

# Specific effect of arachidonic acid on inducible nitric oxide synthase mRNA expression in human osteoblastic cells

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## A B S T R A C T

A specific modulatory effect of PUFAs (polyunsaturated fatty acids) on gene expression of some cytokines involved in bone remodelling has been reported previously. In particular, although a direct action of AA (arachidonic acid) on bone cytokine gene expression has been shown in human osteoblastic cells, OA (oleic acid) and EPA (eicosapentaenoic acid) were ineffective. Since the NO (nitric oxide) system has also been shown to have an important modulatory activity on osteoblasts, osteoclasts and bone metabolism, in the present study we have investigated the effects of PUFAs on iNOS (inducible NO synthase) gene expression in a human osteoblast-like cell line. AA induced a significant increase in iNOS mRNA expression, whereas EPA and OA had no stimulatory effects but instead caused a significant inhibition of AA-induced iNOS gene expression. Blocking of the COX (cyclo-oxygenase) pathway did not inhibit AA-induced iNOS expression. AA action was inhibited instead by the addition of calphostin C and genistein, inhibitors of PKC (protein kinase C) and tyrosine kinases respectively. Experiments performed with specific anti-cytokine antibodies showed a significant decrease in iNOS expression in AA-treated osteoblastic cells, suggesting that both cytokine-dependent and -independent mechanisms account for the effects of AA on iNOS gene expression. In conclusion, our investigation clearly shows specific effects of PUFAs on iNOS expression in human osteoblast-like cells with a cytokine-dependent and -independent mechanism. These results might have clinical relevance and are of interest for understanding the reported beneficial effects of dietary PUFA manipulation on the prevention and/or treatment of primary and secondary bone disease.

## INTRODUCTION

A specific modulatory effect of PUFAs (polyunsaturated fatty acids) on bone metabolism has been suggested by several epidemiological, clinical and experimental studies [1–3]. Our laboratory is extremely interested in the study of the relationships between PUFAs and bone

metabolism and has recently demonstrated [4] that the addition of exogenous AA (arachidonic acid) to a human osteoblast-like cell line induced a time- and dose-dependent increase in mRNA expression of some cytokines involved in bone remodelling, such as IL (interleukin)-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$  (tumour necrosis factor  $\alpha$ ) and M-CSF (macrophage colony-stimulating factor). Two

**Key words:** arachidonic acid, bone metabolism, fatty acid, nitric oxide synthase, osteoblast.

**Abbreviations:** AA, arachidonic acid; ASA, acetyl salicylic acid; COX, cyclo-oxygenase; EPA, eicosapentaenoic acid; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; HI-FCS, heat-inactivated FCS; IL, interleukin; NO, nitric oxide; iNOS, inducible NO synthase; OA, oleic acid; PG, prostaglandin; PKC, protein kinase C; PUFA, polyunsaturated fatty acid; RT, reverse transcription; TK, tyrosine kinase; TNF $\alpha$ , tumour necrosis factor  $\alpha$ .

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other fatty acids, EPA (eicosapentaenoic acid) and OA (oleic acid), an *n*-3 polyunsaturated and a monounsaturated fatty acid respectively, did not have any stimulatory effect, but resulted in a significant inhibition of AA-induced cytokine gene expression [4]. The action of AA appears to be independent of changes in COX (cyclooxygenase) products and to be mediated by the activation of intracellular signalling kinases, such as PKC (protein kinase C) and TKs (tyrosine kinases) [5].

The NO (nitric oxide) system has also been shown to have important modulatory effects on osteoblasts, osteoclasts and bone metabolism [6–11]. In the present study we have therefore investigated the effects of PUFAs on iNOS (inducible NO synthase) gene expression in a human osteoblast-like cell line and the potential cytokine-dependent mechanism accounting for the effects of AA on the NO system.

## METHODS

### Materials

RPMI 1640 medium, FCS (fetal calf serum), penicillin/streptomycin and L-glutamine were purchased from EuroClone. PBS and trypsin/EDTA were from Gibco. AA, OA, EPA, ASA (acetyl salicylic acid), calphostin C, genistein, PMA and anti-human IL-1 $\alpha$ , IL-1 $\beta$  and TNF $\alpha$  antibodies were purchased from Sigma. The GeneAmp RNA PCR kit components were purchased from Applied BioSystems. The specific primers used for the iNOS oligonucleotide were from Primm, whereas those for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were from Clontech Laboratories. All other reagents were from Sigma unless otherwise specified. All chemicals were of the highest grade available.

### Cell culture

The human osteoblast-like MG-63 cells (CRL 1427; A.T.C.C.) were used. This cell line was derived from a human osteosarcoma and shares many properties with normal osteoblasts (alkaline phosphatase activity, secretion of osteocalcin and type I and III collagen, response to parathyroid hormone, 1,25-(OH) $_2$ D $_3$  and cytokines) [12–14]. We used this cell line as an *in vitro* model to examine the functional effect of fatty acids on osteoblastic cells. MG-63 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) HI-FCS (heat-inactivated FCS), 100 international units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mmol/l L-glutamine. Cultures were maintained in T25 flasks in a humidified 5% CO $_2$  atmosphere at 37°C. Cells were seeded on to six-well plates at 80 000 cells/well and treated at subconfluency for the experiments. For the time-dependent studies, the cells were cultured under conditions of 1% (v/v) HI-FCS with AA, OA and EPA (50  $\mu$ mol/l) or vehicle (absolute ethanol) for 1, 3, 6, 12 and 24 h. For the dose-dependent

studies, cells were stimulated with various concentrations of AA, OA and EPA (25, 50, 75 and 100  $\mu$ mol/l) or with the vehicle for 3 h. In addition, co-incubation experiments were performed by treating MG-63 cells for 3 h in the presence of AA (50  $\mu$ mol/l) with increasing concentrations of OA or EPA.

A set of separate experiments was performed to determine the potential biochemical mechanism involved in AA induction of iNOS gene expression. To verify the direct action of AA on iNOS gene expression, MG-63 cells were pretreated for 1 h with ASA (1 mmol/l), a compound that blocks the COX pathway, and then co-incubated with AA (50  $\mu$ mol/l) for 3 h.

To investigate the possible involvement of PKC, MG-63 cells were pretreated for 30 min with calphostin C (0.1 mmol/l), an inhibitor of PKC [15], and subsequently treated with 50  $\mu$ mol/l AA or 0.5  $\mu$ mol/l PMA (used as positive control) for 3 h. In addition, MG-63 cells were preincubated with 0.5  $\mu$ mol/l PMA for 24 h to induce PKC down-regulation, obtained by an increase in PKC proteolysis [16], and then stimulated with 50  $\mu$ mol/l AA or 0.5  $\mu$ mol/l PMA for 3 h. The possible involvement of TKs was investigated using genistein (0.1 mmol/l), an isoflavone compound that possesses a potent TK inhibitory activity [17], for 1 h before treatment with AA for 3 h.

To explore whether the AA effect on iNOS expression was mediated by the AA modulation on bone cytokines as demonstrated previously, experiments with specific antibodies against human IL-1 $\alpha$ , IL-1 $\beta$  and TNF $\alpha$  were performed. MG-63 cells were preincubated with antibodies against IL-1 $\alpha$  (7.8 mg/ml), IL-1 $\beta$  (5 mg/ml) and/or anti-TNF $\alpha$  (10 mg/ml) for 1 h and then stimulated with 50  $\mu$ mol/l AA for 3 h. Concentrations and incubation times of antibody treatment were chosen on the basis of preliminary studies.

### RNA isolation and cDNA synthesis

Total cellular RNA was extracted using the commercial kit RNazolB (Biotecx), according to Chomczynski and Sacchi [18], as described previously [1]. Total RNA (250 ng) was used as template for cDNA synthesis in a 20  $\mu$ l reaction volume containing 2.5 units/ $\mu$ l M-MuLV (moloney murine leukaemia virus) reverse transcriptase, 2.5  $\mu$ mol/l random hexamer, 1 unit/ $\mu$ l RNasin, 1 mmol/l of each dNTP, 5 mmol/l MgCl $_2$  in 50 mmol/l KCl and 10 mmol/l Tris/HCl buffer (pH 8.3). Following subsequent incubations at room temperature for 10 min, 42°C for 30 min, 99°C for 5 min and 5°C for 5 min, the reaction was stopped and cDNAs were stored at –20°C until used.

### PCR analysis

PCR analysis was performed as described previously [1]. Briefly, 1  $\mu$ l of the RT (reverse transcription) reaction product was amplified in 25  $\mu$ l of PCR mixture

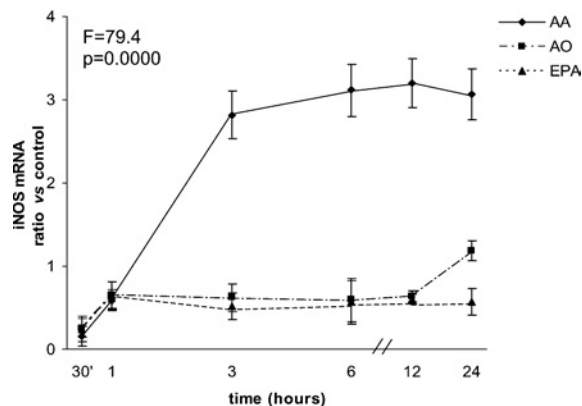
containing  $1 \times$  PCR buffer, 0.2 mmol/l of each dNTP, 1.5 mmol/l  $MgCl_2$  and 0.4  $\mu$ mol/l primers using the Jump-START-*Taq* polymerase (0.2 units/ $\mu$ l) to increase the specificity and the efficiency of the PCR reaction ('hot start' procedure). A semi-quantitative RT-PCR approach was used employing iNOS-specific primers (5'-TGTG-CTCTTTGCCTGTATGCTGAT-3', 3'-CTGAATGTGCTGTTTGCCTCGGAC-5'; the PCR product has a size of 516 bp, and represents nucleotides 1788–2304 in the iNOS gene sequence) and the housekeeping gene GAPDH as the internal control [1,19]. The amplification profile was performed in a programmable thermal cycler (MJ Research), and for selective amplification the touch-down procedure was applied consisting, after an initial 5 min denaturation at 95°C, of 1 min at 94°C, annealing for 1 min at temperatures decreasing from 64 to 59°C, the final annealing temperature (with 1°C decremental steps every two cycles), and ending with an extension step at 72°C for 1 min. The amplification profile for the GAPDH PCR product (5'-TGAAGGTCGGAGTCA-ACGGATTG-3', and 5'-CATGTGGGCCATGAGG-TCCACCAC-3'; PCR product size 983 bp) was 45 s each for melting at 94°C and annealing at 60°C, and 2 min extension at 72°C, after an initial 5 min at 95°C. A final step of 7 min extension at 72°C was also performed. The specific PCR cycles were selected within the exponential phase: 26 cycles for GAPDH and 23 cycles for iNOS. PCR products were electrophoresed on a 7% (w/v) polyacrylamide gel (3% cross-linker; 5% glycerol). Silver staining was used to visualize the gel bands and to quantify the PCR products directly on the gel. Fixed and dried gel images were captured using a scanner and GelPro-Analyzer reading densitometry software (Media Cybernetics). The amount of iNOS was measured and expressed as the ratio between the absorbance of the target gene and the standard gene PCR products.

### Statistical analysis

Results are expressed as the ratio between the level of iNOS expression following different treatments and the level of iNOS expression obtained in controls (cells treated with vehicle alone). Data represent means  $\pm$  S.D. of three different experiments performed in duplicate. Statistical analysis was carried out by ANOVA with a between-within design and by Student's *t* test, as specified.

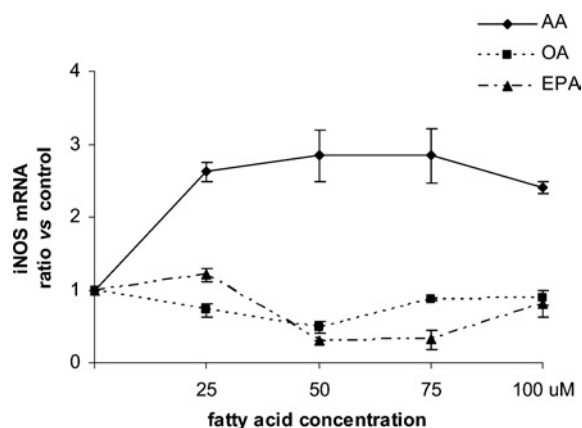
### RESULTS

Figures 1 and 2 show the time- and dose-dependent effects of the different PUFAs on iNOS gene expression in osteoblastic cells respectively. AA at 25  $\mu$ mol/l, after 3 h cell culture incubation, induced the maximum up-regulation of iNOS, which was stabilized for at least 24 h. In the light of these preliminary results, all other



**Figure 1** Time course effects of AA, OA and EPA on iNOS expression in human osteoblastic cells

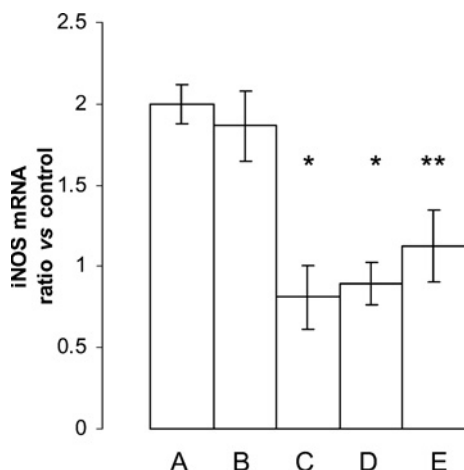
Results are expressed as the ratio between AA, OA and EPA compared with controls (cells treated with vehicle alone) in order to compare the effects of various treatments on iNOS expression. Data are means  $\pm$  S.D. of three culture experiments performed in duplicate. Statistical analysis was carried out using a between-within ANOVA design.



**Figure 2** Dose-dependent effects of AA, OA and EPA on iNOS expression in human osteoblastic cells

Results are expressed as the ratio between AA, OA and EPA compared with controls (cells treated with vehicle alone) in order to compare the effects of various treatments on iNOS expression. Data represent means  $\pm$  S.D. of three culture experiments performed in duplicate. Statistical analysis was carried out using a between-within ANOVA design.

experiments were performed using 50  $\mu$ mol/l AA with an incubation time of 3 h. On the contrary, in the same experimental conditions, OA and EPA did not have any stimulatory effect on iNOS mRNA expression. Moreover, cells treated simultaneously with AA and one of the two fatty acids had a significant inhibitory effect on AA-induced iNOS expression [ $0.73 \pm 0.07$  with AA + OA compared with  $1.87 \pm 0.06$  with AA alone ( $P < 0.001$ , as determined by Student's *t* test);  $0.52 \pm 0.05$  with AA + EPA compared with  $1.87 \pm 0.06$  with AA alone ( $P < 0.001$ )].



**Figure 3** Effects of inhibitors of intracellular signalling pathways on AA-induced iNOS expression

MG-63 cells were treated for 3 h with AA (50 μmol/l) (A), after preincubation for 1 h with ASA (1 mmol/l) (B) and genistein (0.1 mmol/l) (C), for 30 min with calphostin C (0.1 mmol/l) (D), and for 24 h with PMA (0.5 μmol/l) to induce PKC down-regulation (E). Results are expressed as the ratio compared with controls (cells treated with vehicle alone) in order to compare directly the effects of various treatments on iNOS expression. Data are expressed as means  $\pm$  S.D. of three experiments performed in duplicate, and were analysed by Student's *t* test. The inhibitory effects of different compounds on AA-induced iNOS expression were statistically significant (\**P* < 0.005 and \*\**P* < 0.01).

As shown in Figure 3, blocking the COX pathway by pretreatment for 1 h with ASA (1 mmol/l) had no inhibitory effect on iNOS expression induced by treatment with 50 mmol/l AA for 3 h (*P* value was not significant). However, the effect of AA on iNOS mRNA expression was significantly inhibited (*P* < 0.005) by the addition of calphostin C, an inhibitor of PKC, and genistein, an inhibitor of TKs. In addition, down-regulation of cellular PKC, induced by incubation with PMA for 24 h, caused a significant inhibition (*P* < 0.01) of AA-induced iNOS expression.

Pretreatment with specific antibodies against IL-1 $\alpha$ , IL-1 $\beta$  and TNF $\alpha$  together resulted in a significant decrease in iNOS mRNA expression in AA-treated MG-63 cells compared with cells treated with AA alone ( $1.25 \pm 0.12$  compared with  $2.08 \pm 0.21$  respectively; *P* < 0.005, as determined by Student's *t* test).

## DISCUSSION

The results of the present study indicate that the addition of AA to cultures of human osteoblastic cells induces a significant increase in iNOS gene expression. On the contrary, other unsaturated fatty acids, such as OA and EPA, not only had no stimulatory effect, but also prevented AA-induced iNOS gene expression when they were added to cells treated with AA. This suggests that

PUFAs have specific modulatory effects on iNOS gene expression in addition to that reported previously for mRNA expression of some cytokines involved in bone remodelling, such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$  and M-CSF [4,5].

NO, first identified as an endogenous vasodilator agent and as a mediator of macrophage cytotoxicity, is produced by NO synthase. It also plays an important role in the regulation of bone cell function, with effects on both the osteoblast and osteoclast lineage, representing one of the molecules released by osteoblasts which directly regulate osteoclastic activity [6,7,10,11]. In particular, NO derived from the iNOS pathway has been shown to regulate the effects of pro-inflammatory cytokines on bone and to be essential for the stimulatory activity of IL-1 on bone resorption both *in vitro* and *in vivo* [8–10].

Recent studies [20–22] have demonstrated that IL-1, TNF $\alpha$  and IFN $\gamma$  (interferon  $\gamma$ ) stimulate NO production through induction of iNOS expression, suggesting NO as an effector of pro-inflammatory cytokines in several bone diseases [23]. However, as shown in the present study, experiments performed using antibodies against IL-1 $\alpha$  IL-1 $\beta$  and TNF $\alpha$  only have a partial inhibitory effect on AA-induced iNOS expression, suggesting that both a cytokine-dependent and -independent mechanism account for the effects of AA on iNOS.

Hughes et al. [24], investigating the relationship between NO and PG (prostaglandin) synthesis in osteoblasts after cytokine stimulation, found the existence of both NO-dependent and-independent pathways of PG synthesis. In the present study, we observed that the AA-induced iNOS gene expression was independent of the COX pathway. In fact, the addition of ASA, an inhibitor of PG synthesis, had no effects on AA-induced iNOS gene expression.

The biochemical mechanisms responsible for AA-induced iNOS gene expression are, at present, still unclear. Recently, we have reported [5] that AA-induced IL-6 gene expression is mediated by PKC $\alpha$ , one of the conventional isoforms of PKC present in substantial amounts in osteoblasts. A similar mechanism involving PKC could play a role in AA-induced iNOS expression. This is confirmed by our observations in the present study with the PKC inhibitor calphostin C and with PKC depletion (down-regulation studies) [4,5,15,16], which resulted in a significant inhibition of AA-induced activation of iNOS expression. Moreover, it is interesting to note that the addition of genistein, a TK inhibitor [17], resulted in the reduction of AA-induced iNOS expression, also suggesting a possible TK-dependent mechanism underlying the effects of AA on iNOS gene expression in human osteoblast-like cells. On the contrary, OA and EPA not only were unable to induce either PKC activation or IL-6 gene expression, but also prevented both AA-induced PKC $\alpha$  translocation and release of the enzyme from the membranes and its proteolysis once AA-induced PKC $\alpha$

translocation had occurred [5]. Although a mechanism involving PKC $\alpha$  in AA-mediated iNOS expression has not been investigated in the present study, it is reasonable to hypothesize that the down-regulation of PKC $\alpha$  could be a key upstream event in OA- and EPA-induced inhibition of iNOS. However, further studies are required to give more insights into the biochemical mechanisms involving the effects of AA, OA and EPA on iNOS gene expression.

AA is recognized as a second messenger and can affect cell function by modulating intracellular signal transduction [25]. In particular, the important cellular effects are essentially due to the well-known AA-mediated activation of some enzymes, such as TKs and PKC isoenzymes, in different cell types [26,27]. Therefore our data showing an involvement of both PKC and TK in the action of AA on bone metabolism do not seem surprising. Several studies have, in fact, demonstrated that specific phosphorylation of NO synthase isoenzymes at serine sites is required to increase their activity in response to stimuli, including HDL (high-density lipoprotein) [28–32]. Drew et al. [33] reported that HDL and apolipoprotein AI increase eNOS (endothelial NO synthase) activity by multisite phosphorylation changes. Finally, recent studies have shown that the stimulatory action of NO donors on the expression of osteoprotegerin, which is essential in the modulation of osteoclastogenesis, appear to be regulated by a TK-dependent activation [34].

The results obtained from the present study may have important implications, since the cellular phospholipid PUFA composition could be modified by dietary manipulation. Dietary protein content and/or fatty acid quality are able, in fact, to affect the activity of  $\Delta 6$ -desaturase, the rate-limiting step in the biosynthetic pathway of highly unsaturated fatty acids [35–37]. Bone cytokine production, bone NO system modulation and bone remodelling could, therefore, be influenced by the dietary  $n-3/n-6$  PUFA ratio.

In conclusion, the present study has clearly shown the specific effects of PUFAs on iNOS gene expression in human osteoblast-like cells having cytokine-dependent and -independent mechanisms. These results, together with our previous reports demonstrating an analogous action of PUFAs on bone cytokines [4,5], confirm the crucial role of fatty acids in the regulation of bone metabolism. On the whole, our observations might have important clinical/therapeutic implications and are of interest for understanding the reported beneficial effects of diet manipulation by means of PUFAs on the prevention and/or treatment of primary and secondary bone disease, in addition to their well-known benefits on the cardiovascular system. Further *in vivo* and *in vitro* investigations are, however, needed for a better understanding of the complex relationships between PUFAs, the NO system and cytokines in bone metabolism and remodelling.

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