Arachidonic Acid-Induced IL-6 Expression Is Mediated by PKC α Activation in Osteoblastic Cells†

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ABSTRACT: There are several pieces of evidence supporting the important role that essential fatty acids (EFAs) and their metabolites play in regulating calcium and bone metabolism, and their relevance to the pathobiology of bone disease, with particular reference to modulating effects on cytokines. We found that arachidonic acid (AA) triggers a cell signal in osteoblasts and leads to the expression of IL-6. To explore the biochemical pathways involved in AA induction of cytokine gene expression, we evaluated the potential protein kinase C (PKC) dependent mechanism accounting for the AA effect on IL-6 gene expression. The osteoblast-like cell line MG-63 was pretreated with calphostin C, a PKC inhibitor, or phorbol 12-myristate 13-acetate (PMA) for an extended period, a condition which causes PKC downregulation, and subsequently with AA. After these treatments, IL-6 gene expression was no longer evident. We also showed that PKC and, in particular, PKC α, which are both recruited to the particulate fraction, undergo proteolysis and autophosphorylation; all of these steps are required for PKC activation and, subsequently, for AA-induced signaling. It is interesting that other unsaturated fatty acids, such as oleic acid (OA) or eicosapentaenoic acid (EPA), are unable to induce either PKC activation or IL-6 gene expression.

There are several pieces of evidence supporting the important role that essential fatty acids (EFAs) and their metabolites play in regulating calcium and bone metabolism and their relevance to the pathobiology of bone disease (*1*, *2*). EFAs are important factors of calcium transport by mitochondria, isolated brush borders, and basolateral membrane vesicles (*3*). In addition, in idiopathic calcium nephrolithiasis, a relationship has been reported between plasma phospholipid arachidonic acid (AA) content, serum calciotropic hormone concentration, intestinal calcium absorption, calcium urinary excretion, and biochemical markers of bone resorption (*4*). Some researchers have also demonstrated that, in animals, diet supplemented with various EFAs has type EFA-dependent effects both on calcium homeostasis, including calcium intestinal absorption and bone content, and on the development of osteoporosis, suggesting a close link between fatty acids and bone metabolism (*3*, *5*). This hypothesis was recently confirmed by our observations, demonstrating the specific modulatory effect of AA on the gene expression of various cytokines in a human osteoblast-like cell line (*6*). Cell treatment with AA induced an increase in mRNA expression of IL-1α, IL-1β, TNF-α, and M-CSF, which are important local factors regulating the recruitment and activity of osteoclasts and osteoblasts and thus play a crucial role in bone remodeling (*6*, *7*). It is noteworthy that two other fatty acids, oleic acid (OA), a monounsaturated fatty acid, and eicosapentaenoic acid (EPA), an $n - 3$ polyunsaturated fatty acid, had no stimulatory effect on cytokine gene expression (*6*).

Previous data concerning the presence of calphostin C or PMA-induced cellular PKC downregulation have also shown a total inhibition of mRNA expression of various cytokines, further supporting the possible direct PKC involvement in osteoblast gene expression (*6*). PKC has been implicated in the signal pathway of various cell responses (*8*), including bone resorption stimulation (*9*, *10*). According to their structural differences and enzymatic properties, PKC isoforms may be divided into (a) conventional or "classical" isoforms (PKC α, βI/βII, γ) which require both Ca^{2+} and phospholipids for activation, (b) novel Ca^{2+}-independent isoforms (PKC δ, ε, ε′, η, θ), and (c) atypical isoforms (PKC ζ, υ, ν) which require neither Ca^{2+} nor phospholipids for activation and, in contrast with both conventional and novel isoforms, do not respond to phorbol ester (for a review, see refs *11* and *12*). Because of their divergence in structure, the isoforms of the PKC family show manifold sensitivity to activation by DAG, phorbol ester, and fatty acids and are mainly found in several subcellular compartments (*13*).

In exploring the biochemistry underlying AA induction of cytokine gene expression, we evaluated the potential PKC-dependent mechanism causing the effect of AA on IL-6 gene expression, which is a major osteoclast stimulator produced by osteoblasts (*7*). The present study shows that AA-induced gene expression is mediated by PKC and, in particular, by PKC α, which, besides being recruited to the particulate

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fraction, undergoes proteolysis and autophosphorylation. All of these steps are required for PKC activation and, subsequently, for transmission of AA-induced signaling.

EXPERIMENTAL PROCEDURES

Materials

RPMI 1640 medium, FCS, penicillin−streptomycin, and L-glutamine were purchased from EuroClone (CELBIO srl). Calphostin C, arachidonic acid (AA), eicosapentaenoic acid (EPA), and oleic acid (OA) were purchased from Sigma. RNAzolB was purchased from Biotecx. A GeneAmp RNA kit was purchased from Applied Biosystems, and the Jump-START-Taq polymerase was purchased from Sigma. Commercially available IL-6 oligonucleotide RT-PCR primers were obtained from Perkin-Elmer GeneAmp PCR Instrument Systems. The specific primers used for G3PDH were obtained from Clontech Laboratories. [γ-^{32}P]ATP and [^{32}P]-P_i were from Amersham Pharmacia Biotech. Antibodies were from Santa Cruz Biotechnology. The protease inhibitor cocktail and ECL were from Amersham Pharmacia Biotech. All chemicals were of the highest grade available.

Methods

Effects of Arachidonic Acid (AA), Eicosapentaenoic Acid (EPA), and Oleic Acid (OA) on Intact Cells. The human osteoblast-like cell line MG-63 was used. This cell line shares many properties with normal osteoblasts [alkaline phosphatase activity, secretion of osteocalcin and collagen types I and III, response to parathyroid hormone, 1,25-$(OH)_2D_3$, and cytokines] (*14*−*17*) and is widely used as an in vitro model for studies of osteoblast activity. MG-63 cells (ATCC, CRL 1427) were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum (HI-FCS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cell cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C. In all experiments, cells were cultured in conditions of 1% HI-FCS in the presence of OA, EPA, or AA at increasing concentrations from 25 to 100 μM and the vehicle (absolute ethanol) for all other conditions. Final alcohol concentration did not exceed 1:10000.

Cell Treatments. MG-63 cells were seeded into six-well plates at 80000 cells/well and, when subconfluent, washed twice in phosphate-buffered saline (PBS) and treated as described below. Cells were treated for 30 min, 1 h, 3 h, 12 h, and 24 h (in time-dependent studies) or with increasing concentrations (25, 50, 75, and 100 μM) (in dose-dependent studies) of AA, OA, EPA, or the specific vehicle of each compound for 3 h. To determine the potential biochemical mechanism involved in AA induction of cytokine gene expression, the following separate experiments were performed: (1) MG-63 cells were pretreated with 0.1 mM calphostin C, an inhibitor of protein kinase C (PKC) (*18*), for 30 min and subsequently treated with 75 μM AA or 0.5 μM PMA (used as positive control) for 3 h; (2) MG-63 cells were preincubated with 0.5 μM PMA for 24 h to induce PKC downregulation obtained by an increase in PKC proteolysis (*19*) and then stimulated with 75 μM AA or 0.5 μM PMA for 3 h; (3) MG-63 cells were incubated with 75 μM AA and 70 μg/mL calpeptin. When required, MG-63 cells, preincubated for 18 h in the medium supplemented with 25 μCi of [^{32}P]P_i, were incubated in the presence of 1 μM okadaic acid (in order to prevent the action of Ser/Thr-protein phosphatase) for 10 min, then 75 μM AA (or vehicle) was added, and incubation was continued for an additional 3 min.

RT-PCR Analysis. At the end of treatments, cells were washed twice in cold PBS, and total cellular RNA was extracted using the RNAzolB method according to the manufacturer's instructions; cDNA synthesis was carried out as previously described (*6*). Briefly, 250 ng of total RNA was retrotranscribed using a commercially available kit. Semiquantitative RT-PCR using IL-6-specific primers was applied, using the housekeeping gene G3PDH (glyceraldehyde-3-phosphate dehydrogenase) as internal control (*20*−*22*). The "hot start" procedure was applied using Jump-START-Taq polymerase (Sigma, 0.2 unit/μL). The amplification profile was processed in a thermal cycler, and 26 specific cycles were selected for G3PDH and 36 cycles for IL-6. Silver staining was used to visualize gel bands and to quantify the PCR products directly on the gel. In all experiments, the relative amount of cytokine was normalized to the level of controls (cell cultures treated with vehicle) to compare directly the effects of different treatments on IL-6 gene expression.

Preparation of Subcellular Compartments. Cells, treated as described above, were removed from culture dishes by scraping, resuspended in buffer A (20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 2 mM EDTA, 8 mM EGTA, protease inhibitor cocktail), centrifuged at 3000 rpm for 3 min, and washed once in the same buffer. Packed cells were resuspended in 200 μL of buffer A, sonicated for 9 s at 50 mW, and microfuged for 20 min at 14000 rpm at 4 °C. An aliquot of the supernatant was used for protein determination according to Lowry (*23*) and for Western blot analysis. Pellets were resuspended in 200 μL of extraction buffer (20 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Nonidet P-20, 4 mM EGTA, 4 mM EDTA, 20 mM β-mercaptoethanol, protease inhibitor cocktail), shaken for 1 h at 4 °C, and microfuged at 14000 rpm for 20 min. An aliquot of extract−supernatant was used for protein determination and Western blot analysis.

Western Blot Analysis. Samples of supernatant and extract−supernatant were mixed with 2% SDS and 1% β-mercaptoethanol (final concentrations) followed by 5 min treatment at 100 °C and subjected to 0.1% SDS−10% PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes using a Hoefer apparatus at 400 mV for 2 h, immunostained with the appropriate antibody, and visualized by enhanced chemiluminescence.

Anti-PKC α *Immunoprecipitations.* Supernatants and 1:1 buffer B (20 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM NaCl, 50 μM PMSF) diluted membrane extract−supernatants were incubated for 5 h at 4 °C with anti-PKC α antibody bound to protein A−Sepharose. When required, the antibodies were added to the cellular lysate obtained by extracting treated cells with 400 μL of extraction buffer for 2 h in the same conditions described above, diluted 1:1 with buffer B. Immunocomplexes were washed three times in 50 mM Tris-HCl buffer, pH 7.5, containing protease inhibitor cocktail and used for activity assays.

Immunocomplex Kinase Assays. The activity of immunoprecipitated (IP) anti-PKC α was monitored for 10 min at

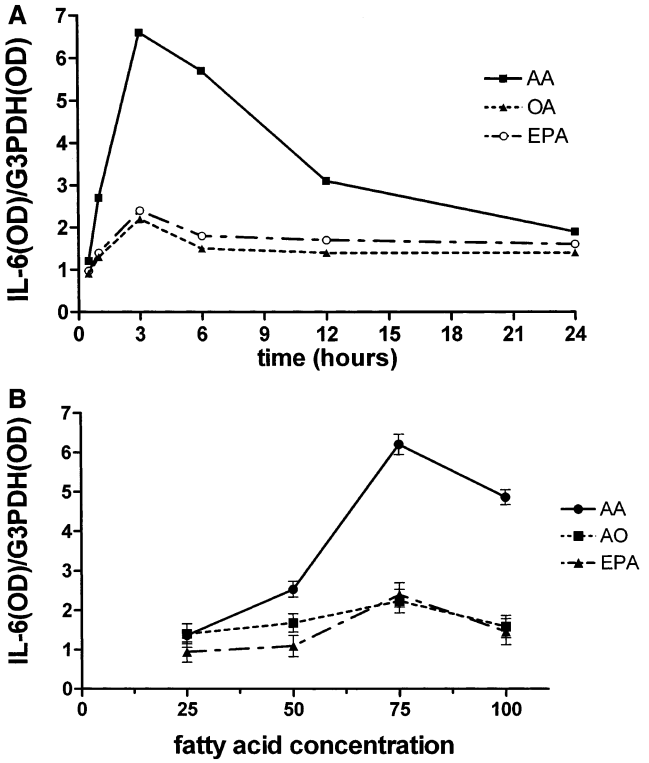

FIGURE 1: Time- (panel A) and dose- (panel B) dependent effects of AA, OA, and EPA on IL-6 gene expression. Cells were incubated with fatty acids, and IL-6 gene expression was evaluated as described in Methods. Values are means of four separate experiments (vertical bars: SE).

30 °C in 30 μL of incubation mixture containing 50 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 20 μM [γ-^{32}P]ATP (specific activity 2000 cpm/pmol), 50 μg/mL phosphatidylserine, and 0.1 mg/mL myelin basic protein, in the presence or absence of 1 mM $CaCl_2$. Reactions were stopped by the addition of 1% SDS and 1% β-mercaptoethanol (final concentrations), followed by 5 min heating at 100 °C. Solubilized proteins were submitted to 0.1% SDS−15% PAGE, dried, and counted for radioactivity in a Packard instant imager.

RESULTS

Effect of AA, OA, or EPA on mRNA Expression of IL-6 in MG-63 Cells. To understand the role of AA on IL-6 mRNA expression, we incubated the osteoblastic cell line MG-63 with AA, at the final concentration of 75 μM, at the time points indicated in Figure 1A. IL-6 gene expression was enhanced after 1 h, reached a maximum after 3 h, and quickly decreased to control levels after 24 h. AA also stimulated IL-6 mRNA in a dose-dependent manner in the range between 25 and 100 μM (Figure 1B) with a maximum effect at 75 μM. Instead, the same type of experiments carried out in the presence of EPA or OA did not stimulate IL-6 gene expression (Figure 1).

PKC Involvement in AA-Mediated Cytokine Expression: Effects of Calphostin C and PMA Long-Term Preincubation on IL-6 Expression. The possibility that AA-induced IL-6 expression depends on PKC activation was then examined. To test this hypothesis, we verified the effect of calphostin C, a strong and specific inhibitor of PKC (*18*), on IL-6 gene expression induced by AA in MG-63 cells. Cells were

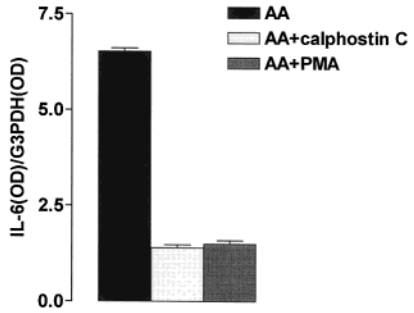

FIGURE 2: Effects of calphostin C and PMA pretreatment on AA-induced IL-6 gene expression. Cells were pretreated with 0.1 mM calphostin C for 30 min, or 0.5 μM PMA for 24 h, as described in Methods and subsequently incubated with 75 μM AA. Control cells were directly incubated with AA for 3 h. Reported values are means of three separate experiments (vertical bars: SE).

preincubated with calphostin C (0.1 μM) for 30 min and subsequently stimulated with 75 μM AA for 3 h. As shown in Figure 2, calphostin C was able to prevent AA-induced gene expression. It is noteworthy that the same results were obtained when cells were incubated with AA after long-term PMA pretreatment, which caused PKC downregulation (*19*) (Figure 2). Both of these experiments indicate that PKC is involved in the AA-induced IL-6 expression.

Phosphorylation Is Involved in AA Stimulation of Osteoblastic Cells. To investigate further the pathway involved in AA-induced cytokine expression, we preincubated osteoblastic cells in a culture medium containing [^{32}P]P_i (as described in Methods). Cells were then treated with 75 μM AA for 3 min. Incubation was stopped by the addition of cold buffer A, and cells were lysed in the same buffer by ultrasound. Supernatant and particulate fractions were obtained by microfuging for 30 min and analyzed by 0.1% SDS−10% PAGE. Compared with the control, in the particulate fraction AA treatment induced a selective increase in phosphorylation of three high molecular mass bands, ranging from 70 to 200 kDa, even with the generalized decrease of other phosphorylated bands (with a loss of total incorporated radioactivity ranging from 20% to 30%).

These data suggest that, in our experimental model, AA treatment is able to modify the phosphorylation state of protein bands. Moreover, since AA increases the phosphorylation level of only one subset of phosphorylable proteins with generalized inhibition of phosphorylation, and since these bands range from 70 to 200 kDa, including an 82 kDa band which may be PKC, an immunoprecipitation assay with anti-PKC antibodies of the cellular lysate from the previously ^{32}P-labeled osteoblastic cells was carried out. Results indicate that the radioactivity of the PKC-IP from the AA-treated ^{32}P cells was 30% higher than in the corresponding control, suggesting that the AA-mediated signal pathway involves PKC autophosphorylation.

PKC and PKC α *Redistribution in AA-Mediated Cytokine Expression.* As the above results explain PKC involvement in the AA-induced signal pathway, we further investigated possible modifications of the PKC status quo after fatty acid treatment. Figure 3A shows that MG-63 cells submitted to AA incubation for 3 min showed a redistribution of PKC from the supernatant, where it is mainly located in resting cells, to the particulate fraction. Since previous studies (*19*) showed that PKC α is present in substantial amounts in

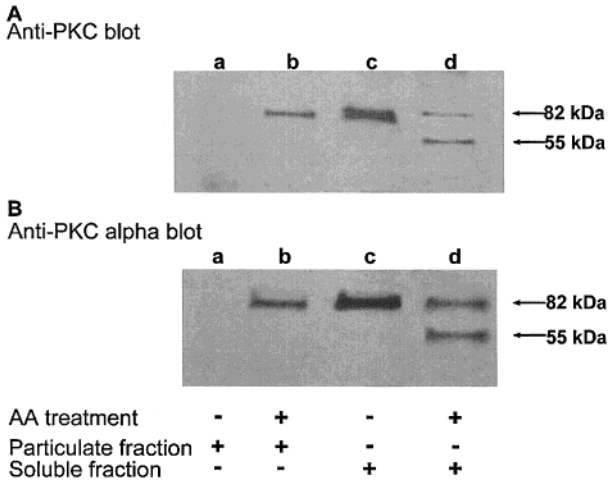

FIGURE 3: Effects of AA on PKC (panel A) and PKC α (panel B) redistribution between particulate (lanes a and b) and soluble (lanes c and d) fractions. Cells were treated for 3 min with 75 μM AA (lanes b and d) or vehicle (lanes a and c), sonicated, and centrifuged as described in Methods. Aliquots (10 μg) of each sample were subjected to 0.1% SDS−10% PAGE, electrotransferred to nitrocellulose membranes, and immunostained with anti-PKC or anti-PKC α antibodies. The figure is representative of three separate experiments.

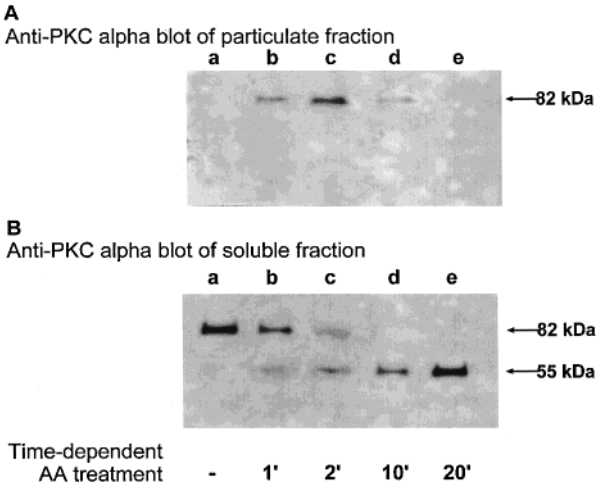

FIGURE 4: Time-dependent effects of AA on PKC α redistribution between particulate (panel A) and soluble (panel B) fractions. Cells were treated with 75 μM AA for 1 (lanes b), 2 (lanes c), 10 (lanes d), and 20 (lanes e) min. Lanes a represent control cells. After incubation, cells were sonicated, and particulate fractions were separated from the soluble counterparts as described in Methods. Aliquots (10 μg) of each sample were analyzed by 0.1% SDS−10% PAGE, electrotransferred, and immunostained with anti-PKC α antibodies. The figure is representative of three separate experiments.

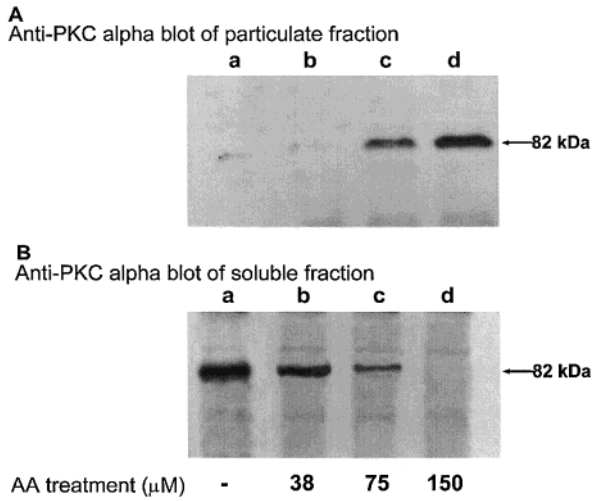

FIGURE 5: Dose-dependent effects of AA on PKC α redistribution between particulate (panel A) and soluble (panel B) fractions. Cells were incubated for 2 min with 38 (lanes b), 75 (lanes c), and 150 (lanes d) μM AA or the vehicle (lanes a) and processed as described above. Aliquots (10 μg) of each sample were submitted to 0.1% SDS−10% PAGE, electrotransferred to nitrocellulose, and immunostained with anti-PKC α antibodies. The figure is representative of three separate experiments.

osteoblastic cells, we tested both supernatant and particulate fractions with anti-PKC α antibodies in Western blot assays. Figure 3 shows that the same redistribution observed for PKC was also evident for PKC α. Moreover, during cell treatment with AA, an additional band, identified by both anti-PKC and anti-PKC α antibodies, was observed at about 55 kDa in the soluble fraction. A similar pattern was obtained by PMA-induced PKC α translocation in human erythrocytes (*24*). This is in line with the previously observed direct effect of unsaturated fatty acid on PKC in the cytosol of human platelets in cell-free conditions without DAG on Ca^{2+} generation (*25*, *26*).

PKC α Response to AA-Induced Cell Activation. Time- and dose-dependent studies were also carried out to better explain PKC α behavior following AA treatment of the cells. MG-63 cells were incubated in 75 μM AA for 1, 2, 10, and 20 min (Figure 4), sonicated, microfuged to separate particulate (Figure 4A) and soluble (Figure 4B) fractions, submitted to Western blot analysis, and immunostained with anti-PKC α antibodies. Results indicate that PKC α, located in the soluble compartment in control cells (Figure 4B), was absent in the corresponding particulate fraction (Figure 4A) and translocated to the membrane fraction in a time-dependent manner, peaking after 2 min of AA treatment.

In the supernatant counterpart (Figure 4B), AA-induced PKC α redistribution was evidenced by the total disappearance of the corresponding band after 2 min treatment, compared with the control supernatant. Moreover, when the incubation time was increased, the PKC α band located in the particulate fraction decreased (Figure 4A) and the enzyme was recovered in the corresponding soluble fractions (Figure 4B), although in a proteolyzed form. In fact, the amount of the lower molecular mass band present in the soluble fraction, revealed by anti-PKC α antibodies, gradually increased during AA treatment. After 20 min (Figure 4B), the soluble compartment showed only the lower molecular mass band instead of the whole PKC α band, suggesting that AA treatment, besides inducing swift translocation of total PKC α from soluble to particulate fractions, also caused PKC α proteolysis after enzyme translocation, simultaneously with enzyme release to the soluble fraction. When different AA concentrations were used for cell treatment, Western blot analysis of soluble (Figure 5A) and particulate (Figure 5B) fractions immunostained with anti-PKC α antibodies revealed the AA dose dependence of PKC α translocation, evidenced by the faster action of the enzyme in leaving the soluble compartment to reach the particulate fraction.

Specific Effect of AA on PKC α Translocation. Other fatty acids, such as OA and EPA, used at the same concentration and time−course incubation as those used for AA treatment,

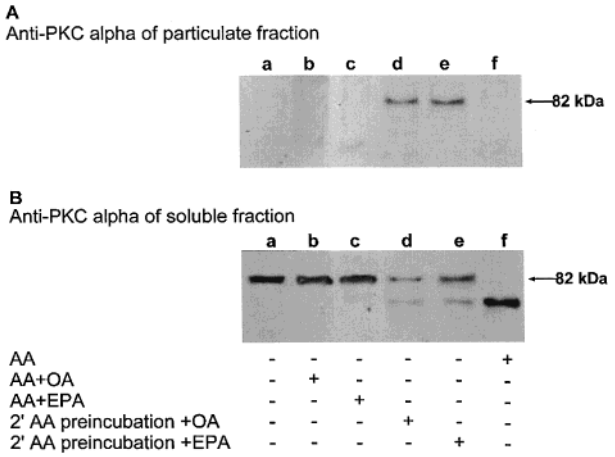

FIGURE 6: Combined effect of OA or EPA added with AA on PKC α redistribution between particulate (panel A) and soluble (panel B) fractions. Cells were incubated with 75 μM AA (lanes b and c) and 75 μM OA (lanes b) or EPA (lanes c) or vehicle (lanes a) for 2 min. Incubation was stopped, and cells were processed as described above. Lanes d and e indicate experiments performed with addition of 75 μM OA (lanes d) or 75 μM EPA (lanes e) to 2 min AA-pretreated cells. Samples were subsequently incubated up to 20 min. Lanes f represent 20 min AA-treated cells (controls). Aliquots (10 μg) of the corresponding particulate and soluble fractions from each sample were analyzed by 0.1% SDS−10% PAGE, electrotransferred, and immunostained with anti-PKC α antibodies. The figure is representative of three separate experiments.

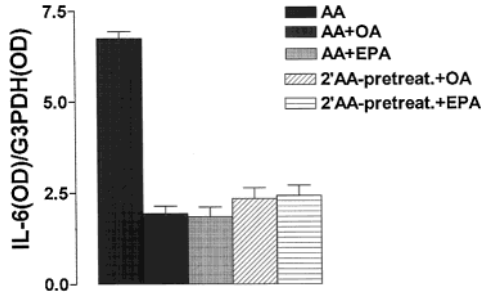

FIGURE 7: Combined effects of OA and EPA added with AA on IL-6 gene expression. Cells were treated with AA, and OA or EPA was added either together with AA at the onset of incubation or after 2 min AA pretreatment. IL-6 gene expression was evaluated as described in Methods. Reported values are means of three separate experiments (vertical bars: SE).

did not cause any PKC α redistribution (data not shown), in agreement with the lack of modification in gene expression (Figure 1). However, when OA or EPA was used together with AA in the cell incubation medium, two different responses were obtained, depending on incubation conditions: if OA (or EPA) was added to osteoblastic cells together with AA for 2 min (Figure 6), no PKC α modification was seen between soluble (Figure 6B) and particulate (Figure 6A) fractions. Simultaneous analysis of IL-6 mRNA expression revealed that no stimulation occurred in the cells (Figure 7).

Instead, if OA and/or EPA were (was) added to the cell culture after 2 min AA treatment and incubation was extended up to 20 min, the pattern of PKC α distribution between soluble and particulate fractions did change (panels B and A, lanes d and e, of Figure 6, respectively). In this case, AA-induced PKC α translocation occurred, as expected, and the proteolyzed enzyme band was found in the soluble fraction (Figure 6B). Surprisingly, after OA or EPA addition

Table 1: PKC α-IP Activity Assayed on MBP[a]

fatty acids	PKC α-IP activity
vehicle	1500 ± 61
AA	8500 ± 218
OA	1800 ± 68
EPA	1750 ± 62

[a] MG-63 cells, treated in the presence of different fatty acids, were processed as described in Methods, and supernatant compartments were immunoprecipitated with anti-PKC α antibodies. PKC α-IP was then incubated with myelin basic protein in the activity assay medium (see Methods) and subjected to 0.1% SDS−15% PAGE. Radioactivity incorporated on the MBP substrate was counted in an Instant Imager and expressed as cpm. Values are mean ± SD of three separate experiments.

and sustained incubation time, the PKC α band in the particulate fraction was unable to return to the cytosol counterpart, as previously shown (lanes f and Figure 5). These findings suggest that OA and EPA can prevent both AA-induced PKC α translocation and release of the enzyme from the membranes and its proteolysis, once AA-induced PKC α translocation has occurred. In these conditions, i.e., with OA and/or EPA added after 2 min AA treatment, cytokine expression (Figure 7) was slightly higher than that of the control, but not comparable with that obtained with AA alone, in contrast with the total absence of mRNA production obtained when OA and/or EPA were (was) added to the cell culture together with AA.

AA-Induced Cytokine Expression Requires PKC α Activation. Taken together, the above results suggest that PKC α translocation from the soluble to particulate fraction is required for AA-induced cytokine expression. In addition, simple translocation is not sufficient to activate cytokine expression but must be accompanied by enzyme proteolysis and its subsequent release into the supernatant, as shown by the above results. However, there are no reports on whether this mechanism also involves PKC α activation, so we assayed the activities of PKC α immunoprecipitates recovered from the cytosol. Results (Table 1) clearly show that PKC α-IP maintained activity 4−5-fold higher compared with the control PKC α-IP, thus confirming that AA-induced action involves a PKC α recruitment mechanism which includes translocation, proteolysis, and enzyme activation. (Extraction and immunoprecipitation of PKC α from the corresponding membranes failed to keep the enzyme in an active form.)

This was further confirmed by data on cells cultured with AA and calpeptin, a selective inhibitor of calpain (*27*), which is thought to be one of the proteolytic enzymes responsible for PKC proteolysis (*28*), because membranous fractions from cells treated with AA and calpeptin maintained their PKC α bands even after 20 min incubation (Figure 8A), compared with the total disappearance of the enzyme with AA alone. The corresponding supernatants showed no proteolytic PKC α form (Figure 8B). Gene expression of AA−calpeptin-treated cells was at the control level (data not shown). These data indicate that PKC α proteolysis is necessary for enzyme activation, which emerged as a condition required for AA-induced IL-6 expression.

DISCUSSION

PKC family isoforms show varying sensitivity to activation by DAG, phorbol ester, and fatty acids. These differences

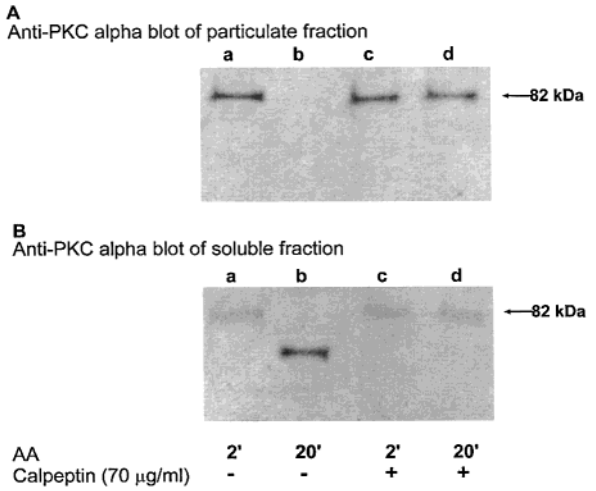

FIGURE 8: Effects of calpeptin on AA-induced PKC α translocation from soluble (panel B) to particulate (panel A) fractions. Cells were incubated in 75 μM AA alone or AA plus 70 μg/mL calpeptin for 2 min (lanes a and c, respectively) or for 20 min (lanes b and d, respectively). Soluble and particulate fractions were obtained as previously described, and 10 μg of each sample was analyzed by 10% SDS−PAGE, electrotransferred, and immunostained with anti-PKC α antibodies. The figure is representative of three separate experiments.

in location and sensitivity create a situation in which fatty acids may act as second messengers in synergy with or independently of DAG and Ca^{2+}. The conventional isoforms are members of the PKC family preferentially activated by fatty acids (*25*, *29*, *30*). However, one direct role of AA-induced signal transduction could no longer be shown. The present results demonstrate that AA is a fatty acid able to induce IL-6 mRNA expression in osteoblastic cells and that PKC is involved in this signal transduction mechanism.

PKC is directly involved in the AA-induced signaling pathway, as clearly shown by the dramatic loss of the AA-induced signal pathway in the presence of calphostin C as well as of PMA-prolonged cell treatment, which causes PKC downregulation (*19*). In both of these conditions of total PKC defeat, no IL-6 expression occurs. Furthermore, the evidence that a modification in the phosphorylation pattern, including PKC autophosphorylation, occurs following AA treatment in osteoblasts indicates that PKC involvement includes enzyme activity. These data are in agreement with previous findings demonstrating that the interaction of unsaturated fatty acids with cytosol PKC may also induce its translocation to the membrane, where it can be activated by DAG (*31*). In addition, a previous report (*25*) shows that AA only induces phosphorylation of a subset of the phosphoproteins which are phosphorylated by phorbol ester activation of PKC in the cytosol of human platelets, raising the possibility that this compound has a particular effect on PKC activity and specificity. All of these data, together with the original observation by McPhail et al. in 1984 (*32*) that the activation of PKC by AA and other unsaturated fatty acids is an effect which is not inhibited by cyclooxygenase or lypooxygenase inhibitors, suggest that AA, and not its metabolites, enhances the activity of PKC (*33*, *34*) as a second messenger in signal transduction (see review in ref *35*).

Recent findings also show that various fatty acids may be differentially involved in the mediating pathway, as indicated by the fact that, whereas arachidonic acid induces slow translocation of some PKC subspecies (ϵ and γ) from the cytoplasm to the perinuclear region, other fatty acids induce rapid translocation to the plasma membrane, thus confirming that each PKC subspecies has a specific target mechanism which depends on extracellular signals (*8*) and that a combination of intracellular activators alters the target site of PKCs (*36*). AA is also involved in PKC stimulation in addition to PKC translocation, as shown by recent findings in polymorphonuclear neutrophils (*26*).

It is noteworthy that, whereas AA induces PKC and, in particular, PKC α rapid translocation from the soluble to the membrane compartment in both time- and dose-dependent manners, other fatty acids such as OA or EPA do not affect PKC distribution, thus resulting in the specific key role of AA in osteoblastic IL-6 production. However, OA and EPA modulate the AA response of cells by preventing PKC translocation, when added together with AA or after 2 min of AA treatment, by preventing PKC release from the membranes, i.e., by exerting a competitive action causing PKC inactivation. Although 2 min AA treatment does induce PKC translocation, the addition of OA or EPA blocks the enzyme in the membrane compartment, preventing its proteolysis and subsequent activation. That proteolysis is necessary for PKC α activation as well as for the PKC α-mediated signal pathway is demonstrated by the absence of IL-6 gene expression in the presence of OA or EPA. The same result is also obtained by adding calpeptin, a specific inhibitor of calpain, which is thought to be responsible for PKC degradation (*27*).

When PKC and PKC α are released into the soluble compartment after AA-induced translocation, they are in a proteolyzed form, have higher phosphorylative activity (about 5 times higher than in controls), and are 30% more phosphorylated than controls. This would account for PKC activation, according to recent findings suggesting that PKC degradation takes place only toward the activated enzyme (*37*). Taken together, these data indicate that AA-induced IL-6 gene expression is mediated by PKC/PKC α, one of PKC's conventional isoforms present in substantial amounts in osteoblasts (*19*). PKC involvement requires enzyme translocation and proteolysis, both necessary for the correct signal pathway to occur. These two preliminary steps include enzyme activation and "auto"-phosphorylation, as confirmed by PKC α-IP experiments. This is in line with observations on platelets, in which AA in microparticles can influence platelet activation in a PKC-dependent manner by activating a membrane-linked signaling cascade culminating in the expression of COX-2 (*38*).

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