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Arachidonic Acid Differentially Affects Basal and Lipopolysaccharide-Induced sPLA$_2$-IIA Expression in Alveolar Macrophages through NF-κB and PPAR-γ–Dependent Pathways

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ABSTRACT

Secretory type IIA phospholipase A_2 (sPLA$_2$-IIA) is a critical enzyme involved in inflammatory diseases. We have previously identified alveolar macrophages (AMs) as the major pulmonary source of lipopolysaccharide (LPS)-induced sPLA$_2$-IIA expression in a guinea pig model of acute lung injury (ALI). Here, we examined the role of arachidonic acid (AA) in the regulation of basal and LPS-induced sPLA$_2$-IIA expression in AMs. We showed that both AA and its nonmetabolizable analog, 5,8,11,14-eicosatetraynoic acid (ETYA), inhibited sPLA$_2$-IIA synthesis in unstimulated AMs. However, only AA inhibited sPLA$_2$-IIA expression in LPS-stimulated cells, suggesting that this effect requires metabolic conversion of AA. Indeed, cyclooxygenase inhibitors abolished this down-regulation. Prostaglandins PGE_2, PGA_2, and 15d-PGJ_2 also inhibited the LPS-induced sPLA$_2$-IIA expression. Nuclear factor-κB (NF-κB) was found to regulate sPLA$_2$-IIA expression in AMs. Both AA and ETYA inhibited basal activation of NF-κB but had no effect on LPS-induced NF-κB translocation, suggesting that suppression of sPLA$_2$-IIA synthesis by AA in LPS-stimulated cells occurs via a NF-κB–independent pathway. 15-Deoxy-$\Delta^{12,14}$-PGJ_2 and ciglitazone, which are, respectively, natural and synthetic ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ), inhibited LPS-induced sPLA$_2$-IIA synthesis, whereas PPAR-α ligands were ineffective. Moreover, electrophoretic mobility shift assay showed PPAR activation by AA and PPAR-γ ligands in LPS-stimulated AMs. Our results suggest that the down-regulation of basal sPLA$_2$-IIA expression is unrelated to the metabolic conversion of AA but is dependent on the impairment of NF-κB activation. In contrast, the inhibition of LPS-stimulated sPLA$_2$-IIA expression is mediated by cyclooxygenase-derived metabolites of AA and involves a PPAR-γ–dependent pathway. These findings provide new insights for the treatment of ALI.

Elevated levels of fatty acids (FAs) are linked to a variety of metabolic and inflammatory diseases including obesity, diabetes, atherosclerosis, and lung inflammation (Horrobin, 1995; Bowton et al., 1997; Arbibe et al., 1998). A key metabolic step in the production of FAs might involve phospholipase A_2 (PLA_2). These enzymes catalyze the hydrolysis of the *sn*-2 fatty acyl chain of phospholipids, thereby generating lysophospholipids and free FAs such as arachidonic acid (AA) (Van den Bosch, 1980). Several mammalian intracellular and secretory PLA_2s (sPLA$_2$s) have been described previously (Six and Dennis, 2000). Among sPLA$_2$s, the type IIA sPLA$_2$ (sPLA$_2$-IIA), also referred to as synovial PLA_2, is a proinflammatory enzyme found to be highly elevated both in the circulation and locally in the tissue, in association with a number of pathological conditions such as atherosclerosis, asthma, and acute lung injury (ALI) (Chilton et al., 1996; Arbibe et al., 1997; Hurt-Camejo et al., 1997). The expression of sPLA$_2$-IIA is found to increase in many inflammatory cells,

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ABBREVIATIONS: FA, fatty acid; PLA_2, phospholipase A_2; AA, arachidonic acid; sPLA$_2$, secretory phospholipase A_2; sPLA$_2$-IIA, secretory type IIA phospholipase A_2; ALI, acute lung injury; AM, alveolar macrophage; IL, interleukin; TNF, tumor necrosis factor; LOX, lipoxygenase; COX, cyclooxygenase; CYP, cytochrome P450 epoxygenase; PG, prostaglandin; NF-κB, nuclear factor-κB; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; ETYA, 5,8,11,14-eicosatetraynoic acid; CAPE, caffeic acid phenethyl ester; EMSA, electrophoretic mobility shift assay; NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; Wy14,643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal.

including guinea pig alveolar macrophages (AM) (Vial et al., 1995), upon stimulation with a variety of proinflammatory stimuli such as cytokines (IL-1β, TNF-α, IL-6) and LPS.

AA, a major metabolically important FA present in mammalian cells, has been implicated in many biological functions as well as in the regulation of gene expression (Khan et al., 1995). Specifically, AA was found to suppress the transcription rate of a number of genes, such as stearoyl-CoA desaturase 1 and glucose transporter 4 in 3T3-L1 adipocytes (Long and Pekala, 1996; Sessler et al., 1996). Stuhlmeier et al. (1996) have shown that AA suppresses gene expression of the adhesion molecules E-selectin and ICAM-1, as well as the IL-8 in stimulated endothelial cells. This FA can act directly as a second messenger or can be further metabolized by three different enzyme systems—cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 epoxygenase (CYP)—to generate eicosanoid signaling molecules including prostaglandins (PGs), leukotrienes and thromboxanes (Shimizu and Wolfe, 1990). Recently, several works have reported that FAs and PGs are able to regulate gene expression through transcription factors such as NF-κB or peroxisome proliferator-activated receptors (PPARs) (Camandola et al., 1996; Kliewer et al., 1997; Thommesen et al., 1998). Camandola et al. (1996) reported that AA-induced NF-κB activation is mediated by the metabolization of AA to PGs and leukotrienes. PLA$_2$s have also been shown to be involved in the TNFα-mediated NF-κB activation via the generation of AA and other mediators (Thommesen et al., 1998).

In the last decade, the critical role of PPARs in the regulation of lipid metabolism and inflammation has become increasingly apparent. PPARs are members of the nuclear receptor superfamily of ligand-dependent transcription factors, which, upon heterodimerization with the 9-*cis*-retinoic acid receptor, bind specific DNA sequence elements termed PPAR response elements (PPREs), thus regulating the expression of target genes (Chinetti et al., 2000). PPREs have been identified in the regulatory regions of a variety of genes that are involved in lipid metabolism, as well as in rat sPLA$_2$-IIA promoter (Lemberger et al., 1996; Couturier et al., 1999). Three different subtypes have been described: PPAR-α, PPAR-δ (also called -β or NUC-I), and PPAR-γ (Chinetti et al., 2000). Recently, PPAR-γ has been shown to be important in the modulation of inflammatory responses in peripheral macrophages and monocytes (Jiang et al., 1998; Ricote et al., 1998). Like other nuclear hormone receptors, the ability of PPAR-γ to function as a transcription factor depends on its binding to a ligand. 15-Deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) and the thiazolidinediones, such as ciglitazone, can act as direct ligands for PPAR-γ (Spiegelman, 1998).

We have shown previously that unsaturated FAs inhibit basal sPLA$_2$-IIA expression in guinea pig AMs (Alaoui-El-Azher et al., 2000). In the present study, we focus on the signaling mechanisms involved in the regulation of sPLA$_2$-IIA expression by AA in both unstimulated and LPS-stimulated cells. We demonstrate that the NF-κB pathway is required for the induction of sPLA$_2$-IIA gene expression. Direct alteration of the NF-κB translocation by AA contributes to a negative regulation of the basal sPLA$_2$-IIA expression. In contrast, inhibition of LPS-induced sPLA$_2$-IIA synthesis by AA is not related to the inhibition of nuclear translocation of NF-κB. This process is mediated by COX metabolites of AA and involves a PPAR-γ–dependent mechanism.

Experimental Procedures

Materials. Male Hartley guinea pigs were obtained from Elevages Saint-Antoine (Pleudaniel, France). RPMI 1640 medium, antibiotics, and PBS without Ca^{2+} and Mg^{2+} were from Invitrogen (Cergy-Pontoise, France). Fetal calf serum was from Jacques Boy (Reims, France). Aspirin, flurbiprofen, benzamidine, aprotinin, leupeptin, *N*-ethylmaleimide (NEM), soybean trypsin inhibitor, PMSF, EDTA, DTT, arachidonic acid, 5,8,11,14-eicosatetraynoic acid (ETYA), eicosapentaenoic acid, and oleic acid were from Sigma (St. Louis, MO). All PGs were from Cayman (Massy, France). *Escherichia coli* O55:B5 LPS was from Difco Laboratories (Detroit, MI). 1-Aminobenzotriazole, NS-398, caffeic acid phenethyl ester (CAPE), rabbit polyclonal anti-PPAR-γ, ciglitazone, and Wy14,643 were from Biomol (Le Perray-en-Yvelines, France). Polyclonal anti-p50 and anti-p65 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxidase-linked donkey anti-rabbit IgG and an enhanced chemiluminescence Western blotting detection system was purchased from Amersham Biosciences (Little Chalfont, Buckinhamshire, UK). Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) was from Calbiochem (Meudon, France). *N*-(3-Phenoxycinamyl)-acetohydroxamic acid was from Wellcome Foundation (Beckenham, UK). Sodium pentobarbital was from SANOFI Research Center (Montpellier, France). Products for staining cytocentrifuge smears (modified May-Grünwald-Giemsa) were from Diff-Quik (Düdingen, Switzerland). Fluorescent phospholipid [1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-monomethyl-phosphoglycerol] was from Interchim (Montluçon, France). Nylon membranes were purchased from Schleicher & Schuell (Dassel, Germany). RNeasy kit for total RNA extraction was from QIAGEN GmbH (Hilden, Germany).

Bronchoalveolar Lavage and Macrophage Culture. Male Hartley guinea pigs weighing approximately 500 g were anesthetized by i.p. injection of sodium pentobarbital (20 mg/kg). Ten successive bronchoalveolar lavages were performed aseptically with 10-ml aliquots of saline that were injected with a plastic syringe through a polyethylene cannula inserted into the trachea. The cell suspensions were centrifuged at 475*g* for 10 min at 25°C, and the pellets were resuspended in RPMI 1640 medium containing 50 μg/ml streptomycin, 50 U/ml penicillin, and 3% fetal calf serum. Cells were adjusted at 3×10^6 cells/ml. The AMs constituted more than 85% of harvested cells, as assessed by cytocentrifugation and modified May-Grünwald-Giemsa stains. Cells allowed to adhere in 6- or 12-well plates for 1 h at 37°C in 5% CO_2/95% air. At this step, the cell population of adherent cells consisted of 95 to 99% macrophages. The plates were then washed twice with medium and incubated in serum-free RPMI 1640 for the indicated time with appropriate agents as described in figure legends. All drugs were used at concentrations similar to those reported in the literature and had no toxic effect on AMs. The cell lysis was controlled by the measurement of lactate dehydrogenase activity released after the end of incubations using a commercial kit from Roche Molecular Biochemicals (Mannheim, Germany). No increase in lactate dehydrogenase activity was observed in any of the experiments performed.

Preparation of Cell Lysates. At the end of incubation, culture supernatants were harvested, centrifuged at 1500*g* for 5 min at 4°C to remove detached cells, and stored at −20°C until use. Adherent macrophages were scraped in PBS containing 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 mM EDTA. Cells were then lysed by ultrasonication (30 s, 150 W) in an ice bath using an MSE (Annemasse, France) sonifier and stored at −20°C until use.

Measurement of sPLA$_2$ Activity. The measurement of sPLA$_2$ activity was carried out in the pellets and supernatants of AMs using the fluorometric assay shown to be selective for low-molecular-weight sPLA$_2$s (Hidi et al., 1993). Furthermore, the PLA$_2$ activity measured in guinea pig AMs was totally blocked by LY311727, an inhibitor of low-molecular-weight sPLA$_2$ activity (Arbibe et al., 1997).

Extraction and Analysis of mRNA Levels. Cells were isolated and cultured as described above. Total RNAs were extracted by an RNeasy kit, electrophoresed (10 μg/lane), and transferred to a nylon membrane. The blots were hybridized at 65°C using α-^{32}P-labeled (random priming) full-length guinea pig sPLA$_2$-IIA cDNA (Vial et al., 1995) as a probe. Finally, blots were washed off and rehybridized with murine β-actin cDNA at 62°C.

Nuclear Extracts and EMSA. Nuclear proteins were extracted from 3×10^6 cells. Briefly, AMs were washed once and scraped in PBS containing 1 mM PMSF and 2 mM benzamidine before centrifugation for 5 min at 700*g*. The cells were resuspended in 20 mM HEPES, pH 7, 10 mM KCl, 0.15 mM EDTA, 0.15 mM EGTA, 25% glycerol, 1% Nonidet P-40, and antiproteases; incubated for 5 min at 4°C; and then centrifuged for 5 min at 1250*g* at 4°C. The pellet (nuclear fraction) was resuspended in 10 mM HEPES, pH 8, 400 mM NaCl, 0.1 mM EDTA, 25% glycerol, and antiproteases; incubated for 30 min at 4°C under agitation; and centrifuged for 10 min at 15,000*g* at 4°C. Supernatant corresponding to the nuclear extract was quickly frozen at −80°C. The NF-κB double-stranded oligonucleotides corresponded to an NF-κB binding site consensus sequence of 5′-GATCATGGGGAATCCCCA-3′. The PPRE double-stranded oligonucleotides corresponded to a PPAR-binding site consensus sequence from acyl-CoA oxidase gene 5′- GGGAACGTGACCTTTGTCCTGGTCCC-3′ (Couturier et al., 1999). The overhanging ends were γ-^{32}P-labeled with T4 polynucleotide kinase. Binding reactions were performed in a total volume of 20 μl for 20 min at room temperature by adding 5 μg of nuclear extract, 10 μl of 2× binding buffer [40 mM HEPES, pH 7, 140 mM KCl, 4 mM DTT, 0.02% Nonidet P-40, 8% Ficoll, 200 μg/ml bovine serum albumin, 1 μg of poly(dI:dC)], and 1 μl of labeled probe. In certain experiments, nuclear extracts were incubated for 20 min with 50-fold excess of unlabeled probe or irrelevant oligonucleotide corresponding to Oct-1 (5′-ATGCAAAT-3′) before the addition of labeled probe. For supershift assay, 2 μg of polyclonal anti-p50 or anti-p65 antibodies were added, and the mixtures were incubated at room temperature for 20 min. The reaction mixtures were separated on a 5% polyacrylamide gel in 0.5× Tris/borate/EDTA buffer at 150 V for 2 h. Gels were dried and exposed for 2 to 12 h.

Protein Extraction and Western Blot Analysis. Adherent macrophages were washed twice and scraped in PBS before centrifugation for 5 min at 700*g*. The cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 3 mM EDTA, 100 μM leupeptin, 100 μM aprotinin, 1 μM soybean trypsin inhibitor, 5 mM NEM, 1 mM PMSF, and 5 mM benzamidine, pH 7.4) to which 1% Triton X-100 was added. Protein extraction was performed at 4°C for 30 min with occasional vortex mixing, and cell homogenates were collected by centrifugation for 15 min at 15,000*g* at 4°C. The supernatant was then made soluble by the addition of a one-fifth volume of a buffer (12% SDS, 30 mM NEM, and 10 mM Tris-HCl, pH 6.8) and heated at 100°C for 5 min; protein concentrations were determined using the Pierce assay from Interchim (Montluçon, France). SDS-polyacrylamide gel electrophoresis was performed according to the procedure described by Laemmli (1970). Total proteins (50 μg/lane) were electrophoresed under reducing conditions (sample buffer containing 10 mM DTT). Proteins were transferred onto nitrocellulose membranes by semidry transfer (25 mM Tris, 192 mM glycine, 20% methanol). Nonspecific binding sites were blocked overnight with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20. Blots were probed for 1 h with anti-PPAR-γ (1:1000 dilution). The primary antibody was removed, and immunoreactive bands were visualized using a peroxidase rabbit immunoglobulin antibody (1:10,000 dilution) followed by enhanced chemiluminescence reagent.

Calculations and Statistical Analysis. Data are expressed as mean ± S.E.M. of separate experiments, and statistical analyses were performed using the unpaired Student's *t* test.

Results

Suppression of LPS-Induced sPLA$_2$-IIA Expression by AA but Not by Its Nonmetabolizable Analog ETYA. We have shown previously that cultured AMs spontaneously synthesized an enzyme with characteristics similar to those of the low-molecular-weight sPLA$_2$ (Hidi et al., 1993). The molecular cloning of this enzyme allowed us to identify it as an sPLA$_2$-IIA (Vial et al., 1995), the expression of which was enhanced after LPS stimulation (Arbibe et al., 1997). In addition, this enzyme was found in the cell homogenates and supernatants, and its catalytic activity was completely abolished by LY311727, a specific inhibitor of the low-molecular-weight sPLA$_2$ activity (Arbibe et al., 1997). Taken together, these findings allowed us to conclude that the observed enzymatic activity was mainly associated with the sPLA$_2$-IIA (Arbibe et al., 1997).

Here, we examined the signaling pathways involved in the regulation by AA of sPLA$_2$-IIA expression in both unstimulated (basal synthesis) and LPS-stimulated AMs. The pretreatment of cells with AA (30 μM) for 1 h markedly reduced both basal and LPS-stimulated sPLA$_2$-IIA activity and mRNA levels after 20 h of incubation (Fig. 1, A and B). This effect was concentration-dependent with an IC$_{50}$ of ≈7.5 and 12.5 μM in unstimulated and LPS-stimulated AM, respectively. A similar decrease was observed in cell-associated and released sPLA$_2$-IIA activity (data not shown). To test whether AA itself is involved in this inhibition, we used a nonmetabolizable AA analog, ETYA. AMs were pretreated with ETYA for 1 h and then incubated for 20 h in the presence or absence of LPS. In unstimulated cells, a similar pattern of inhibition was observed with ETYA compared with AA. However, ETYA had no effect on LPS-induced sPLA$_2$-IIA expression, suggesting that the inhibitory effect of AA is mainly mediated by its metabolites in LPS-stimulated cells (Fig. 1, A and B). Moreover, the specificity of AA (C20:4) in suppressing sPLA$_2$-IIA expression was investigated using other unsaturated FAs. The results reported in Table 1 indicate that eicosapentaenoic (C20:5) and oleic (C18:1) acids were able to reduce basal sPLA$_2$-IIA activity, but they were ineffective on LPS-induced sPLA$_2$-IIA activity. These results therefore showed that both AA and its nonmetabolizable analog inhibit basal sPLA$_2$-IIA synthesis in unstimulated AM, whereas only AA inhibits this expression in LPS-stimulated cells.

Inhibition by AA of LPS-Induced sPLA$_2$-IIA Is Mainly Mediated by Cyclooxygenase Pathway. AA is known to be rapidly converted to a number of eicosanoids, which exert potent biological activities. To identify the metabolic pathway involved in the inhibitory effect of AA on LPS-induced sPLA$_2$-IIA expression, inhibitors of the enzyme systems were used. Previous studies have shown that 5-LOX is the major lipoxygenase isoform operating in guinea pig AMs (Hirata et al., 1990). Pretreatment of AMs with selective inhibitors of 5-LOX and CYP pathways, respectively, *N*-(3-phenoxycinamyl)-acetohydroxamic acid or 1-aminobenzotriazole did not alter the AA-induced suppression of sPLA$_2$-IIA activity in LPS-stimulated AMs (data not shown). In contrast, pretreatment of AMs for 30 min with aspirin and flurbiprofen, dual COX-1/COX-2 inhibitors, abolished the inhibitory effect of AA on LPS-induced sPLA$_2$-IIA activity and mRNA levels (Fig. 2, A and B). These results indicate that

COX metabolites rather than AA itself or 5-LOX/CYP products are the suppressive agents in the down-regulation of LPS-induced sPLA$_2$-IIA expression by AA. To further investigate which isoform of COX is involved in this process, AMs were treated with NS-398, a specific COX-2 inhibitor, before the addition of AA. The results show that NS-398 reversed only partially the inhibition by AA of LPS-induced sPLA$_2$-IIA expression (Fig. 2, A and B). It should be also noted that in the absence of exogenous AA, pretreatment of AMs with aspirin, flurbiprofen, or NS-398 led to a marked increase in LPS-induced sPLA$_2$-IIA expression. These COX inhibitors enhanced sPLA$_2$-IIA expression at similar levels (Fig. 2, A and B). We concluded from these experiments that LPS-induced sPLA$_2$-IIA is inhibited mainly by AA through the cyclooxygenase pathway.

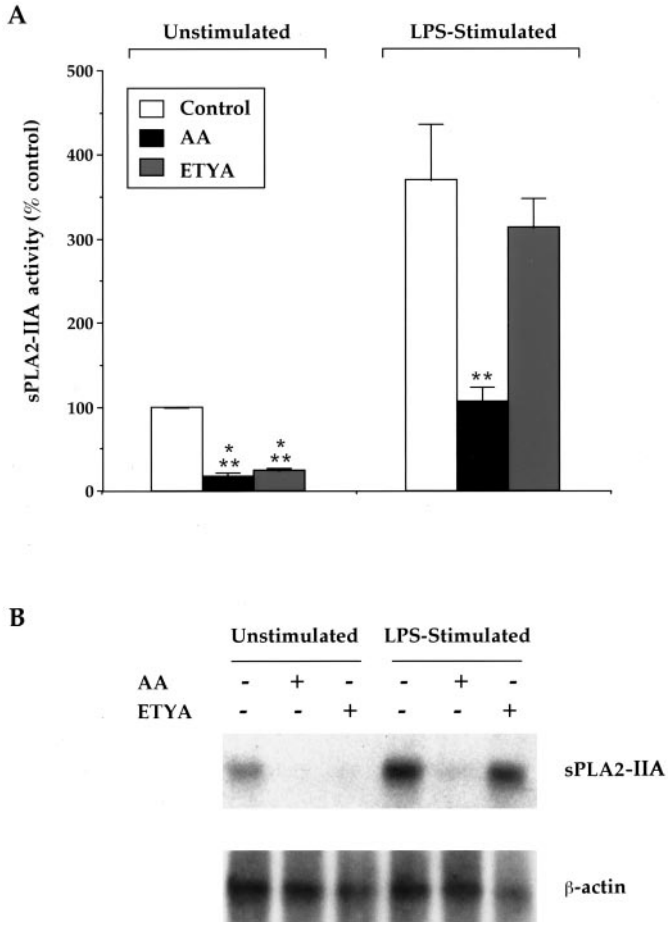

Fig. 1. Effect of AA and ETYA on sPLA$_2$-IIA synthesis in unstimulated and LPS-stimulated AMs. AMs were pretreated for 1 h with AA (30 μM) or ETYA (20 μM) and then incubated for 20 h with or without LPS (25 μg/ml). A, total sPLA$_2$-IIA activity is calculated as the sum of activity in the pellet and supernatant and is expressed as the percentage of the control value determined in unstimulated AMs ($n = 4$). **, $P < 0.01$ and ***, $P < 0.001$ compared with corresponding controls. B, sPLA$_2$-IIA mRNA expression. Total RNA was extracted and analyzed as described under *Experimental Procedures*. The Northern blot analysis shown is representative of two separate experiments.

TABLE 1

Effect of unsaturated fatty acids on sPLA$_2$-IIA activity

AMs were preincubated for 1 h with 30 μM concentrations of the indicated fatty acids and then incubated for 20 h with or without LPS (25 μg/ml). The results show total sPLA$_2$-IIA activity calculated as the sum of activity in the pellet and supernatant. They are expressed as the percentage of the control value determined in unstimulated AM ($n = 4–6$).

Fatty Acids	sPLA$_2$ Activity	
	Unstimulated	LPS-Stimulated
	% control	
Control	100	402.91 ± 66.14
Arachidonic acid	12.03 ± 4.69[a]	110.77 ± 15.95[b]
Eicosapentaenoic acid	41.76 ± 4.87[a]	354.38 ± 89.96
Oleic acid	40.15 ± 5.58[a]	402.45 ± 97.71

[a] $P < 0.001$.
[b] $P < 0.01$.

Effect of Exogenously Added PGs on LPS-Induced sPLA$_2$-IIA Expression. The results shown above led us to examine the ability of a panel of COX products to mimic the inhibitory effect of AA on LPS-induced sPLA$_2$-IIA expression. AMs were pretreated for 1 h with 3 μM PGs before stimulation with LPS for 20 h. We found that PGE$_2$, PGA$_2$, and 15d-PGJ$_2$ markedly decreased sPLA$_2$-IIA activity and mRNA levels in LPS-stimulated cells, whereas PGD$_2$ and PGF$_{2\alpha}$ had

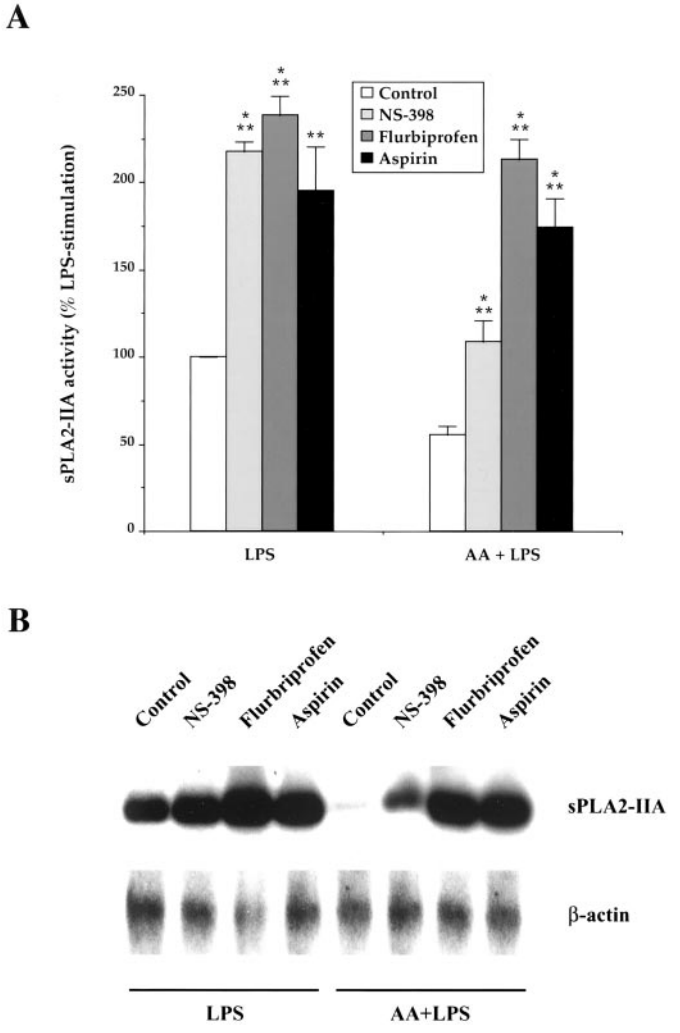

Fig. 2. Cyclooxygenase inhibitors reversed the inhibitory effect of AA on LPS-induced sPLA$_2$-IIA synthesis. AMs were pretreated for 30 min with aspirin (200 μM), flurbiprofen (100 nM), or NS-398 (10 μM) before incubation with AA (10 μM) for 1 h. Cells were then stimulated with LPS (25 μg/ml) for 20 h. A, total sPLA$_2$-IIA activity is calculated as the sum of activity in the pellet and supernatant. It is expressed as the percentage of the value determined in LPS-stimulated AMs ($n = 4$). **, $P < 0.01$ and ***, $P < 0.001$ compared with LPS-treated cells. B, sPLA$_2$-IIA mRNA expression. Total RNA was extracted and analyzed as described under *Experimental Procedures*. The Northern blot shown is representative of two separate experiments.

no effect (Fig. 3, A and B). However, it should be noted that at higher concentration (25 μM), PGD_2, which is the natural precursor of 15d-PGJ_2 (Hirata et al., 1988), displayed a significant inhibitory effect on LPS-induced $sPLA_2$-IIA (data not shown).

Exploration of the Involvement of NF-κB in the Inhibitory Effect of AA on $sPLA_2$-IIA Expression. Previous studies have reported that the transcription factor NF-κB is an essential component of the up-regulation of $sPLA_2$-IIA gene transcription in rat mesangial and vascular smooth muscle cells (Walker et al., 1997; Couturier et al., 1999). We thus examined the effect of CAPE and MG-132, two potent inhibitors of NF-κB activation acting at different steps of the NF-κB signaling pathway, on the $sPLA_2$-IIA synthesis in our cell system. AMs were pretreated with drugs for 2 h and then incubated in the presence or absence of LPS for 20 h. The results shown in Fig. 4 indicate that $sPLA_2$-IIA activity was reduced by CAPE and MG-132 with a maximal effect at 10 μM and 100 nM, respectively, in both unstimulated and LPS-stimulated cells. We also found that CAPE completely blocked basal and LPS-induced $sPLA_2$-IIA mRNA accumulation and NF-κB translocation as assessed by Northern blot analysis and EMSA, respectively (data not shown).

We examined whether AA interferes with NF-κB translocation in AMs by the use of EMSA. The nuclear extracts from untreated cells gave one major complex with labeled oligonucleotides bearing NF-κB consensus site (Fig. 5 and 6). Specificity of this complex was verified by inhibition with an excess of unlabeled oligonucleotides and failure of irrelevant oligonucleotide Oct-1 to block the complex formation (Fig. 5A). Moreover, supershift studies showed that antibodies directed against p50 and p65 subunits constituting NF-κB displaced this band, thus confirming that these complexes belong to the NF-κB family (Fig. 5B). When the cells were treated with AA or ETYA, a marked inhibition of basal NF-κB translocation was observed. However, stimulation of AMs with LPS led to a strong activation of NF-κB, which was not affected by prior treatment of the cells with AA or ETYA (Fig. 6A). In addition, COX-derived metabolites of AA, PGE_2, PGA_2, and 15d-PGJ_2 failed to interfere with LPS-induced NF-κB translocation (Fig. 6B). We showed here that NF-κB regulates $sPLA_2$-IIA expression in AM and that both AA and ETYA inhibited its basal level of activation but had no effect on LPS-induced NF-κB translocation.

Involvement of PPAR-γ in the Inhibitory Effect of AA on LPS-Induced $sPLA_2$-IIA Expression. 15d-PGJ_2 is a naturally occurring ligand of PPAR-γ (Spiegelman, 1998). This PG inhibited $sPLA_2$-IIA expression in LPS-activated AMs, suggesting the involvement of PPAR-γ in this process. In contrast, ETYA, which is known to activate PPAR-α had no effect on LPS-induced $sPLA_2$-IIA synthesis. To determine PPAR-γ expression in AM, we performed a Western blot

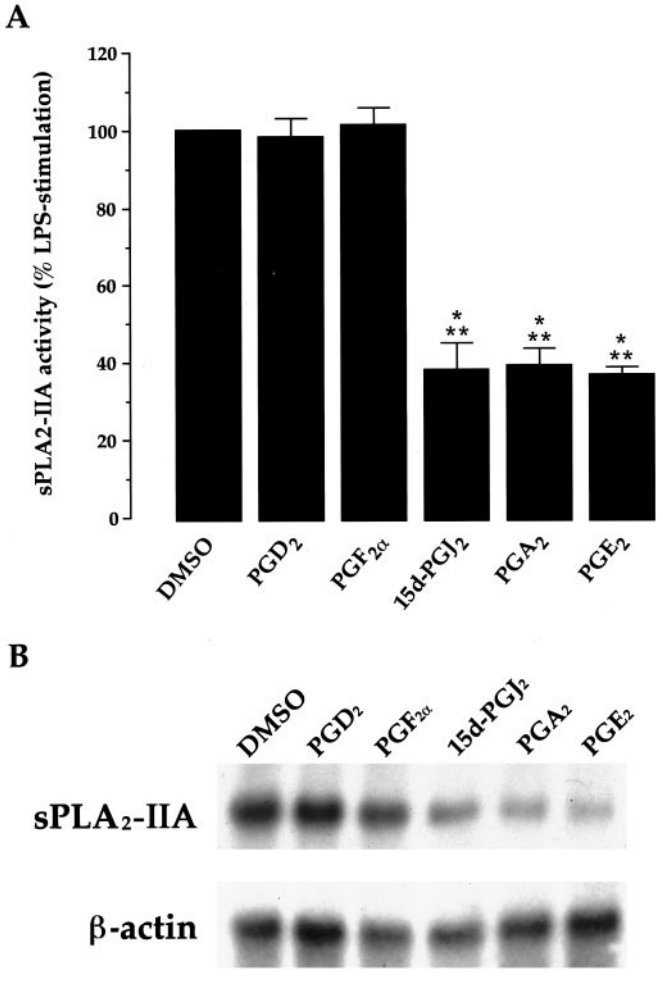

Fig. 3. PGE_2, PGA_2, and 15d-PGJ_2 suppress LPS-induced $sPLA_2$-IIA synthesis. AMs were pretreated with 3 μM of PGD_2, $PGF_{2\alpha}$, 15d-PGJ_2, PGA_2, or PGE_2 for 1 h and then were stimulated with LPS (25 μg/ml) for 20 h. A, total $sPLA_2$-IIA activity is calculated as the sum of activity in the pellet and supernatant, and is expressed as the percentage of the value determined in LPS-stimulated AMs (n = 3–4). ***, $P < 0.001$ compared with LPS-treated cells. B, total RNA was extracted from AMs, and Northern blotting was carried out as described under *Experimental Procedures*. The results are representative of two separate experiments.

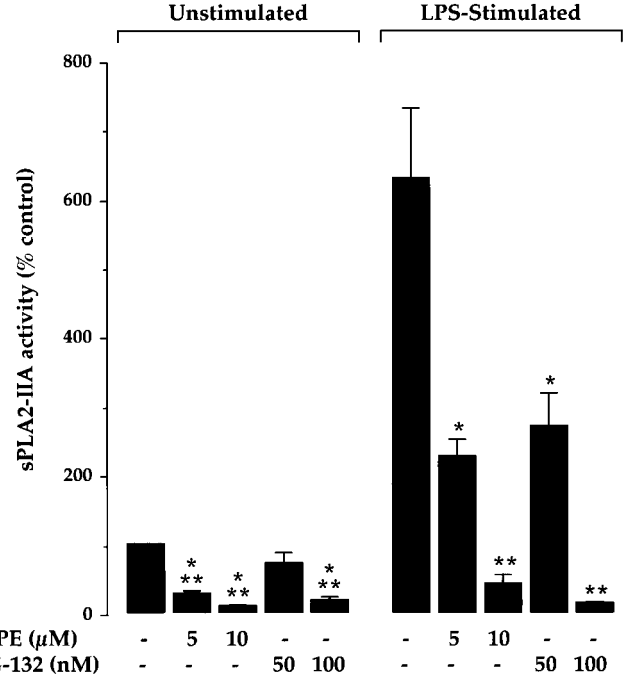

Fig. 4. NF-κB regulates basal and LPS-induced $sPLA_2$-IIA synthesis by AMs. AMs were pretreated for 2 h with NF-κB inhibitors, CAPE, or MG-132 and then incubated for 20 h with or without LPS (25 μg/ml). The results show total $sPLA_2$-IIA activity calculated as the sum of activity in the pellet and supernatant. They are expressed as the percentage of the control value determined in unstimulated AMs (n = 3–4). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with corresponding controls.

analysis using a PPAR-γ–specific antibody. The presence of appreciable amounts of PPAR-γ protein was detected in AMs cultured for 20 h (Fig. 7A). The level of PPAR-γ protein was not modified after LPS stimulation, indicating that the latter did not interfere with the synthesis of this transcription factor in our cell system. To further confirm the function of PPAR-γ in AM, we used a synthetic PPAR-γ ligand, the thiazolidinedione reagent ciglitazone. Pretreatment of AMs for 1 h with 3.5 μM ciglitazone resulted in a dramatic decrease of sPLA$_2$-IIA activity and mRNA levels in LPS-stimulated AM, whereas a selective PPAR-α ligand, Wy14,643 (10 μM), was ineffective (Fig. 8, A and B). Neither Wy14,643 nor ciglitazone interfered significantly with the basal sPLA$_2$-IIA expression (data not shown). On the other hand, we performed an EMSA to assess whether AA and PPAR-γ ligands modify the binding of PPAR to PPRE consensus sequence. Nuclear extracts from unstimulated AMs form a major complex with labeled oligonucleotides bearing a PPAR binding site, which is slightly activated upon LPS stimulation (Fig. 7, B and C). An excess of cold PPRE displaced this complex, whereas the irrelevant oligonucleotide Oct-1 had no effect (Fig. 7C). The pretreatment of LPS-stimulated AMs for 1 h with AA, 15d-PGJ$_2$, or ciglitazone resulted in an increase of binding of the PPAR to its responsive element (Fig. 7B). These data therefore demonstrate the involvement of PPAR-γ in the inhibitory effect of AA in LPS-induced sPLA$_2$-IIA expression.

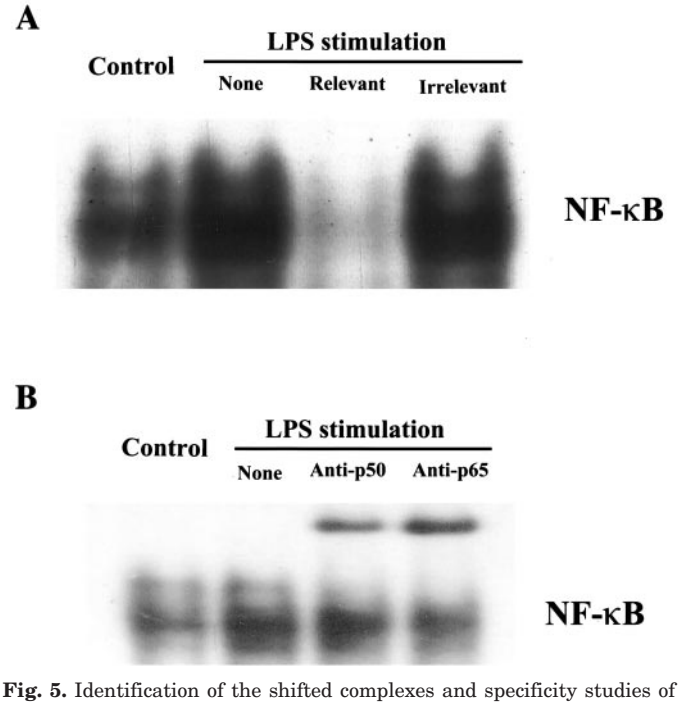

Fig. 5. Identification of the shifted complexes and specificity studies of NF-κB in LPS-stimulated AMs. A, nuclear extracts of AM, prepared as indicated under *Experimental Procedures,* were incubated for 20 min with 50-fold excess of unlabeled oligonucleotide corresponding to NF-κB binding site, or 50 fold excess of irrelevant oligonucleotide corresponding to the Oct-1 binding site. B, for supershift assay, 2 μg of polyclonal anti-p50 or anti-p65 antibodies were added and the mixtures were incubated at room temperature for 20 min. The results are representative of three separate experiments.

Discussion

We demonstrated here that AA down-regulates sPLA$_2$-IIA expression by distinct signaling pathways in unstimulated and LPS-stimulated guinea pig AMs. The nonmetabolizable AA analog ETYA decreases sPLA$_2$-IIA expression in unstimulated AMs, but it had no effect on this expression in LPS-activated AMs. This suggests that AA metabolism is required for the inhibition of LPS-induced sPLA$_2$-IIA expression but not for that of basal expression. Consistently, COX inhibitors abolished the inhibitory effect of AA. In contrast, the 5-LOX and CYP pathways seem not to be involved in this process because potent inhibitors of these enzymes failed to reverse the inhibitory effect of AA. Hence, inhibition of LPS-induced sPLA$_2$-IIA synthesis by AA is mainly mediated by its COX-derived metabolites. COX products, particularly PGE$_2$ and cyclopentenone PGs (15d-PGJ$_2$ and PGA$_2$), decreased LPS-induced sPLA$_2$-IIA expression, suggesting that they may be intermediate metabolites in this gene expression regulation. The inhibition of LPS-induced sPLA$_2$-IIA expression by AA was completely reversed by aspirin and flurbiprofen, dual COX-1/COX-2 inhibitors, and only partially by NS-

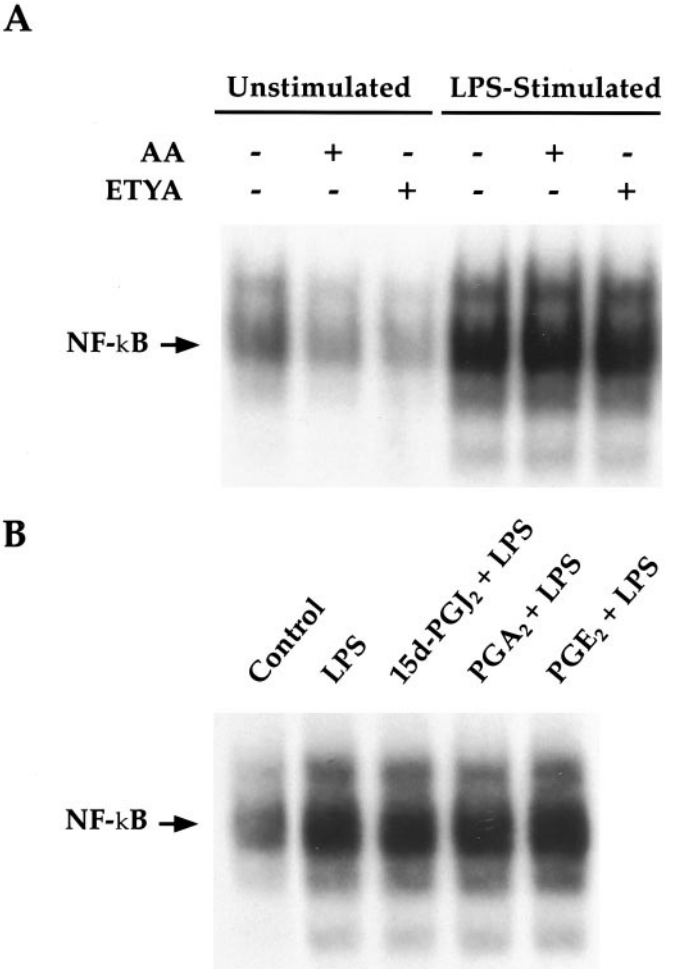

Fig. 6. Effect of AA, ETYA, and PGs on nuclear translocation of NF-κB. A, AMs were pretreated for 1 h with AA (30 μM) or ETYA (20 μM) and then incubated for 1 h with or without LPS (25 μg/ml). B, AMs were pretreated for 1 h with 3 μM 15d-PGJ$_2$, PGA$_2$, or PGE$_2$, then stimulated with LPS (25 μg/ml) for 1 h. Nuclear extracts were prepared and assayed for NF-κB activation as described under *Experimental Procedures.* Similar results were obtained in three other experiments.

398, a specific COX-2 inhibitor. This suggests that the inhibitory effect of exogenous AA was mainly mediated by COX-1 pathway. Our results also showed that, in the absence of exogenous AA, pretreatment of AMs with COX inhibitors led to a marked increase in LPS-induced $sPLA_2$-IIA expression. Under these conditions, NS-398 enhanced $sPLA_2$-IIA expression at a level similar to that with aspirin and flurbiprofen. These findings suggest that endogenous AA inhibits the expression of $sPLA_2$-IIA in LPS-stimulated AMs and that COX-2–derived metabolites play an important role in this process. Together, these results led us to postulate that in LPS-stimulated AMs, exogenous AA inhibits $sPLA_2$-IIA expression mainly via the COX-1 pathway, whereas endogenous AA uses predominantly a COX-2 pathway to inhibit this expression. This might be caused by different compartmentation of these two COX isoforms, leading to different metabolization of endogenous versus exogenous AA. Alternatively, these enzymes may have different catalytic properties for the conversion of AA. Indeed, it has been recently shown that COX-1 preferentially metabolizes high concentrations of AA (Murakami et al., 1999), which generally correspond to those of exogenously added AA. However, only COX-2 was functional at low AA concentrations (Shitashige et al., 1998). Thus, it is likely that endogenous AA, whose concentrations are 10 to 20 times lower than those of exogenously added AA in LPS-stimulated AMs (data not shown), is preferentially metabolized by COX-2, thus involving this COX isoform in the inhibition of $sPLA_2$-IIA expression in the absence of exogenous AA.

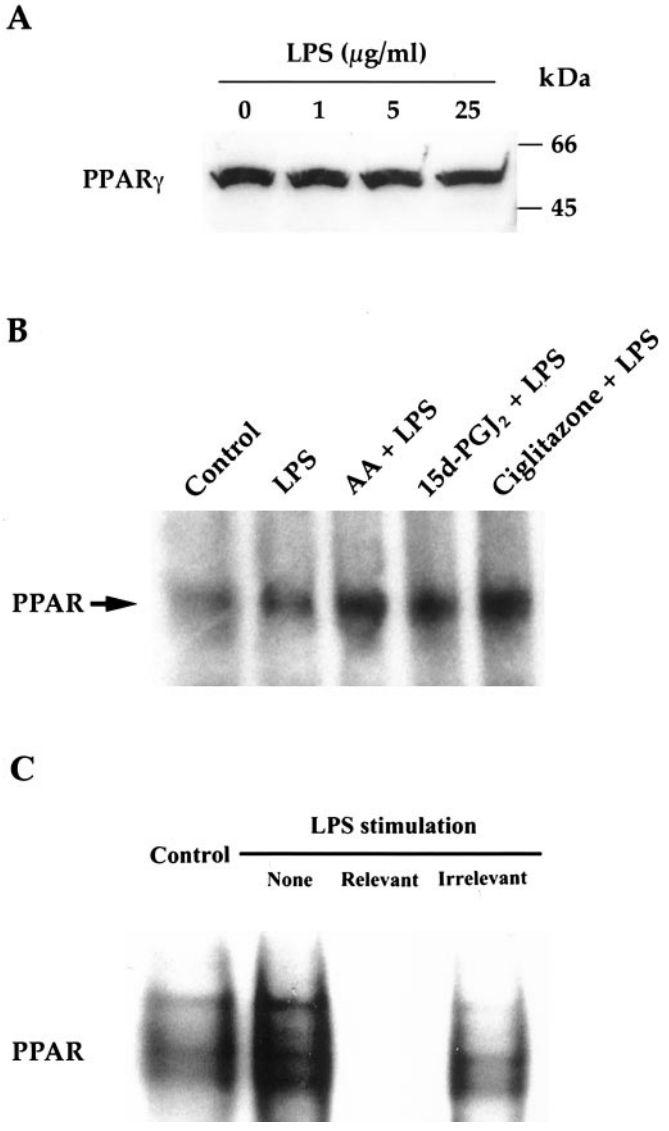

Fig. 7. Expression and activation of PPAR-γ in LPS-stimulated AMs. A, PPAR-γ expression in AMs. Cells were incubated for 20 h with increasing concentrations of LPS (1, 5, and 25 μg/ml). Proteins were extracted from AMs, and Western blot was performed as described under *Experimental Procedures* using polyclonal anti-PPAR-γ antibody. B, AMs were pretreated for 1 h with AA (30 μM), 15d-PGJ_2 (3 μM), or ciglitazone (3.5 μM) and then stimulated with LPS (25 μg/ml) for 16 h. C, nuclear extracts of AMs were incubated for 20 min with 50-fold excess of an unlabeled oligonucleotide corresponding to PPRE binding site or 50-fold excess of an irrelevant oligonucleotide corresponding to Oct-1 binding site. Nuclear extracts were prepared and assayed for PPAR activation as described under *Experimental Procedures*. Similar results were obtained in two separate experiments.

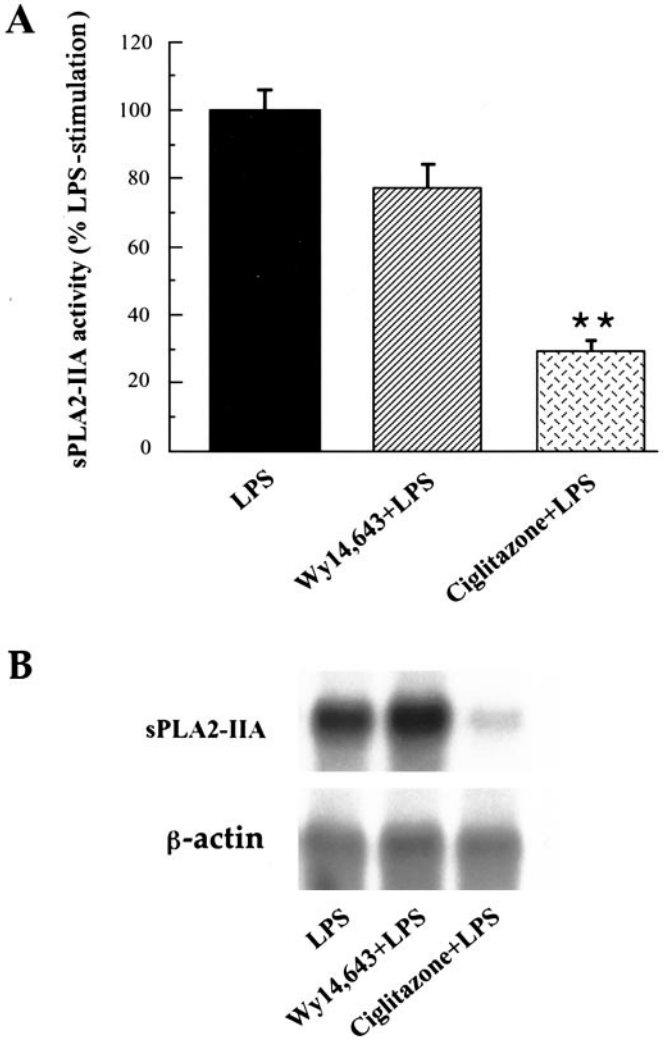

Fig. 8. Role of PPAR-γ activation in the inhibition of LPS-induced $sPLA_2$-IIA synthesis by AA. AMs were pretreated for 1 h with Wy14,643 (10 μM) or ciglitazone (3.5 μM), agonists of PPAR-α and PPAR-γ, respectively. Cells were then stimulated with LPS (25 μg/ml) for 20 h. A, total $sPLA_2$-IIA activity is calculated as the sum of activity in the pellet and supernatant. It is expressed as the percentage of the value determined in LPS-stimulated AMs ($n = 4$); **, $P < 0.01$ compared with LPS-treated cells. B, total RNA was extracted and analyzed by Northern blot as described under *Experimental Procedures*. The results are representative of two separate experiments.

Transcriptional up-regulation of proinflammatory genes is strongly dependent on NF-κB activation. This transcription factor mediates IL-1β–induced sPLA$_2$-IIA expression in rat mesangial and vascular smooth muscle cells (Walker et al., 1997; Couturier et al., 1999). In several cell systems, it has been suggested that AA interferes directly or via its metabolites with NF-κB activation (Camandola et al., 1996; Stuhlmeier et al., 1997; Thommesen et al., 1998). We thus evaluated the possible occurrence of such a mechanism in our cell system. We first investigated whether NF-κB is involved in sPLA$_2$-IIA gene expression in AMs and found a basal NF-κB binding activity, which dramatically increased after LPS stimulation. Potent NF-κB inhibitors, MG-132 and CAPE, markedly reduced basal and LPS-induced sPLA$_2$-IIA synthesis. These findings indicate that sPLA$_2$-IIA is under transcriptional control of NF-κB in guinea pig AMs. To confirm the nature of the complexes involved in the major retarded band showed in Fig. 6, we found that both anti-p50 and anti-p65 antibodies decreased this band and caused a supershift, suggesting that p50/p65 heterodimers are activated in AMs after LPS stimulation. Our studies also showed that both AA and ETYA reduced NF-κB translocation in unstimulated AMs. This suggests that AA can inhibit by itself the basal translocation of NF-κB, which might explain its effect on sPLA$_2$-IIA expression in unstimulated AMs. In contrast, LPS-induced NF-κB translocation was not affected by AA or by ETYA. Thus, the inhibition of LPS-induced sPLA$_2$-IIA synthesis by AA requires its metabolic conversion by COX pathway and is independent from NF-κB translocation in AMs. These findings contrast with those reported by Stuhlmeier et al. (1996, 1997) showing that AA suppresses directly TNFα-induced NF-κB translocation, a process resulting in the inhibition of proinflammatory genes in endothelial cells. Recently, several studies have reported that cyclopentenone PGs inhibit NF-κB transcriptional activity by preventing nuclear translocation and/or DNA binding of NF-κB complex (Rossi et al., 2000; Straus et al., 2000). This mechanism seems not to occur in our cell system because the PGs that inhibited sPLA$_2$-IIA synthesis (e.g., 15d-PGJ$_2$, PGA$_2$, and PGE$_2$) had no effect on NF-κB translocation.

Taken together, these findings suggest that AA inhibits sPLA$_2$-IIA synthesis by two distinct mechanisms, depending on the activation state of AMs. In unstimulated AMs, AA can inhibit sPLA$_2$-IIA synthesis by itself, and the impairment of NF-κB translocation contributes to this down-regulation. In contrast, AA exerts its inhibitory effect on LPS-induced sPLA$_2$-IIA expression essentially via its COX products (e.g., 15d-PGJ$_2$, PGA$_2$, and PGE$_2$) without interfering with NF-κB activation. This suggests that the signaling pathways involved in the activation of NF-κB in unstimulated and LPS-stimulated cells may be different.

Previous studies have demonstrated that PGD$_2$ metabolites are major products of AA metabolism in macrophages and specialized antigen-presenting cells (Urade et al., 1989). The prostanoid PGD$_2$ dehydration product, 15d-PGJ$_2$, is a natural ligand for PPAR-γ (Spiegelman, 1998), which has been demonstrated to inhibit the induction of genes involved in inflammatory response, including the inducible nitric oxide synthase and TNF-α genes in a PPAR-γ–dependent manner during monocyte/macrophage activation (Jiang et al., 1998; Ricote et al., 1998). Taken together, these studies suggested that in our cell system, a PPAR-γ–mediated pathway could be involved in the regulation of LPS-induced sPLA$_2$-IIA expression by AA. In fact, Western blot experiments showed that guinea pig AMs expressed PPAR-γ, and the thiazolidinedione PPAR-γ agonist ciglitazone caused significant inhibition of LPS-induced sPLA$_2$-IIA synthesis in AMs. The involvement of PPAR-γ in the down-regulation of sPLA$_2$-IIA expression by AA was supported by the stimulatory effect of AA as well as PPAR-γ ligands on the binding of nuclear factors to a PPRE consensus sequence. The PPAR-α isoform has also been shown to exhibit anti-inflammatory roles (Devchand et al., 1996; Staels et al., 1998). However, we found that the PPAR-α activator Wy14,643 failed to exert any effect on LPS-induced sPLA$_2$-IIA expression at a concentration (10 μM) known to selectively activate PPAR-α but not PPAR-γ (Chinetti et al., 1998). This is consistent with the fact that ETYA, which is also known to activate PPAR-α, failed to inhibit LPS-induced sPLA$_2$-IIA synthesis in our cell system.

In the present study, we have not examined the mechanism by which PGE$_2$ may function to suppress sPLA$_2$-IIA synthesis, but we have previously shown that PGE$_2$ inhibits sPLA$_2$-IIA expression in AMs via cAMP-dependent process (Vial et al., 1998). cAMP has recently been shown to interfere with CCAAT/enhancer-binding protein for the regulation of sPLA$_2$-IIA in rat vascular smooth muscle cells (Couturier et al., 2000). Further investigations will allow for clarification of the role of this transcription factor in regulating sPLA$_2$-IIA expression in our cell system.

Although these studies clearly showed that PPAR-γ down-regulates the synthesis of sPLA$_2$-IIA in AM, the mechanisms by which this transcription factor inhibits sPLA$_2$-IIA gene transcription in our cell system is still unclear. It is likely that PPAR-γ modulates the synthesis of sPLA$_2$-IIA by interfering with the activity of transcription factors (such as NF-κB) involved in the regulation of sPLA$_2$-IIA gene expression, rather via a direct effect on sPLA$_2$-IIA promoter. Indeed, Ricote et al. reported that PPAR-γ down-regulates the expression of genes involved in inflammatory responses in murine macrophages by interfering negatively with NF-κB, activator protein-1, and signal transducer and activator of transcription signaling pathways (Ricote et al., 1998). Chinetti et al. (1998) also showed that PPAR-γ ligand binding inhibited the transcriptional activity of the NF-κB p65/RelA subunit in macrophages. On the other hand, Couturier et al. (1999) recently showed that NF-κB and PPAR-γ cooperate at the enhanceosome-coactivator level to regulate the transcription of the sPLA$_2$-IIA gene in rat vascular smooth muscle cells (Couturier et al., 1999). Cloning of guinea pig sPLA$_2$-IIA promoter will allow us to perform studies to examine this hypothesis in our cell system.

It should be noted, however, that in rat vascular smooth muscle cells, PPAR-γ agonists stimulate the expression of sPLA$_2$-IIA gene (Couturier et al., 1999), in contrast to our cell system, in which PPAR-γ inhibits this expression. This discrepancy might be due to cell type-specific signal transduction or to differences in the organization of regulatory elements of sPLA$_2$-IIA promoter.

In summary, these studies demonstrate that in guinea pig AM, AA down-regulates sPLA$_2$-IIA expression by two distinct mechanisms: in unstimulated AMs, AA directly inhibits sPLA$_2$-IIA expression via a process involving, at least in part, the impairment of NF-κB activation, and in LPS-stimulated

AM, AA effect is mediated via its oxidative metabolism to the COX metabolites and the subsequent PPAR-γ activation by 15d-PGJ_2. Recent studies demonstrating the presence of 15d-PGJ_2 in an acute lung inflammation model provided evidence for an anti-inflammatory role of this PG (Gilroy et al., 1999). Because AMs are the major pulmonary source of $sPLA_2$-IIA in LPS-induced ALI (Arbibe et al., 1997), the present study suggests that PPAR-γ ligands may be useful in the treatment of ALI.

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