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

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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). Acute phase of ALI, AMs often release different kinds of pro-inflammatory cytokines, such as cyclooxygenase-2 (COX-2), IL-1\(\beta\), IL-8, IL-6, GM-CSF, and TNF-\(\alpha\), 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-inflammatory activity, including anti-inflammatory activity. 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-\(\kappa\)B 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)
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Aspirin inhibits the LPS-induced COX-2 mRNA and PGE2 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) PGE2 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 PGE2 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.

PKC and PTP were involved in the suppressive effect of aspirin on LPS-stimulated COX-2 and PGE2 production
To further explore the mechanism by which aspirin inhibited LPS-induced COX-2 expression and PGE2 production in p-AMs, we determined the effect of LPS on PKC and PTP activity. As shown in Fig. 2A, LPS stimulation elevated PKC activity, particularly at 10 min post-stimulation. In contrast, the activities of PTP were significantly reduced at 10-15 min post-stimulation in p-AMs. Treatment with aspirin minimized the effect of LPS on PKC and PTP activities in p-AMs (18.90 ± 2.43 ng/ml vs. 18.38 ± 0.99 ng/ml), while aspirin mitigated the LPS-induced PGE2 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).

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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 activation significantly increased the levels of COX-2 mRNA in LPS-stimulated p-AMs (Fig. 2E). Consistently, the level of PGE2 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 PGE2 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 PGE2 upregulation compared with aspirin alone (Fig. 2E and 2F).
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activation and IκBζ 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 IκBζ 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 up-regulation 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 IκBα. 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 IκBζ 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 IκBζ 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 IκBζ 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 IκBζ 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 alveolar 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 (MEM) supplemented with 10% FBS, 2 mM L-glutamine (BDH Chemicals, Poole, England), 1% non-essential amino acids (Gibco BRL, Invitrogen), 1 mM sodium
pyruvate, and a mixture of antibiotics in a humidified 5% CO₂ 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.

**Western blotting**

Total protein was extracted as previously described (21). The bound antibodies were detected with antibodies 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.

**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'-ttt gat gtc acg cac gat ttc c-3' and reverse 5'-ttt gat gtc agc 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 PGE₂ 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.

**Acknowledgements**

This work was supported by the National Nature Science Foundation of China (NSFC No. 39800064, 81071844), and the Nature Science Foundation Project of Chongqing (CSTC No. 2009BB5146).

**REFERENCES**


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