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## ORIGINAL ARTICLE

# Arachidonic acid inhibits osteoblast differentiation through cytosolic phospholipase $A_2$-dependent pathway

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**OBJECTIVE: Arachidonic acid, a precursor of prostaglandins (PGs), is released by phospholipase $A_2$ ($PLA_2$) and plays an important role in biological reactions. We examined the roles of arachidonic acid on the pathway of PG synthesis and osteoblast differentiation by using clone MC3T3-E1 cells.**

**MATERIALS AND METHODS: The effect of arachidonic acid was evaluated by the measurement of alkaline phosphatase activity, cells shape, production of arachidonic acid and the expression of cyclooxygenase (COX).**

**RESULTS: Arachidonic acid dose dependently decreased alkaline phosphatase activity and increased $PGE_2$ production in MC3T3-E1 cells. The cell shape changed from polygonal to fibroblastic following treatment with arachidonic acid. These effects were recovered by the treatment of NS-398 and indomethacin. Arachidonic acid increased the expression of COX-2 mRNA and the $PGE_2$ production. The exogenous arachidonic acid induced the release of cellular arachidonic acid in MC3T3-E1 cells. Moreover, methylarachidonyl fluorophosphonate suppressed the arachidonic acid release and the expression of COX-2 mRNA.**

**CONCLUSION: The present results indicate that exogenous arachidonic acid stimulated the activity of $PLA_2$, leading to the new release of membranous arachidonic acid. The amplified arachidonic acid enhanced $PGE_2$ production by COX-2, which inhibits the differentiation of MC3T3-E1 cells. Our results provide a new insight into the molecular mechanisms by which exogenous arachidonic acid plays a role as a paracrine/autocrine amplifier of $PGE_2$ biosynthesis by coupling with $PLA_2$ and COX-2.**

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## Introduction

Arachidonic acid, a *cis*-polyunsaturated essential fatty acid, plays important roles in biological processes such as cell growth (Peppelenbosch *et al*, 1995; Graber *et al*, 1996), chemotaxis (Siegel *et al*, 1982), inflammation (Irvine, 1982) and signal transduction (Axelrod *et al*, 1988). Arachidonic acid has also been reported to be a potent activator of several intracellular signaling kinases such as protein kinase C, extracellular signal-regulated kinases and c-Jun N-terminal kinase (McPhail *et al*, 1984; Rao *et al*, 1994; Hii *et al*, 1995; Cui and Douglas, 1997). Arachidonic acid is a component of membrane phospholipids and is released by phospholipase $A_2$ ($PLA_2$) from the cell membrane in response to a wide range of stimuli (Davidson and Dennis, 1990; Exton, 1994). $PLA_2$ regulates the production of prostaglandins (PGs) and leukotrienes (Van den Bosh, 1980) as a rate-limiting enzyme, and exogenous $PLA_2$ increases the arachidonic acid release and PG biosynthesis (Shinohara *et al*, 1999). $PLA_2$ catalyzes the hydrolysis of membrane phospholipids at the sn-2 position to release arachidonic acid. The released arachidonic acid is converted into PGs, thromboxanes and leukotorienes by cyclooxygenases (COXs) (Vane, 1994; Otto and Smith, 1995).

Osteoblasts produce prostaglandin $E_2$ ($PGE_2$) as a major prostanoid among the several arachidonate metabolites (Rodan *et al*, 1981). $PGE_2$ is described as a bone resorption factor both *in vitro* and *in vivo* (Klein and Raisz, 1970). However, bone-forming activity of $PGE_2$ was also demonstrated in some reports (Jee *et al*, 1985; Nagata *et al*, 1994). MC3T3-E1 cells, a mouse osteoblastic cell line, produce only $PGE_2$ as arachidonic acid metabolites (Yokota *et al*, 1986). In MC3T3-E1 cells, exogenous $PGE_2$ induces both stimulatory and inhibitory effects on cell growth and osteoblastic differentiation depending on the concentrations (Hakeda

*et al*, 1985). Recently, the presence of multiple $PGE_2$ receptors was clarified and their differences were associated with the multiple actions of $PGE_2$ in osteoblasts (Farndale *et al*, 1988; Muallem *et al*, 1989; Hagel-Bradway *et al*, 1991; Suzawa *et al*, 2000).

Although the $PGE_2$ action has been focused upon bone tissues, there are few reports about arachidonic acid release and its concerning enzymes in bone tissues. In the present study, we investigated the effects of arachidonic acid on the expression of bone metabolic markers and examined the effects of exogenous arachidonic acid on the pathway of cytosolic phospholipase $A_2$ and COXs in MC3T3-E1 cells.

## Materials and methods

*Materials*

Alfa-Modified Eagle's minimum essential medium (α-MEM) was obtained from ICN Biosciences (Aurora, OH, USA) and fetal bovine serum (FBS) from Wako Pure Chemicals (Osaka, Japan). Arachidonic acid was purchased from Sigma Chemical Co. (St Louis, MO, USA). NS-398, indomethacin, methylarachidonyl fluorophosphonate (MAFP) and STAT-Prostaglandin $E_2$ Enzyme Immunoassay Kit were from Cayman (Ann Arbor, MI, USA); TRIZOL reagents were from Invitrogen (Carlsbad, CA, USA). Hybond-N+ membrane, [5,6,8,9,11,12,14,15-$^3$H] arachidonic acid and [$^{32}$P] dCPT were from Amersham Life Science (Little Chalfont, UK). DNA-labeling kit was from Takara (Kyoto, Japan). All other materials used were commercial products of the highest grade available.

*Cell culture*

MC3T3-E1 (Sudo *et al*, 1983), $3.0 \times 10^5$ cells, were suspended in α-MEM containing 10% FBS and 100 units $ml^{-1}$ of penicillin G and plated in 90-mm plastic dishes. Cultures were maintained at 37°C in a humidified atmosphere consisting of 5% $CO_2$ in air. The medium was changed every 2 days. After confluence, cells were plated at 6200 cells per $cm^2$ in a 35-mm culture dish and maintained for 7 days under the same condition.

*Determination of alkaline phosphatase activity*

For the assay of alkaline phosphatase (ALP) activity, cells were cultured for 7 days and then incubated for 24 h with or without arachidonic acid. After the incubation, cells were washed in phosphate-buffered saline (PBS), scraped in ice-cold 50 mM Tris–HCl buffer (pH 7.4) and sonicated twice for 20 s, using a sonifier-cell disrupter (Model UR-20P; Tomy, Tokyo, Japan). The sonicates were vortexed for 10 min at 4°C and centrifugation was performed at 10 000 *g* for 10 min. ALP activity in the supernatant was then determined with *p*-nitrophenyl phosphate as a substrate according to the method of Lowry *et al* (1954).

*Northern blot analysis*

Total cellular RNA was extracted from cell layers by the TRIZOL reagent method indicated by the manufacturer. Ten micrograms of RNA was separated by electrophoresis in 1% agarose gels containing 2 M formaldehyde and transferred onto nylon membranes. The cDNA probes were labeled with [$^{32}$P] dCPT using a random-primer DNA-labeling kit. Prehybridization was performed for more than 4 h at 42°C in 50% formamide–5 × SSPE [1 × SSPE: 0.18 M NaCl, 10 mM $NaH_2PO_4$ and 1 mM EDTA (pH 7.7)], 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 200 $\mu g\ ml^{-1}$ of salmon sperm DNA. Hybridization was performed at 42°C for 18–24 h in the same solution with $10^6$ dpm $ml^{-1}$ of $^{32}$P-labeled cDNA probes. The membrane was washed twice in 2 × SSPE–0.1% SDS for 20 min at 42°C. The relative radioactivity of each band was estimated using a BAS2000 imaging analyzer (Fujix, Tokyo, Japan). The mRNA levels of COX-1 and COX-2 were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

*Measurement of $PGE_2$ secretion*

Aliquots of culture medium from arachidonic acid-treated cells were assayed for $PGE_2$ secretion using a STAT-Prostaglandin $E_2$ Enzyme Immunoassay Kit. This assay is based on competition between $PGE_2$ and a $PGE_2$–ALP conjugate for a limited amount of $PGE_2$ monoclonal antibody. Briefly, 50 $\mu l$ of culture medium was added to 96-well plates. $PGE_2$ in the medium bound to $PGE_2$–ALP conjugate and $PGE_2$ monoclonal antibody, and the antibody–$PGE_2$ complex bound to a goat anti-mouse polyclonal antibody, which was attached to the 96-well plate. The plate was washed to remove unbound reagents and then *p*-nitrophenyl phosphate was added to the wells. The product of this enzymatic reaction showed a distinct yellow color and was quantified with 405 nm using a plate reader (Bio Rad, Richmond, CA, USA).

*Measurement of [$^3$H]-arachidonic acid metabolites*

Confluent cells were treated with 18.5 MBq $\mu l^{-1}$ of [$^3$H]-arachidonic acid for 18 h. To remove the free radio-labeled arachidonic acid, which was not incorporated into cellular lipids, cells were washed three times with serum-free medium containing 0.5 mg $ml^{-1}$ bovine serum albumin (BSA) and then treated with 100 $\mu$M arachidonic acid for 18 h. The culture medium was assayed to measure the radioactivity of the released [$^3$H]-arachidonic acid metabolites with a liquid scintillation counter (Aloka, Tokyo, Japan). Aliquots of culture media were separated by thin-layer chromatography (TLC) in a mixture of ether/petroleum ether/acetic acid (85:15:0.1, v/v) at 4°C and the plate was heated for 1 h in an oven at 100°C. Separated arachidonic acid and $PGE_2$ were detected by UV and silica gels collected from the spots on the TLC plate were assayed for radioactivity with a liquid scintillation counter as described above.

*Statistical analysis*

Data were expressed as mean values ± s.e.m. and were analyzed by Student's *t*-test calculations by StatView

(Abacus Concept, Berkeley, CA, USA). The acceptable level of significance was $P < 0.01$.

## Results

*Effect of exogenous arachidonic acid on ALP activity and $PGE_2$ secretion in MC3T3-E1 cells*

MC3T3-E1 cells reached confluency on day 5 after the plating. ALP activity then began to increase and became constant on days 5–12. At 7 days after confluency, MC3T3-E1 cells were treated for 24 h with various concentrations of arachidonic acid (1, 10 and 100 $\mu$M). Figure 1A shows that arachidonic acid decreased the ALP activity in a dose-dependent fashion, showing 83% and 48% of the control value at 10 and 100 $\mu$M, respectively. Similar suppression of ALP activity was observed in the cells on day 10 after confluency (data not shown). Arachidonic acid increased the $PGE_2$ secretion dose dependently with maximum concentration being 100 $\mu$M. As shown in Figure 1B, the cell shape was changed from polygonal to fibroblastic by the treatment with arachidonic acid. In the 100 $\mu$M arachidonic acid-treated cells, most cells show fibroblastic morphology.

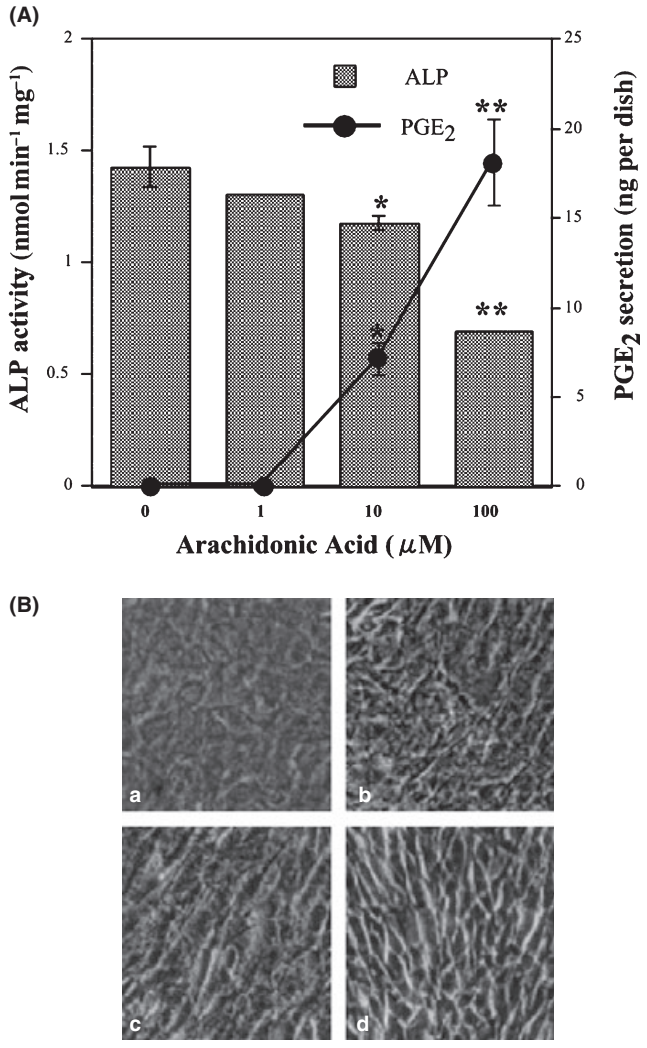


**Figure 1** Effects of exogenous arachidonic acid on alkaline phosphatase (ALP) activity and prostaglandin $E_2$ ($PGE_2$) secretion in MC3T3-E1 cells. MC3T3-E1 cells were plated at 6200 cells per $cm^2$ in 35-mm dishes and cultured for 7 days. (**A**) Various concentrations of arachidonic acid were then exposed for 24 h, followed by the determination of ALP activity and $PGE_2$ production. The values are mean values ± s.e.m. from four separate samples. The experiments were performed three times and the most representative data were presented. Asterisks show significant differences from the control value ($*P < 0.05$ and $**P < 0.005$). (**B**) The morphologic change of MC3T3-E1 cells treated without (a) or with arachidonic acid at 1 $\mu$M (b), 10 $\mu$M (c) and 100 $\mu$M (d) was examined

*Effect of exogenous arachidonic acid in the presence of COX inhibitors*

Before the addition of arachidonic acid, NS-398 (a specific inhibitor for COX-2) or indomethacin (an inhibitor for both COX-1 and COX-2) was pre-incubated for 30 min. As shown in Figure 2A, ALP activity which was suppressed by 100 $\mu$M arachidonic acid treatment was completely recovered to the control level by the NS-398 and indomethacin treatments. Moreover, $PGE_2$ secretion stimulated by 100 $\mu$M arachidonic acid was suppressed in accordance with the recovery of ALP activity. The morphology of the cells treated with NS-398 is shown in Figure 2B. While arachidonic acid-treated cells changed from polygonal to fibroblastic, NS-398 dose dependently recovered the cells to polygonal shape. Similar result was observed in the indomethacin-treated cultures (data not shown).

*Effect of arachidonic acid on COX mRNA expression and $PGE_2$ secretion*

The effects of 100 $\mu$M arachidonic acid on the expression of COX-1 and COX-2 mRNA were investigated (Figure 3a). The expression of COX-1 mRNA was not affected by the arachidonic acid treatment by 6 h, whereas a slight increase was detected at 12–24 h (approximately twofold). Arachidonic acid increased the expression of COX-2 mRNA in a time-dependent manner until 3 h. The maximal effect was observed at 2–3 h, showing a more than 60-fold increase, and then the expression level rapidly decreased up to 24 h. The expression level of COX-2 mRNA in control cells were consistently low during the treatment. Figure 3b shows that secretion of $PGE_2$ was markedly increased by the treatment with 100 $\mu$M arachidonic acid. The marked increase of $PGE_2$ was observed at 3–9 h after arachidonic acid treatment.

*Effects of exogenous arachidonic acid on the secretion of radiolabeled arachidonate metabolites*

MC3T3-E1 cells were prelabeled with [$^3$H]-arachidonic acid to investigate the mobilization of arachidonic acid metabolites. Figure 4a shows that radiolabeled arachidonic acid metabolites were secreted to the culture medium by treatment with exogenous arachidonic acid. When the cells were treated with 100 $\mu$M arachidonic acid, the secretion of [$^3$H]-arachidonic acid metabolites increased in a time-dependent manner, reached a maximum at 3 h and retained at higher level until 18 h (Figure 4a). The secreted [$^3$H]-arachidonic acid metabolites were separated into arachidonic acid (Figure 4b)

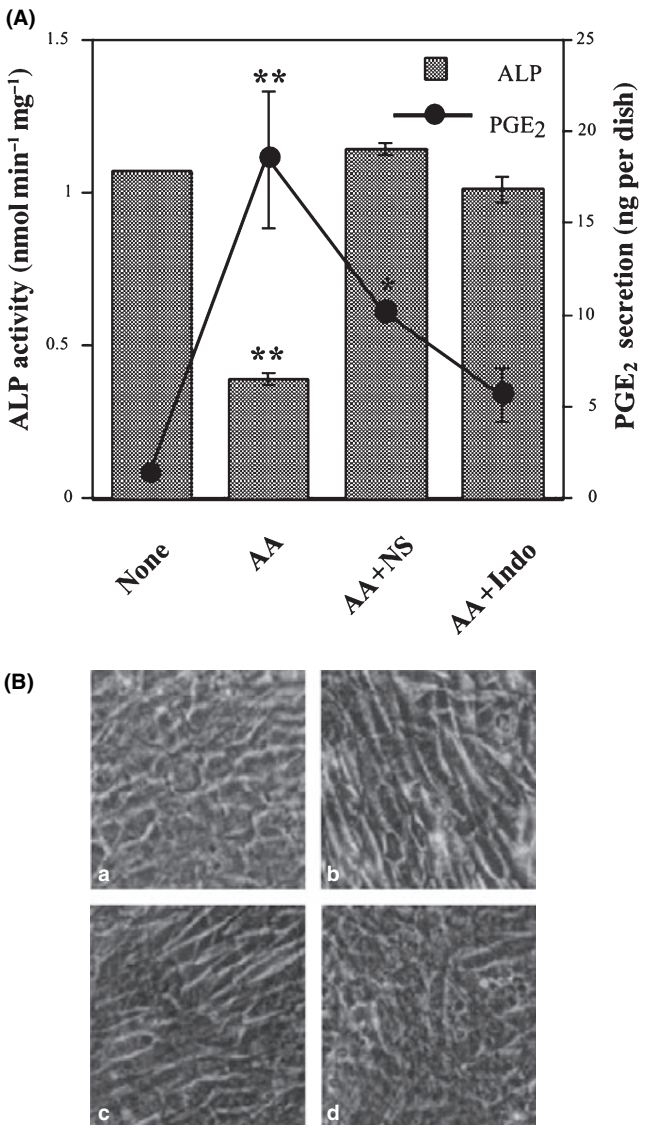


**Figure 2** Effects of cyclooxygenase (COX) inhibitors on alkaline phosphatase (ALP) activity and prostaglandin $E_2$ ($PGE_2$) secretion in MC3T3-E1 cells. The cells were cultured for 7 days and pretreated for 30 min with 5 $\mu$M NS-398 (NS) or 5 $\mu$M indomethacin (Indo) before treatment with 100 $\mu$M arachidonic acid for 24 h (AA). (**A**) ALP activity and $PGE_2$ were determined and shown as mean values ± s.e.m. from four separate samples. The experiments were performed three times and the most representative data were presented. Asterisks show significant differences from the control value (none). *$P < 0.05$ and **$P < 0.005$. (**B**) Morphologic change of cells treated without (a) or with 100 $\mu$M arachidonic acid (b), pretreated for 30 min with NS-398 at 0.5 $\mu$M (c) or 5 $\mu$M (d) before the treatment with 100 $\mu$M arachidonic acid

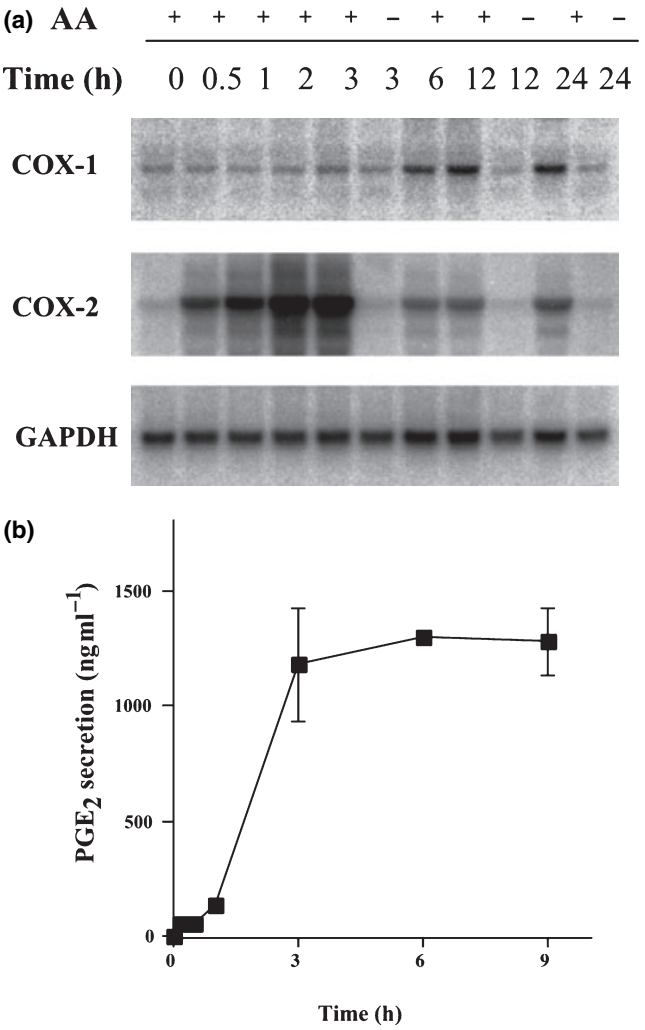


**Figure 3** Effect of exogenous arachidonic acid on cyclooxygenase (COX) mRNA and prostaglandin $E_2$ ($PGE_2$) secretion in MC3T3-E1 cells. (**a**) MC3T3-E1 cells were plated at 4700 cells per cm$^2$ in 90-mm dishes, cultured for 7 days and then treated with 100 $\mu$M arachidonic acid (AA) for the indicated time points. The levels of COX-1, COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by Northern blot hybridization. (**b**) MC3T3-E1 cells were plated at 6200 cells per cm$^2$ in a 35-mm dishes, cultured for 7 days and treated with 100 $\mu$M arachidonic acid for various times. The secretion of $PGE_2$ was measured as described in the Materials and methods section. The values are mean values ± s.e.m. from four separate samples. The experiments were performed three times and the most representative data were presented

and $PGE_2$ (Figure 4c) by TLC. The level of [$^3$H]-arachidonic acid increased in a time-dependent manner, reached a maximum at 3 h and then decreased. After 6 h, the labeled radioactivity was not detected. Secretion of [$^3$H]-arachidonic acid was detected as early as 5 min of exogenous arachidonic acid treatment (Figure 4b). The amount of secreted radiolabeled $PGE_2$ increased up to 3 h and then retained at higher level until 24 h, the profile of the secretion was similar to that of the secreted [$^3$H]-arachidonic acid metabolites described in Figure 4a (Figure 4c). Within 30 min, no radiolabeled $PGE_2$ was detected in the culture media (Figure 4c). These results indicate that within 30 min, the secreted [$^3$H]-arachidonic acid metabolite is arachidonic acid.

*Effect of inhibitors of COX and cPLA$_2$ on arachidonate mobilization*

To further determine the nature of released free arachidonic acid we examined the effect of cytosolic $PLA_2$ ($cPLA_2$), which facilitates the release of arachidonic acid from cell membrane phospholipids, on the arachidonic acid release. As shown in Figure 5a, pretreatment of

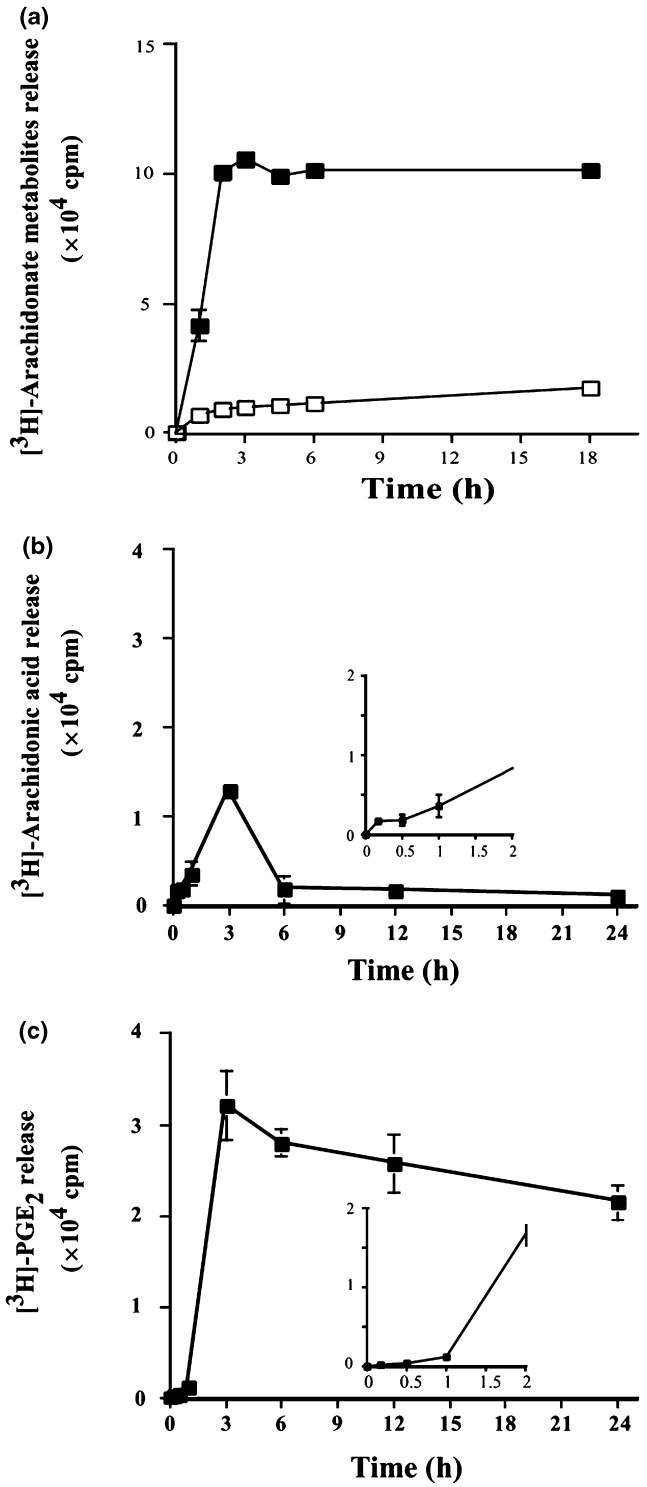


**Figure 4** Effect of exogenous arachidonic acid on secretion of radiolabeled arachidonate in MC3T3-E1 cells. (**a**) The cells were cultured for 7 days and radiolabeled with 18.5 MBq $\mu l^{-1}$ of [$^3$H]-arachidonic acid for 18 h. The cells were then untreated (open square) or treated with 100 $\mu$M arachidonic acid (closed square) for various terms indicated and radioactivity was measured. Secreted arachidonic acid (**b**) and prostaglandin $E_2$ ($PGE_2$) (**c**) were separated by thin-layer chromatography, collected and radioactivity measured

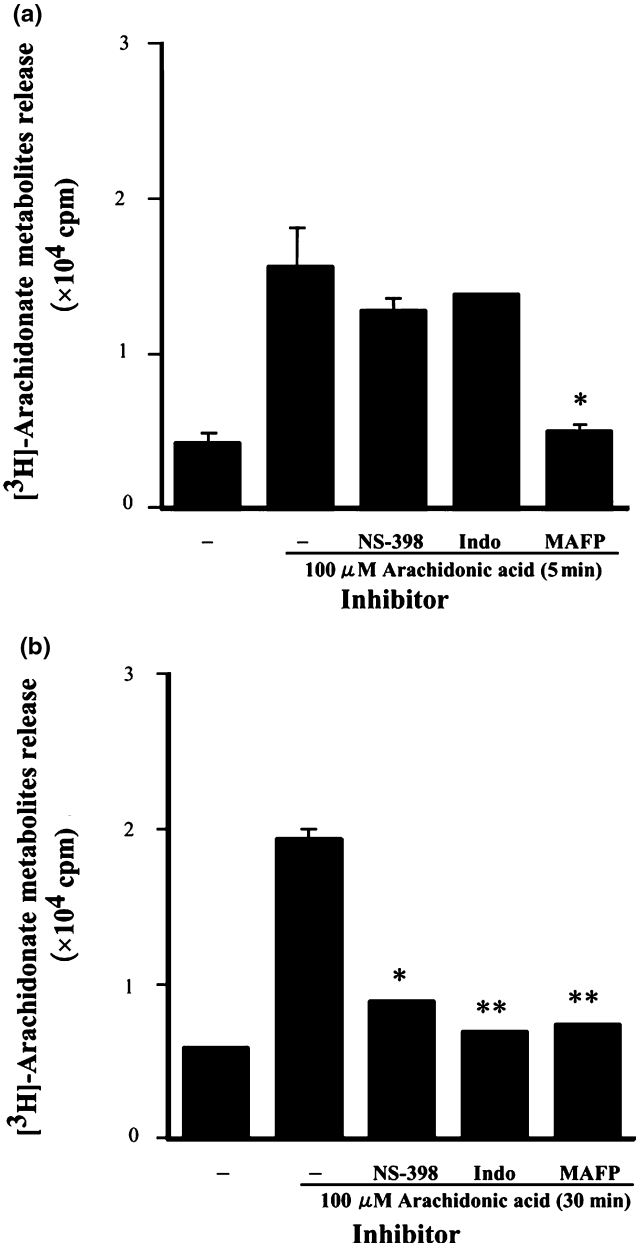


**Figure 5** Effect of cyclooxygenase (COX) and cytosolic phospholipase $A_2$ ($cPLA_2$) inhibitors on secretion of radiolabeled arachidonate in MC3T3-E1 cells. MC3T3-E1 cells were cultured for 7 days and labeled with 18.5 MBq $\mu l^{-1}$ of [$^3$H] arachidonic acid for 18 h. The cells were then pre-incubated with 5 $\mu$M NS-398 (NS), 5 $\mu$M indomethacin (Indo) or 25 $\mu$M methylarachidonyl fluorophosphonate (MAFP) for 30 min. The cells were then treated with 100 $\mu$M arachidonic acid for 5 min (**a**) or 30 min (**b**). The experiments were performed three times and the most representative data were presented. The values are mean values ± s.e.m. from four separate samples. Asterisks show significant differences from the single treatment with 100 $\mu$M arachidonic acid. *$P < 0.001$ and **$P < 0.0001$

MAFP ($cPLA_2$ inhibitor) completely inhibited the secretion of arachidonate metabolites within 5 min after the addition of exogenous arachidonic acid. However, this inhibition was not observed in the treatment

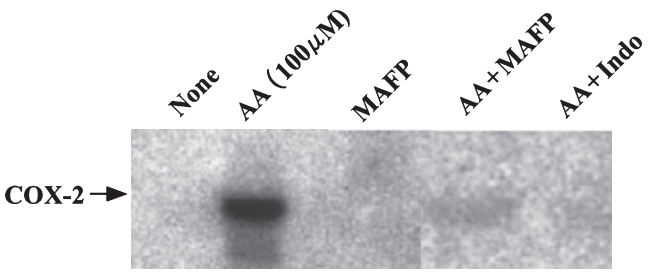


**Figure 6** Effect of cytosolic phospholipase $A_2$ ($cPLA_2$) inhibitor on cyclooxygenase-2 (COX-2) mRNA expression in MC3T3-E1 cells. The cells were plated at 4700 cells per $cm^2$ in 90-mm dishes and cultured for 7 days, pre-incubated with 25 $\mu$M methylarachidonyl fluorophosphonate (MAFP) or 5 $\mu$M indomethacin (Indo) for 30 min and treated for 3 h with 100 $\mu$M arachidonic acid (AA). The levels of COX-2 mRNA was analyzed by Northern blot hybridization

of NS-398 and indomethacin. These results indicate that arachidonate metabolites released within 5 min were free arachidonic acid. On the other hand, at 30 min after addition of arachidonic acid, all these inhibitors completely blocked the secretion of arachidonate metabolites, suggesting that free arachidonic acid produced by $cPLA_2$ was converted into $PGE_2$ (Figure 5b).

*Effect of $cPLA_2$ inhibitor on COX-2 mRNA*

Finally, we examined the effect of MAFP on COX-2 mRNA expression. As shown in Figure 6, the expression of COX-2 mRNA was augmented by 100 $\mu$M arachidonic acid treatment. This effect was almost completely suppressed by MAFP as well as by indomethacin. These results indicate that COX-2 induction by exogenous arachidonic acid was dependent on $cPLA_2$ activity.

## Discussion

In this study, we demonstrated that exogenous arachidonic acid stimulated $cPLA_2$ activity, and then induced new release of membranous arachidonic acid. This excessive arachidonic acid augmented the $PGE_2$ production through induction of COXs. Our present results suggest that arachidonic acid can regulate eicosanoids production by amplifying their own release in the first step of the arachidonic acid cascade. The exogenous arachidonic acid decreased the ALP activity and changed the cell shape from polygonal to fibroblastic. MC3T3-E1 cells are fibroblastic in the growth stage and become polygonal with multiple layers in the differentiation stage (Sudo *et al*, 1983). ALP activity is also detected in the early developmental stage and possibly plays a primary role in mineralization as a progression marker in osteoblastic differentiation (Beck *et al*, 1998; Fedde *et al*, 1999). Based on these findings, exogenous arachidonic acid inhibited differentiation of MC3T3-E1 cells in the present study. Moreover, ALP activity and cell shape were regulated in parallel with the production of $PGE_2$, and these effects were recovered by COX inhibitors which block the $PGE_2$ production, indicating that $PGE_2$ closely correlates with the differentiation of osteoblasts. It was reported that $PGE_2$ has multiple actions in osteoblasts: bone-resorbing activity (Klein and Raisz, 1970; Kajii *et al*, 1999) and bone-forming activity (Yang *et al*, 1993). This discrepancy of $PGE_2$ actions is thought to be mediated by subtypes of PGE receptors (EP1, EP2, EP3 and EP4) present in osteoblastic cells. It has been reported that EP1 and EP4 are detected in MC3T3-E1 cells, and EP1 agonist decreased the ALP activity and changed the cell shape to a fibroblastic appearance (Suda *et al*, 1996). Thus, the exogenous arachidonic acid should regulate the osteoblastic differentiation in the pathway downstream of EP1.

COX is an enzyme that converts arachidonic acid to the intermediate PG precursor and then regulates the PG biosynthetic pathway. Two isozymes, COX-1 and COX-2, were identified. COX-1 is constitutively expressed in several mammalian tissues (DeWitt, 1991) and COX-2 is undetectable under physiologic conditions but markedly induced by several cytokines and growth factors (Kujubu *et al*, 1991; O'Banion *et al*, 1991; Harrison *et al*, 1994; Kawaguchi *et al*, 1995; Onoe *et al*, 1996). In the present study, MC3T3-E1 cells slightly expressed COX-2 mRNA in the control cells at day 7 after confluency. This may be because of the presence of FBS because FBS induces the COX-2 mRNA level in MC3T3-E1 cells (Pilbeam *et al*, 1995). In the present study, arachidonic acid induced the new release of [$^3$H]-labeled arachidonic acid metabolites. Our results with TLC separation revealed that the early stage release of arachidonate metabolites is arachidonic acid rather than $PGE_2$. The release of arachidonate metabolites was also suppressed within 5 min by the inhibitor of $cPLA_2$. In contrast, COX inhibitors did not affect the release of arachidonic acid metabolites in 5 min, but blocked it in 30 min. These results indicate that the induced [$^3$H]-labeled arachidonic acid metabolites at early stage are arachidonic acid, and then COXs convert them to $PGE_2$ after 30 min. MC3T3-E1 cells are genetically deficient in functional type IIA $cPLA_2$ (Kennedy *et al*, 1995). Therefore, our results suggest that $cPLA_2$ is required for the new release of arachidonic acid induced by exogenous arachidonic acid, leading to the production of $PGE_2$. Interestingly, arachidonic acid could not induce the release of free arachidonic acid or the expression of COX-2 mRNA in the presence of MAFP (Figures 5 and 6). These results indicate that the induction of arachidonic acid by $cPLA_2$ is necessary to activate the pathway downstream of arachidonic acid. Thus arachidonic acid acts as an amplifier of itself by coupling with a $cPLA_2$ in our model. Our results are in agreement with some reports showing that $cPLA_2$-dependent arachidonic acid release is essential for the $PGE_2$ synthesis induced by IL-1$\alpha$ in human fibroblasts and mouse osteoblastic cells (Lin *et al*, 1992; Chen *et al*, 1997).

In conclusion, the present study provides a new insight into the molecular mechanisms that the exogenous arachidonic acid plays a role as a paracrine/autocrine amplifier of $PGE_2$ biosynthesis by coupling with $cPLA_2$ and COX-2.

## Acknowledgements

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