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Aspirin inhibits lipopolysaccharide-induced COX-2 expression and PGE_2 production in porcine alveolar macrophages by modulating protein kinase C and protein tyrosine phosphatase activity

Yuzhong Duan, Fanglin Chen, Anmei Zhang, Bo Zhu, Jianguo Sun, Qichao Xie & Zhengtang Chen*
Cancer Institute of PLA, Xinqiao Hospital, Third Military Medical University, Chongqing 40037, China

Aspirin has been demonstrated to be effective in inhibiting COX-2 and PGE_2 in Alveolar macrophages (AMs). However, the mechanisms have not been fully understood. In the present study, we found that pretreatment with aspirin inhibited LPS-induced COX-2 and PGE_2 upregulation, IκBα degradation, NFκB activation and the increase of PKC activity, but elevated LPS-induced the decrease of PTP activity. The PKC inhibitor calphostin C dramatically reduced the COX-2 mRNA and PGE_2 levels, but the PTP inhibitor peroxovanadium (POV) significantly increased the COX-2 mRNA and PGE_2 levels. Furthermore, the PTP inhibitor mitigated the inhibitory effect of aspirin on COX-2 and PGE_2 upregulation and NF-κB activation, whereas the PKC inhibitor enhanced the inhibitory effects of aspirin on the production of COX-2 and PGE_2. Our data indicate a novel mechanism by which aspirin acts as a potent anti-inflammatory agent in alveolus macrophages and ALI. [BMB Reports 2014; 47(1): 45-50]

INTRODUCTION

Acute lung injury (ALI), which is characterized by hypoxemia, widespread capillary leakage, pulmonary edema and inflammatory infiltration, is an inflammatory condition culminating in respiratory failure (1). Many pulmonary and extrapulmonary insults can result in ALI. However, the earliest phases of ALI feature severe neutrophil-rich alveolar inflammation regardless of the precipitating cause (2). Therefore, a therapeutic strategy that specifically decreases local inflammation would be highly valuable in treating ALI (3).

Alveolar macrophages (AMs) are key participants in the pathogenic process of pulmonary inflammation (4). In the acute phase of ALI, AMs often release different kinds of pro-inflammatory cytokines, such as cyclooxygenase-2 (COX-2), IL-1β, IL-8, IL-6, GM-CSF, and TNF-α, etc (5, 6). COX-2, a principal inflammatory mediator, and is crucial for the development of ALI. During the process of ALI, COX-2-related high levels of prostaglandins (PG) production promote alveolar inflammation (7). PGs are characterized as important regulators and mediators in general inflammatory reactions (8) and PGE_2 is a vital mediator of pulmonary edema in ALI (9). Since macrophages are the main producers of PGE_2 (10), macrophage-related alveolar inflammation may be mediated by producing high levels of COX-2 and PGE_2.

Aspirin (acetylsalicylic acid), a potent inhibitor of COX, has a broad spectrum of pharmacological activity, including anti-inflammation. Recently, aspirin has been shown to work effectively against many factors related to induction of ALI (11, 12). Inhibition of COX-2 activity with aspirin reduces inflammatory PG production in human umbilical vein endothelial cells (13). Furthermore, aspirin can down-regulate ERK and subsequent NF-κB activation, inhibiting COX-2 expression in neonatal rat ventricular cardiomyocytes (14). However, the effects and the underlying mechanisms of aspirin on the COX-2 expression and PGE_2 production in AMs have not been clearly explored. Therefore, in this study, we employed the purified porcine AMs (p-AMs) to explore the effects of aspirin on LPS-induced COX-2 expression and PGE_2 production and the potential mechanisms underlying the effects of aspirin.

RESULTS

Aspirin inhibited LPS-induced COX-2 mRNA expression and PGE_2 production in p-AMs

Although aspirin is considered to be an inhibitor of COX-2, its inhibitory effects on COX-2 are controversial in different cell lines (13, 15). To determine the effect of aspirin on COX-2 expression in p-AMs, semi-quantitative RT-PCR was performed (Fig. 1A, B). LPS stimulation significantly elevated the levels of COX-2 mRNA at indicated times (2.33, 4.73, 6.2, 3.4, 2.63-fold increase at 1, 2, 4, 6 and 8 h, respectively). Pretreatment with 3 mM aspirin for 30 min abrogated LPS-induced COX-2 mRNA upregulation in p-AMs (0.94-fold *vs.* 3.18-fold)

*Corresponding author. Tel: +86-23-68774484; Fax: +86-23-68774631; E-mail: yuzhong.duan@gmail.com

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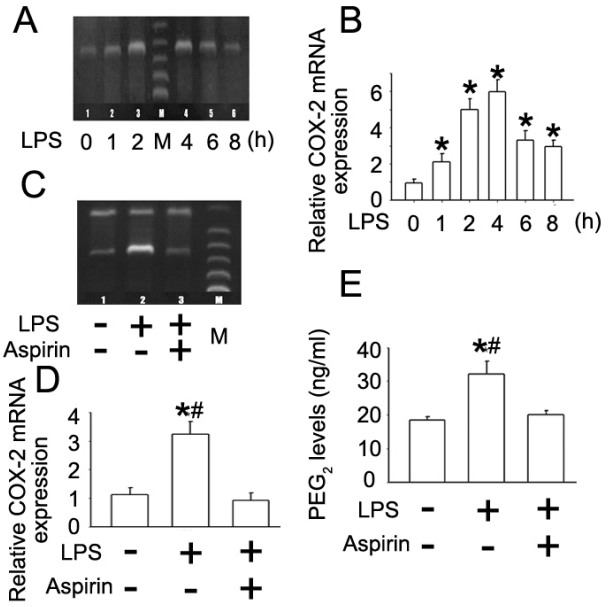

Fig. 1. Aspirin inhibits the LPS-induced COX-2 mRNA and PGE_2 production *in vitro*. (A) The effect of LPS on COX-2 mRNA levels. p-AMs were treated with LPS (1 μg/ml) for indicated times. Then the total RNA was isolated and the levels of COX-2 mRNA were determined by RT-PCR. (C) The effect of aspirin on LPS-induced COX-2 mRNA expression. The cells were pretreated with aspirin (3 mM) for 30 min and then treated with LPS (1 μg/ml) for the indicated times. (B, D) The relative expression of COX-2 mRNA compared with control. (E) The effect of aspirin (3 mM) on LPS-stimulated (1 μg/ml) PGE_2 production. The cells were pretreated with aspirin (3 mM) for 30 min and then treated with LPS (1 μg/ml) for 12 h. The levels of PGE_2 in the supernatants were determined by competitive ELISA method. Data are expressed as mean ± SE from 3 separate experiments. $^*P < 0.05$, compared with control, $^{\#}P < 0.05$, compared with aspirin group.

(Fig. 1C, D). COX-2 is expressed by activated macrophages and responsible for producing high levels of PGE_2 (16). As shown in Fig. 1E, LPS treatment significantly increased PGE_2 production 2 h post stimulation (32.12 ± 1.23 ng/ml *vs*. 18.38 ± 0.99 ng/ml), while aspirin mitigated the LPS-induced PGE_2 production in pAMs (27.85 ± 1.19 ng/ml *vs*. 32.12 ± 1.23 ng/ml). Notably, treatment with 3 mM aspirin alone for 24 h did not significantly alter the COX-2 mRNA levels and the viability of pAMs (data not shown).

PKC and PTP were involved in the suppressive effect of aspirin on LPS-stimulated COX-2 and PGE_2 production

To further explore the mechanism by which aspirin inhibited LPS-induced COX-2 expression and PGE_2 production in p-AMs, we determined the effect of LPS on PKC and PTP activity. As shown in Fig. 2A, LPS stimulation elevated PKC activities, particularly at 10 min post-stimulation. In contrast, the activities of PTP were significantly reduced at 10-15 min post-stimulation in p-AMs (Fig. 2B). Treatment with aspirin

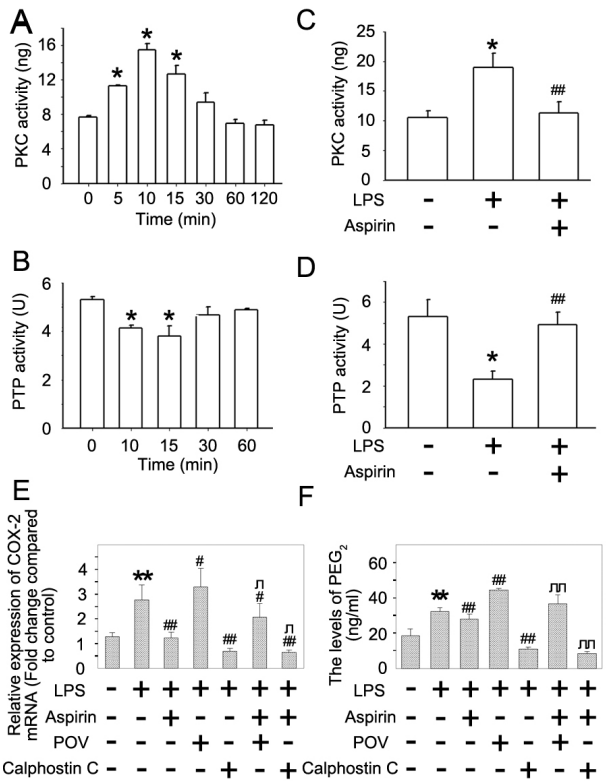

Fig. 2. PKC and PTP were involved in the suppressive effect of aspirin on LPS-stimulated COX-2 and PGE_2 production. (A, B) The effect of LPS (1 μg/ml) on the activity of PKC and PTP at the indicated times, respectively. (C, D) The effect of aspirin (3 mM) on the LPS-enhanced PKC activity and reduced PTP activity, respectively. The effect of calphostin C and POV on the suppressive effect of aspirin on LPS-induced COX-2 mRNA (E) and PGE_2 (F) expression. p-AMs were pretreated with the PKC inhibitor calphostin C (0.5 μmol/L), the PTP inhibitor POV (20 μmol/L), aspirin (3 mM), or combination of aspirin and calphostin C or POV, and then stimulated with LPS (1 μg/ml) for 1 h or 12 h for COX-2 mRNA and PGE_2 determination respectively. The levels of COX-2 mRNA and PGE_2 were determined as described in the methods section. Each bar represents mean ± SE from 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, compared with control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with LPS group, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared with aspirin group.

minimized the effect of LPS on PKC and PTP activities in p-AMs (18.90 ± 2.43 ng *vs*. 12.18 ± 1.39 ng; 2.19 ± 0.32 U *vs*. 4.91 ± 0.48 U for PKC and PTP in LPS and aspirin groups respectively) (Fig. 2C and 2D).

To further determine how the alternation of PKC and PTP activities affects LPS-induced COX-2 and PGE_2 upregulation, p-AMs were pretreated with PKC inhibitor calphostin C or PTP inhibitor peroxovanadium (POV), with or without aspirin, and then stimulated with LPS. Calphostin C-induced inhibition of PKC activity dramatically reduced the levels of COX-2 mRNA in LPS-stimulated p-AMs, but POV-induced inhibition of PTP

activity significantly increased the levels of COX-2 mRNA in LPS-stimulated p-AMs (Fig. 2E). Consistantly, the level of PGE_2 was also downregulated by Calphostin C and upregulated by POV in LPS-stimulated p-AMs (Fig. 2F). Furthermore, treatment with POV mitigated the inhibitory effect of aspirin on LPS-induced COX-2 and PGE_2 upregulation in p-AMs (Fig. 2E and 2F). In contrast, treatment with both calphostin C and aspirin appeared to further inhibit LPS-induced COX-2 and PGE_2 upregulation compared with aspirin alone (Fig. 2E and 2F).

Aspirin inhibited LPS-induced NF-κB activation

As shown in Fig. 3A, LPS treatment induced NF-κB activation in p-AMs. However, aspirin pretreatment mitigated LPS-induced NF-κB activation in p-Ams (Fig. 3B). Given that the NF-κB activation is dependent on the phosphorylation and subsequent degradation of IκBα (17), we further examined the effect of LPS and aspirin on the degradation of IκBα in p-AMs. The IκBα levels in LPS-treated cells were gradually reduced, particularly at 15-90 min post-stimulation (Fig. 3C). Aspirin pretreatment also abrogated LPS-induced degradation of IκBα in p-AMs.

PKC and PTP were involved in the suppressive effect of aspirin on LPS-induced NF-κB activation

Finally, we examined whether PKC and PTP were involved in the suppressive effect of aspirin on LPS-induced NF-κB activation and IκBα degradation. Although combination of POV and LPS did not significantly increase the activation of NF-κB (Fig. 4A), and it significantly decreased the level of IκBα compared with treatment with LPS alone (Fig. 4B). In contrast, treatment with calphostin C significantly abrogated LPS-induced NF-κB

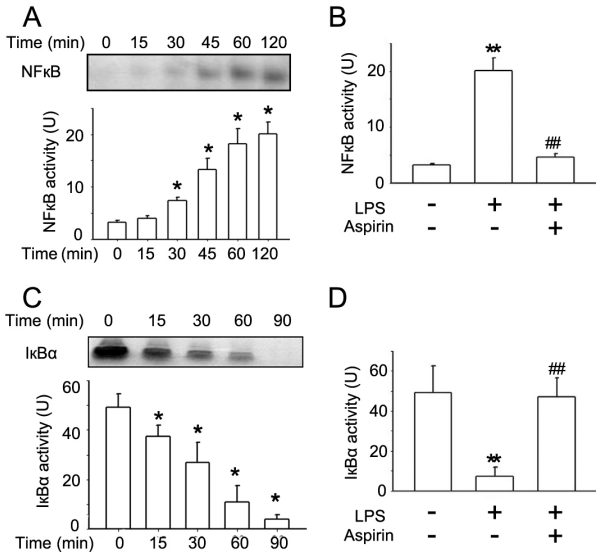

Fig. 3. Aspirin inhibited LPS-induced NF-κB activation. (A) LPS stimulated NF-κB activation. Cells were treated with LPS (1 μg/ml) for indicated times. Then the DNA binding activity of NF-κB was measured by EMSA. (B) Aspirin treatment inhibited LPS-stimulated NF-κB activation. Cells were pretreated with aspirin (3 mM) for 30 min and then treated with LPS (1 μg/ml) for 60 min. Then the DNA binding activity of NF-κB was measured by EMSA. (C) The effect of LPS treatment on the level of IκBα at the indicated times. Cells were treated with LPS (1 μg/ml) for indicated times. Then the levels of IκBα were determined by western blot. (D) Aspirin treatment inhibited LPS-induced degradation of IκBα. Cells were pretreated with aspirin (3 mM) for 30 min and then treated with LPS (1 μg/ml) for 1 h. Then the levels of IκBα were determined by western blot. Each bar represents mean ± SE from six independent experiments. *P < 0.05, **P < 0.01 compared with control, #P < 0.05, ##P < 0.01 compared with aspirin group.

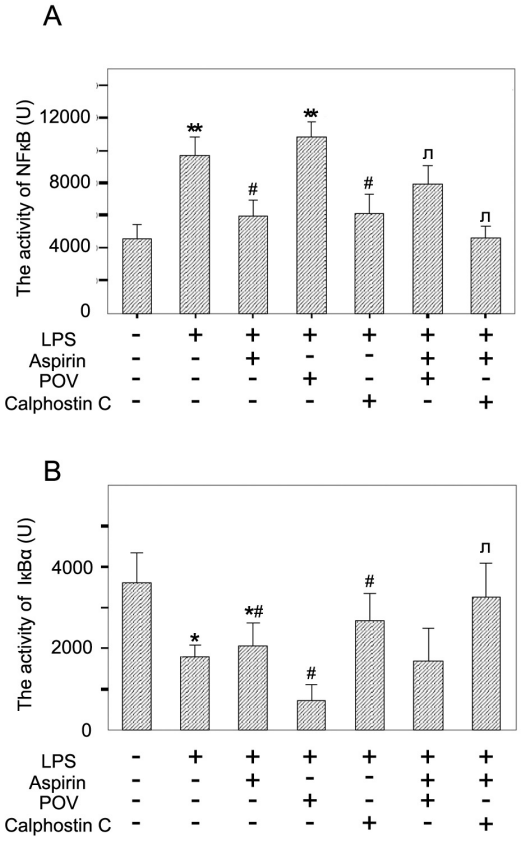

Fig. 4. PKC and PTP were involved in the suppressive effect of aspirin on LPS-induced NF-κB activation. The effect of calphostin C and POV on the suppressive effect of aspirin on LPS-induced NF-κB activation (A) and IκBα degradation (B). The p-AM cells were pretreated with calphostin C (0.5 μmol/L), POV (20 μmol/L), aspirin (3 mM), or combination of aspirin and calphostin C or POV for 30 min and then treated with LPS (1 μg/ml) for 1 h or 10 min for NF-κB and IκBα determination respectively. Then the activity of NF-κB and the level of IκBα were determined by EMSA and western blot, respectively. Each bar represents mean ± SE from 3 independent experiments. *P < 0.05, **P < 0.01, compared with control group, #P < 0.05, ##P < 0.01 compared with LPS group, лP < 0.05, ллP<0.01 compared with aspirin group.

activation and IκBα degradation (Fig. 4A and 4B).

The inhibitory effects of aspirin on LPS-induced NF-κB activation was blocked by the co-treatment with POV, whereas there was no significant difference in the activation of NF-κB between aspirin group and combination of aspirin and POV group. However, the inhibitory effects of aspirin on NF-κB activation and IκBα degradation were significantly enhanced by co-treatment with calphostin C (Fig. 4A and 4B).

DISCUSSION

ALI is a very severe illness involved many inflammation-related molecular processes, including the seminal macrophage activation. In this study, our results showed that pretreatment with aspirin abrogated LPS-induced COX-2 and PGE_2 upregulation in p-AMs. Furthermore, treatment with aspirin abrogated the effect of LPS on PKC and PTP activities and NF-κB activation. Moreover, PKC and PTP were shown to be involved in the inhibitory effect of aspirin on LPS-stimulated COX-2 and PGE_2 upregulation and NF-κB activation.

LPS is able to induce the secretion of many proinflammatory cytokines, such as TNF-α, IL-1β, and COX-2 (18, 19). Suppression of the proinflammatory cytokines production by the other constituents, such as salvianolic acid B, and ginsenosides, are suggested to improve several inflammatory diseases (20, 21). Upon LPS stimulation, the secretion of COX-2 and PGE_2 was significantly elevated and aspirin significantly inhibited the upregulation of COX-2 and PGE_2, suggesting a potential anti-inflammatory effect of aspirin on ALI. In the present study, LPS enhanced PKC activity but inhibited PTP activity. Treatment with a PKC inhibitor mitigated LPS-induced COX-2 and PGE_2 upregulation, while treatment with a PTP inhibitor enhanced LPS-induced COX-2 and PGE_2 upregulation in p-AMs, indicating that PKC and PTP are both crucial mediators of LPS-induced COX-2 and PGE_2 upregulation in p-AMs. More importantly, pretreatment with aspirin completely eliminated LPS-modulated PKC and PTP activity, implicating aspirin exerts an inhibitory effect on LPS-induced COX-2 and PGE_2 upregulation in p-AMs through regulating the PKC and PTP activity. Our findings extend previous observations that PKC or PTP inhibitor alone is capable of modulating LPS- and cytokine-induced COX-2 expression in macrophages (16, 22). Therefore, modulation of the PKC and PTP activity may be a promising strategy for therapeutic intervention of pulmonary inflammation.

Many studies have shown that NF-κB pathway plays a pivotal role in ALI (23). The activation of NF-κB positively regulates the expression of a wide variety of genes, particularly in the inflammatory process. In unstimulated cells, NF-κB is bound to IκBa. Upon stimulation, IκBα is phosphorylated and degraded via the ubiquitin proteasome pathway, resulting in the activation of NF-κB and thereby the upregulation of inflammatory cytokines (24). In the present study, treatment with LPS rapidly reduced the levels of IκBα and enhanced NF-κB activation. Furthermore, treatment with the PKC inhibitor abrogated LPS-induced IκBα degradation and NF-κB activation, while treatment with the PTP inhibitor enhanced LPS-induced IκBα degradation and NF-κB activation in p-AMs, suggesting that PKC and PTP are regulators of LPS-induced IκBα degradation and NF-κB activation. Therefore, our findings may provide new insights into the molecular mechanisms underlying LPS-mediated inflammation. Similarly, treatment with aspirin also blocked LPS-induced IκBα degradation and NF-κB activation in p-AMs. More importantly, pretreatment with the PTP inhibitor, but not the PKC inhibitor, blocked the effect of aspirin on LPS-induced IκBα degradation and NF-κB activation, suggesting modulating the activity of PTP and PKC and subsequently inhibiting the activation of NF-κB may contribute to the anti-inflammatory effect of aspirin on ALI.

Previous studies have reported conflicting data on the effect of aspirin on NF-κB activation. Aspirin was shown to promote the nuclear translocation of NF-κB complexes by inducing IκBα phosphorylation and degradation in colon cancer cells, but had no effect on IκBα in embryonic kidney cells and lung adenocarcinoma cells (25). In contrast, another study found that treatment with aspirin reduced the DNA binding activity of NF-κB by inhibiting the IκB kinases (IKK) activity (26). Furthermore, aspirin was demonstrated to block glutamate-induced NF-κB activation (27). In the current study, aspirin suppressed LPS-induced IκBα degradation and NF-κB activation by down-regulating PKC activity, but enhancing PTP activity, and thereby down-regulated the COX-2 expression and PGE_2 production in p-AMs. Collectively, these findings suggest the regulatory effect of aspirin on the IκBα degradation and NF-κB activation is dependent on cell type.

In summary, our study demonstrated that aspirin was effective in LPS-induced inflammatory response through modulating PKC and PTP activity and subsequent IκBα degradation and NF-κB activation in p-AMs. Our data indicate a novel mechanism by which aspirin acts as a potent anti-inflammatory agent in alveolus macrophages.

MATERIALS AND METHODS

Materials

LPS, aspirin, calphostin C, and POV were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The PepTag PKC assay kit was purchased from Promega (Madison, WI, USA). Antibody against IκBα was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Isolation of p-AMs and cell culture

p-AMs were obtained from bronchoalveolar lavage fluid of 4- to 6-week-old conventional Belgian Landrace pigs, as described previously (28). p-AMs were cultured in Earle's modified Eagle's medium (EMEM) supplemented with 10% FBS, 2 mM L-glutamine (BDH Chemicals, Poole, England), 1% nonessential amino acids (Gibco BRL, Invitrogen), 1 mM sodium

pyruvate, and a mixture of antibiotics in a humidified 5% CO_2 atmosphere at 37°C.

Determination of PKC activity in p-AMs

p-AMs were washed twice with phosphate-buffered saline (PBS) and resuspended in 50 μl of buffer A (20 μM Tris, 2 μM EDTA, 0.5 μM EGTA, and 1 μM PMSF, pH 7.4) containing 50 μM 2-mercaptoethanol and 25 μl of 10% NP-40, followed by brief sonication with a Branson Model Sonifier for 45 s at 20 W. The cell lysates were centrifuged at 100,000 g for 1 h and stored at −20°C. The PKC activity was determined by a non-radioactive PKC assays using the PepTag PKC assay kit, according to manufacturer's instructions.

Determination of protein phosphotyrosine phosphatase activity

The PTP activity was determined using the p-nitrophenyl phosphate (pNPP) as a colorimetric substrate. Briefly, PTP assay was carried out in a 200 μl reaction mixture containing 4 μl of PTPs, 10 μM pNPP as the substrate, 25 μM NaAc-HAc buffer (pH 3.8), 1 μM EDTA and 5 μM DTT at 37°C for 10 min and terminated by addition of 10 μl of 200 μM NaOH. Activity was measured at absorbance (optical density, OD) of 405 nm using a microplate reader (Thermo Scientific Corporation, Waltham, MA, USA). According to Lambert-Beer's law, the PTPase activity was determined by the following formula: $[(OD405 \times 910)/(10 \times 1.73 \times 10^4)]$.

Western blotting

Total protein was extracted as previously described (21). Briefly, the cells were lyzed with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) and then centrifuged at 12,000 g for 15 min at 4°C. The supernatants were collected for the next western blot. Protein concentration was determined with BCA method. Protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, US). After blocking with 5% fat-free milk, the membranes were probed with antibodies against IκBα (1 : 800 dilution; C-21, sc-371) or against β-tubulin (1 : 10,000) overnight at 4°C. The bound antibodies were detected with HRP-conjμgated goat anti-mouse IgG (1:2000) for 1 h at room temperature and visualized using enhanced chemiluminescence detection kit (ECL; Amersham International, Buckinghamshire, UK). The relative levels of IκBα to β-tubulin were determined by densitometric analysis (GS-800 densitometer; Bio-Rad, Hercules, CA, USA).

RT-PCR

Total RNA was extracted from p-AMs using the Trizol reagent according to the manufacturer's instructions. cDNA was synthesized using MMLV reverse transcriptase (Promega). Subsequently, the relative levels of COX-2 mRNA were determined by quantitative PCR. The sequences of primers were: COX-2, forward 5'-aag act tgc cag gct gaa ct-3', reverse 5'-ctt ctg cag tcc agg ttc aa-3'; β-actin, forward 5'-tgt gat ggt ggg aat ggg tca g-3' and reverse 5'-ttt gat gtc acg cac gat ttc c-3'. PCR amplifications were performed in duplicate under the following thermal cycling conditions: initial denaturation at 94°C for 4 min; 35 cycles of amplification at 94°C for 50 s, 55°C for 50 s, and 72°C for 90 s. The relative levels of COX-2 mRNA were normalized to β-actin.

Enzyme-linked immunosorbent assay (ELISA)

The levels of PGE_2 in the supernatants were determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA)

The activation of NF-κB was determined by EMSA, as described previously (29). Briefly, nuclear extracts were prepared from pAMs and 5 μg of nuclear extract from individual samples by incubating with 10 μl of binding buffer, containing 1 ng of the 32P-labeled DNA probe (40,000 cpm) and 1 μg of poly (dI-dC), for 30 min at room temperature. A 100-fold excess of the unlabeled oligonucleotide and 1 μg of anti-p50 antibodies were used as a competitor and for supershift, respectively. The DNA-protein complex was resolved on native 5% polyacrylamide gels in TBE buffer and subjected to autoradiography.

Statistical analysis

Data are expressed as mean ± SE. Statistical significance was determined using SPSS 11.0 for Windows. One-way ANOVA was performed for multiple comparisons followed by Fisher LSD post-hoc comparisons. Differences were deemed significant if $P < 0.05$.

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