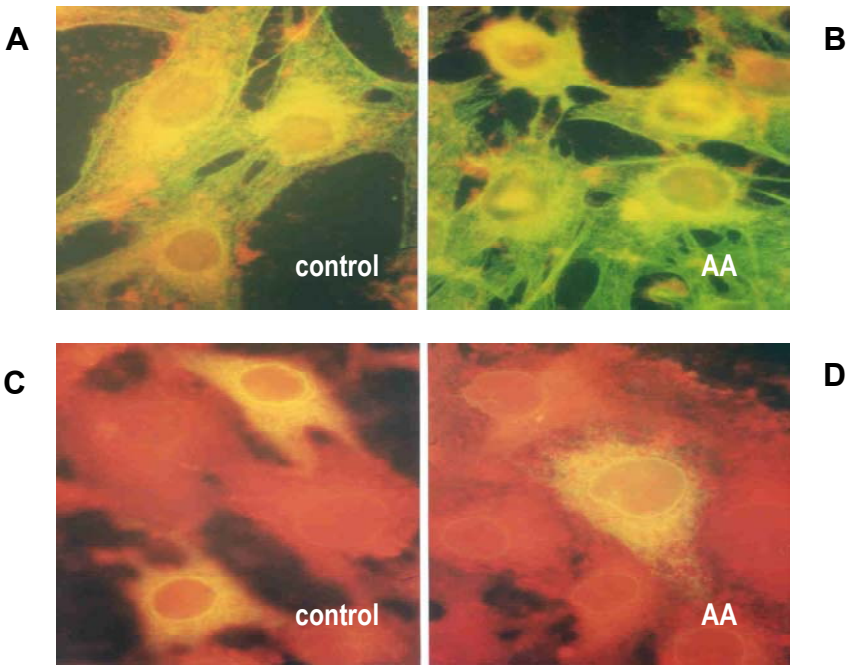

Figure 4.3 Immunofluorescent staining for COX-1 and COX-2 in control and arachidonic acid-activated MG-63 cells

After MG-63 cells were cultured with DMEM with 10% FCS for 24 hours, fresh growth medium containing vehicle only (control) or 20 μg/ml AA was added to near confluent layers. Cells were exposed for four hours at 37ºC. At the end of the experiment, cells were subjected to indirect immunofluorescence staining using mouse monoclonal antibody against human COX-1 (A and B) or COX-2 (C and D) and then biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in FITC-conjugate diluent. Photographs were taken with 400 ASA film on a fluorescent microscope. (Original magnification: 400x).

4.3.3 Effects of arachidonic acid, parathyroid hormone and oestrogen on prostaglandin E_2 production in MG-63 and MC3T3-E1 cells

Effects of arachidonic acid and parathyroid hormone on prostaglandin E_2 production in MG-63 cells

Figure 4.4 depicts the effects of AA and PTH on PGE_2 production in MG-63 cells. Exposure to PTH (10^{-7} M) for four hours resulted in a 30% increase in PGE_2 production compared to control. Simultaneous exposure of these cells for four hours to AA and PTH did not increase PGE_2 secretion above the AA-stimulated PGE_2 production. It therefore seems that AA-PTH co-treatment had no synergistic effect on PGE_2 production in this model. Pre-incubation of MG-63 cells with the COX- blockers indomethacin or NS-398 prior to the combined PTH-AA treatment, yielded results similar to those observed when blockers were added prior to AA treatment only. Therefore no PTH-mediated COX stimulation could be observed under the conditions used in this assay.

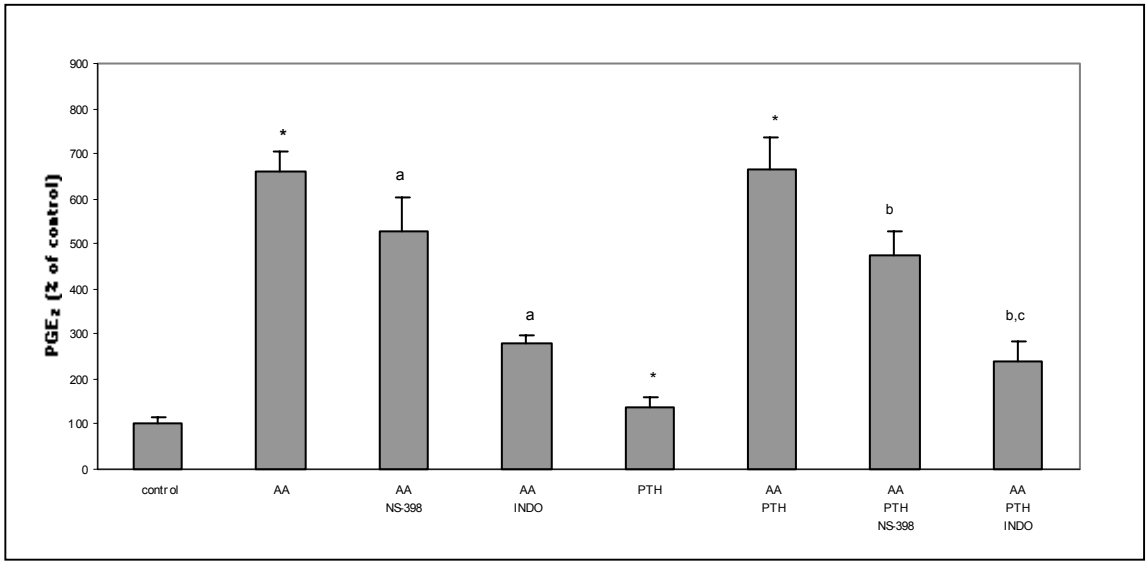

Figure 4.4 Effects of arachidonic acid and parathyroid hormone on prostaglandin E_2 production in MG-63 cells

MG-63 cells were treated with vehicle (0.2% ethanol), AA (20 μg/ml) and PTH (10^{-7} M) for four hours before sampling of the media. Indomethacin (INDO)(1μM) and NS-398 (0.1μM) were added 45 minutes prior to AA treatment. PGE_2 radioimmunoassay was performed as described in the Materials and Methods and expressed as percentage of control.
* Significant difference from control; **a** significant difference from AA; **b** significant difference from (AA + PTH); **c** significant difference from (AA + PTH + NS-398), $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

Effects of arachidonic acid and oestrogen on prostaglandin E_2 production in MG-63 cells

Figure 4.5 depicts the effects of AA and oestrogen (E2) on PGE_2 production in MG-63 cells. Exposing MG-63 cells to oestrogen (10^{-6} M) for four hours did not affect PGE_2 production compared to control. Exposing cells to AA and oestrogen simultaneously did not enhance PGE_2 production above that already observed after AA exposure only. Pre-incubation of MG-63 cells with the COX-blockers indomethacin or NS-398 prior to the combined oestrogen-AA treatment, yielded similar results to those observed when blockers were added prior to AA treatment only. Therefore, oestrogen was not able to enhance AA-stimulated PGE_2 synthesis under the conditions used in this assay.

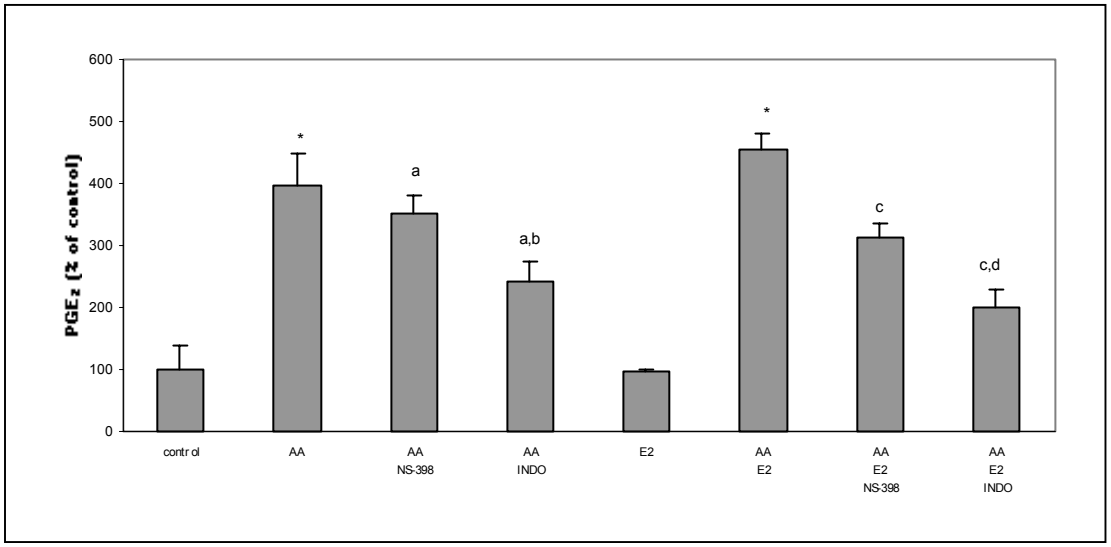

Figure 4.5 Effects of arachidonic acid and oestrogen on prostaglandin E_2 production in MG-63 cells

MG-63 cells were treated with vehicle (0.2% ethanol), AA (20 μg/ml) or E2 (10^{-6} M) for four hours before sampling of the media. Indomethacin (INDO)(1 μM) and NS-398 (0.1μm) were added 45 minutes prior to AA treatment. PGE_2 radioimmunoassay was performed as described in the Materials and Methods and expressed as percentage of control. * Significant difference from control, **a** significant difference from AA; **b** significant difference from (AA + NS-398); **c** significant difference from (AA + E2); **d** significant difference from (AA+E2+NS-398), $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

Effects of arachidonic acid, oestrogen and parathyroid hormone on prostaglandin E_2 production in MC3T3-E1 cells

The MG-63 osteoblast is an osteosarcoma derived cell line. These cells have undergone an extended period of abnormal growth *in vivo*.[305] Concern has been expressed that cell lines derived from tumor tissue are different from their normal counterparts because cell regulatory mechanisms in permanently transformed cells might differ from those in normal cells. Therefore, one comparative experiment to examine PG synthesis following exposure to AA, and systemic hormones, oestrogen and PTH was conducted on normal murine osteoblast-like cells.

Figure 4.6 depicts the effects of AA, oestrogen and PTH on PGE_2 production in MC3T3-E1 murine osteoblasts. Exposing MC3T3-E1 cells to AA for four hours stimulated PGE_2 secretion significantly. PGE_2 synthesis in the MC3T3-E1 cells was amplified two and a half times over and above what was observed in the MG-63 cells after exposure to AA. Compared to control, AA-stimulated PGE_2 secretion was 16 fold in MC3T3-E1 cells, compared to six fold in MG-63 cells (Figure 4.2 and Figure 4.6). No stimulation of PGE_2 secretion was observed after exposure of the MC3T3-E1 cells to PTH or oestrogen.

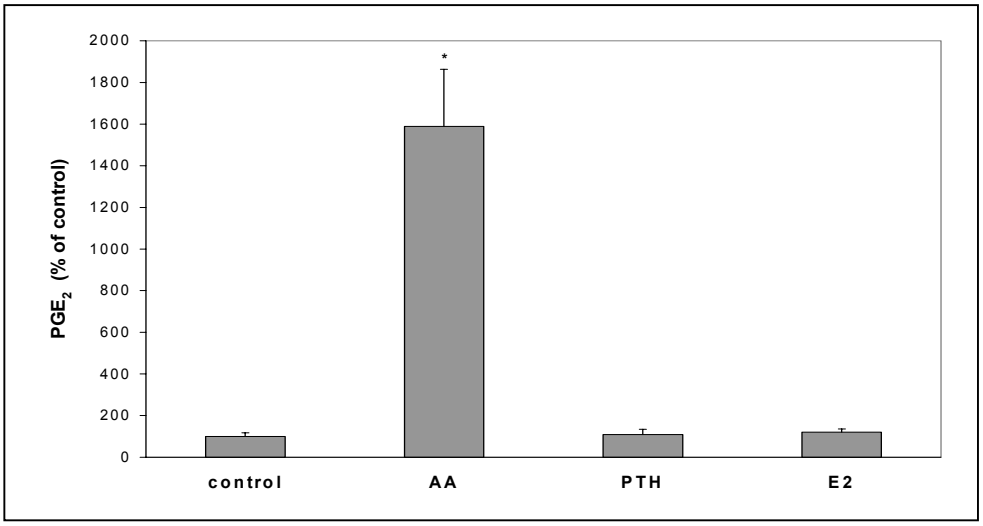

Figure 4.6 Effects of arachidonic acid, parathyroid hormone and oestrogen on prostaglandin E_2 production in MC3T3-E1 cells

Cells were treated with vehicle (0.2% ethanol), AA (20 μg/ml), oestrogen (E2) (10^{-6} M) and PTH (10^{-7} M) for 4 hours before sampling of the media. PGE_2 radioimmunoassay was performed as described in the Materials and Methods and expressed as percentage of control. * Significant difference from control, $P<0.05$, n=4. Data are from a representative experiment. The experiment was repeated three times, each experiment yielding comparable data.

4.4 Discussion

The aim of this study was to determine whether AA, oestrogen and PTH modulate PGE_2 synthesis in MG-63 and MC3T3-E1 osteoblast-like cell lines, and whether COX-1 or COX-2 is involved. Exposure to AA enhanced PGE_2 production significantly in both MG-63 and MC3T3-E1 cell lines (Figure 4.2 and Figure 4.6). Since AA is the natural substrate for PGE_2 synthesis[170] and PGE_2 the major prostaglandin produced by osteoblastic cells,[228,229] this observation was expected. The AA-stimulated production of PGE_2 by MC3T3-E1 cells however, was significantly higher than that in MG-63 cells (Figure 4.2 and Figure 4.6). This observation might reflect a difference in the stimulatory mechanisms involved in PGE_2 production in these cell lines. PGE_2 may autoamplify its own production by inducing COX-2 in bone.[243-246,314] Suda *et al* (1998) showed that autoamplified production of PGE_2 is mediated via the EP_1 subtype of PGE receptors in mouse MC3T3-E1 osteoblasts.[232] It is not known whether MG-63 cells express the EP_1 receptor.

Prostaglandins act on the parent cell and/or neighbouring cells in an autocrine and/or paracrine fashion through specific prostanoid receptors thereby affecting changes in the levels of second messengers.[170] There are at least four distinct receptors for PGE_2 with differential signaling pathways: EP_1 with Ca^{2+} mobilisation; EP_2 and EP_4 with stimulation of cAMP production; and EP_3 mainly with the inhibition of cAMP production.[235,236] The expression patterns of PG receptors differ in various cell types, differentiation status of these cells, tissues, and species. MC3T3-E1 mouse osteoblastic cells have been reported to predominantly express EP_1 and EP_4 receptors,[229] whilst human mesenchymal stem cells express EP_4 receptors.[149] (Refer to 2.11.1)

To elucidate whether AA increased PGE_2 via COX-1 and/or COX-2, COX-blockers were included before exposure to AA. Indomethacin is known to block both COX-1 and COX-2, while low concentrations of NS-398 are responsible for COX-2 specific inhibition.[248,253] Exposing MG-63 control cells to either NSAIDS inhibited PGE_2 production significantly. As both blockers are reponsible for COX-2 inhibition and the PGE_2 inhibitory effects by these blockers were similar, it is possible that the observed inhibition could be attributed mainly to COX-2 suppression. In our

experiment, cells were cultured in DMEM with 10% heat-inactivated FCS, as measurable PGE_2 was not produced by cells in serum-free media.[248] Fetal calf serum contains AA in its composition, however, the concentration thereof may be different for different batches.[56] It has been reported that COX-1 requires higher concentrations of AA for its optimal function than does COX-2, implying that the amount of AA supplied critically influences which COX isozymes are utilised.[233] One can therefore speculate that in basal conditions, with low AA concentrations present in the culture media, COX-2 could be the major cyclooxygenase responsible for AA metabolism.

In the MG-63 cells pre-incubation with either indomethacin or NS-398 prior to AA supplementation resulted in a decrease in PGE_2 production compared to AA-stimulated PGE_2 production (Figure 4.2). The degree of inhibition of the blockers however was not the same. The use of indomethacin resulted in a much larger inhibitory effect than NS-398, suggesting that both COX-1 and COX-2 were involved in AA-stimulated PGE_2 production. When PGE_2 production was stimulated by exogenous AA supplementation, pre-incubation with COX-blockers did not reduce PGE_2 production to the same low levels observed when these blockers were applied to basal culture conditions (Figure 4.2). Hamilton *et al* (1999) reported similar results and showed that increasing the supply of AA in rats *in vivo* reduces the effectiveness of NSAIDS as inhibitors of prostanoid production.[319]

In order to confirm the observation that both COX-1 and COX-2 isoenzymes are involved in AA-stimulated PGE_2 synthesis, indirect immunofluorescence was applied to determine the presence and location of the COX enzymes in MG-63 cells. Exposing MG-63 cells to either vehicle (0.2% ethanol) or AA in the presence of FCS, resulted in detection of both COX-1 and COX-2 enzymes. Morita *et al* (1995) reported that COX-1 and COX-2 are both located in the endoplasmic reticulum as well as the nuclear envelope.[234] COX-2 however, is more highly concentrated on the nuclear membrane than COX-1.[234] Our study confirmed this observation (Figure 4.3). Indirect immunofluorescence did not show any difference in the densities of either COX-1 or COX-2 in control or AA-treated cells, suggesting that the experimental conditions applied did not affect distribution of these enzymes. However, FCS itself can induce COX-2 expression.[234,248] The lack of effect of AA on COX distribution in our experiment may therefore be masked by the FCS-stimulated

effect on COX-2 expression. In order to confirm this observation, cells should also be cultured in FCS- free conditions.

Systemic hormones can influence prostaglandin production.[170,313] In order to determine whether the bone active hormones oestrogen and PTH affected PGE_2 production in osteoblast-like cells, MG-63 and MC3T3-E1 cells were exposed to these hormones. Although it was reported by others that oestrogen inhibits osteoblastic production of PGE_2 in primary osteoblasts, human osteosarcoma osteoblasts and organ culture,[108] oestrogen exposure did not affect PGE_2 production in either MG-63 or MC3T3-E1 cell lines in our study. However, it is well documented that oestrogen acts through oestrogen receptors (ER) located on the nuclear membrane, thereby affecting intracellular pathways.[35] It might be necessary to determine whether the cell lines used in our study express sufficient ER levels to be affected by oestrogen treatment. One can therefore speculate that the cells investigated in our study might be ER-deficient or may express very low levels of ER. Low ER expression levels in MC3T3-E1 cells have been reported[320] and could explain the inability of these cells to respond to oestrogen treatment.

In our study, PTH-treatment stimulated PGE_2 production significantly in MG-63 cells (Figure 4.4). Others confirmed this observation.[313-315] The mechanisms implicated in this observation are not clear. PTH stimulation of PGE_2 synthesis has been attributed to induction of COX-2[313,314] as well as increased release of incorporated AA from cell membranes.[321] PTH could act through cAMP, which can increase PG production in bone.[322] In our study, PTH however, had no stimulatory effect on PGE_2 production by MC3T3-E1 cells. Suda *et al* (1983) reported the presence of PTH receptors in this cell line,[285] however, subcloning of this original nontransformed cell line, may have resulted in loss or modification of the PTH receptor.

In conclusion, our study confirms that exogenously added AA (20 μg/ml) stimulates PGE_2 production significantly in both the MG-63 human osteosarcoma cell line as well as the nontransformed murine MC3T3-E1 osteoblast-like cell line. Stimulated PGE_2 production by MC3T3-E1 cells, however, was significantly higher than that of MG-63 cells. This observation might be attributed to autoamplification, as PGE_2 autoamplification is mediated via the EP_1 subtype of PGE receptors in mouse MC3T3-E1 osteoblasts.[232] Although others reported a stimulatory effect of PTH on

PGE_2 production in osteoblasts,[313-315] we could only demonstrate a stimulatory effect in the MG-63 cell line. Rickard (1999) reported that oestrogen inhibits PGE_2 production in osteoblast-like cells[108] but we were unable to confirm these effects in the cell lines used and experimental conditions. Variation in responses may reflect species differences, differences in the mechanism of cell line immortalisation, or differences in the degree of osteoblastic differentiation. Further work, including receptor studies, needs to be done to elucidate these responses or lack thereof.

Animal as well as human studies has shown that PUFAs such as AA (representative of the n-6 PUFA family) and DHA (representative of the n-3 PUFA family) affect the structure and strength of bone.[25-29,31] Experimental work was therefore included in this study to investigate the effects of these PUFAs on osteoblastic cell proliferation (Chapter 5) as well as differentiation of these cells into mature mineralising osteoblasts (Chapter 6).