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Salvianolic Acid B Attenuates Cyclooxygenase-2 Expression In Vitro in LPS-Treated Human Aortic Smooth Muscle Cells and In Vivo in the Apolipoprotein-E-Deficient Mouse Aorta

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Abstract Inflammation plays an essential role in atherosclerosis and post-angioplasty restenosis and the synthesis and release of inflammatory cytokines from vascular smooth muscle cells is an important contributor to these pathologies. It is assumed that drugs that prevent the overproduction of inflammatory cytokines may inhibit cardiovascular disorders. In the present study, the effects of a water-soluble antioxidant, salvianolic acid B (Sal B), derived from a Chinese herb, on the expression of cyclooxygenase (COX) in lipopolysaccharide (LPS)-treated human aortic smooth muscle cells (HASMCs) and in the aortas of cholesterol-fed apoE deficient mice were investigated. In unstimulated HASMCs, COX-2 mRNA and protein were almost undetectable, but were strongly upregulated in response to LPS. In contrast, HASMCs with or without LPS treatment showed constitutive expression of COX-1 mRNA and protein. The activation of COX-2 protein synthesis in LPS-stimulated HASMCs was shown to involve the activation of the extracellular-signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase (JNK), and p38 mitogen-activated protein kinase pathway. Incubation of HASMCs with Sal B before LPS stimulation resulted in pronounced downregulation of COX-2 expression. Sal B treatment suppressed ERK1/2 and JNK phosphorylation and attenuated the increase in prostaglandin E_2 production and NADPH oxidase activity in LPS-treated HASMCs. When apoE-deficient mice were fed a 0.15% cholesterol diet with or without supplementation with 0.3% Sal B for 12 weeks, the intima/media area ratio in the thoracic aortas was significantly reduced in the Sal B group ($0.010 \pm 0.009\%$) compared to the apoE-deficient group ($0.114 \pm 0.043\%$) and there was a significant reduction in COX-2 protein expression in the thickened intima. These results demonstrate that Sal B has anti-inflammatory properties and may explain its anti-atherosclerotic properties. This new mechanism of action of Sal B, in addition to its previously reported inhibition of LDL oxidation, may help explain its efficacy in the treatment of atherosclerosis. J. Cell. Biochem. 98: 618–631, 2006.

Key words: salvianolic acid B; smooth muscle cells; COX-2; MAPKs; apoE-deficient mice

There is increasing evidence that inflammation plays a central role in the cascade of events that eventually results in the formation of atherosclerotic plaques and restenosis [van der Wal et al., 1994]. Cyclooxygenase (COX), one of the inflammatory cytokines synthesized

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and secreted by vascular smooth muscle cells, may play an important role in the formation of atherosclerotic plaques and restenosis [Cipollone et al., 2004]. Two isoforms of COX have been identified, namely COX-1 and COX-2 [DeWitt and Smith, 1995]. COX-1 is constitutively expressed and is responsible for the biosynthesis of prostaglandins involved in vascular homeostasis. In contrast, expression of COX-2 is induced in response to growth factors, inflammatory stimuli, and phorbol esters, suggesting that this enzyme is involved in the generation of prostaglandins involved in inflammatory diseases [Simon, 1999]. Consistent with the hypothesis that COX-2 contributes to the clinical instability of coronary artery disease, COX-2 is expressed in atherosclerotic lesions, its levels increase after vascular injury, and it has been detected in the myocardium of patients with congestive heart failure [Cipollone et al., 2001]. Overexpression of COX-2 has been suggested to be involved in the pathogenesis of inflammatory and neoplastic diseases [Cipollone et al., 2004]. The signaling pathways that lead to induction of COX-2 in LPS-stimulated vascular smooth cells, the major component cells of the neointima, are not clearly defined. In addition, COX-2 levels may reflect the degree of inflammation and provide a measure for assessing the effect of drugs on the inflammatory process. The development of COX-2 inhibitors has been a major advance in the therapy of inflammatory processes and their use includes the prevention or treatment of disorders associated with the induction of this enzyme.

The herb, *Salvia miltiorrhiza* Bunge (abbreviated as SM), is often used in folk medicine in China, Japan, and Taiwan for the treatment of cardiovascular disorders (called blood stasis in traditional Chinese medicine) [Lei and Chiou, 1986]. Treatment with SM reduces intimal thickness in the air-injured carotid artery in rats and inhibits the proliferation of isolated rabbit arterial smooth muscle cells [Zhou et al., 1996]. A *Salvia miltiorrhiza* extract (SME) inhibits LDL oxidation in vitro and ex vivo and reduces atherosclerosis in hypercholesterolemic rabbits [Wu et al., 1998]. Sal B, a water-soluble polyphenolic antioxidant fraction of SME, scavenges 1,1-diphenyl-2-picryhydrazyl (DPPH) radicals and inhibits LDL oxidation more effectively than the antioxidant, probucol [Wu et al., 1998]. Sal B attenuates the expression of VCAM-1 and ICAM-1 in TNF-α-stimulated human aortic endothelial cells by partial blockage of NF-κB expression and also significantly inhibits adhesion of the human monocytic cell line, U937, to human aortic endothelial cells [Chen et al., 2001a]. Given that Sal B has cytoprotective and antioxidant effects, we evaluated its effects on LPS-inducible COX-2 mRNA and protein expression in human aortic smooth muscle cells (HASMCs). In addition, we chose cholesterol-fed apo-E deficient mouse model to study the effects of Sal B on intimal thickening and COX expression because it offers an accepted model for studying atherosclerosis and inflammation. Our study shows that Sal B attenuates the expression of COX-2 both in vitro and in vivo, and that this effect is mediated by partial blockage of MAPKs phosphorylation.

MATERIALS AND METHODS

Reagents

Sal B was purified as described previously [Chen et al., 2001a]. In brief, dry roots of SM were ground to a powder, which was then extracted for 24 h at room temperature with 10 volumes of a 4:1 (vol/vol) mixture of water and ethanol. After filtration of the mixture through filter paper, the solvent was evaporated under reduced pressure and the solid material (SME) stored at -70°C. The concentration of Sal B in the SME, determined by reversed-phase HPLC, was 4.4% by weight. Since Sal B is water-soluble and has low pKa values similar to that of a carboxylic acid, the content of Sal B was further increased by adjustment of the pH of the concentrated SME and precipitation with acetone-water. After ion exchange and Sephadex LH-20 column chromatography, Sal B was obtained at greater than 98% purity. For use, it was dissolved as a 14 mM stock solution in phosphate-buffered saline, pH 7.4 (PBS). Unless otherwise specified, all reagents were from Sigma (Missouri).

Culture of HASMCs

HASMCs, purchased as cryopreserved tertiary cultures from Cascade Biologics (OR, USA), were grown in culture flasks in smooth muscle cell growth medium, M231 (Cascade Biologics, Inc.) supplemented with fetal bovine serum (FBS, 5%), human epidermal growth

factor (EGF, 10 ng/ml), human basic fibroblast growth factor (bFGF, 3 ng/ml), insulin (10 μg/ml), penicillin (100 U/ml), streptomycin (100 pg/ml), and Fungizone (1.25 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere. The growth medium was changed every other day until confluency, when the cells were passaged by division between four Petri dishes and again grown to confluency. Cells were used between passages 3 and 8. The purity of the HASMC cultures was verified by immunostaining with a monoclonal antibody against smooth muscle-specific α-actin. Before treatment with lipopolysaccharide (LPS), the cells were serum and growth factor (EGF, bFGF, and insulin)-starved for 24 h.

Effect of LPS and Sal B on Cell Viability

HASMCs were plated at a density of 10^4 cells/well in 96-well plates. After overnight growth, the cells were treated with a different concentrations of LPS or Sal B for various times, then cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MTT (0.5 mg/ml) was applied to the cells for 4 h to allow the conversion of MTT into formazan crystals, then, after washing with PBS, the cells were lysed with dimethyl sulfoxide and the absorbance read at 530 and 690 nm with a DIAS Microplate Reader (Dynex Technologies, VA). The reduction in optical density caused by LPS or drug treatment was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

Testing of the Effect of Preincubation With Sal B on the Effects of LPS

HASMCs (10^6 cells in 5 ml of medium in a 10 cm Petri dish) were incubated with the indicated concentration of Sal B or medium for the indicated time, then medium or the indicated concentration of LPS was added and incubation continued for the indicated time.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time-PCR

Total RNA was extracted using the TRIzol reagent method (Invitrogen) and stored at −80°C until use. For RT-PCR studies, RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen), and single-stranded cDNA was used as a template in PCR. cDNA aliquots were amplified with primers specific for COX-1 and COX-2 and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in a Gene-Amp PCR system 9600 (Perkin Elmer, CA) and the PCR products were run on 2% tri-borate ethylenediaminetetraacetic acid (TBE) agarose gels. Quantitative PCR was performed using continuous fluorescent monitoring of PCR amplification (Light Cycler). Each reaction mix (20 μl final volume) contained 4 mmol/L $MgCl_2$, 0.4 μmol/L each primer, 1× SYBRGreenI, 1× Titanium Tdq DNA polymerase, and template cDNA. The COX-1-specific oligonucleotide primer pair consisted of the sense primer 5′-GAG CAT CTC TCG GAT GAA GG and the antisense primer 5′-GCC AGT GAA TCC CTG TTG TT, the corresponding COX-2-specific primers being 5′-ATG AGA TTG TGG GAA AAT TGC T and 5′-GAT CAT CTC TGC CTG AGT ATC. To quantify and prove the integrity of the isolated RNA, RT-PCR analysis for GAPDH was carried out using the specific sense and antisense primers TGC CCC CTC TGC TGA TGC C and CCT CCG ACG CCT GCT TCA CCA C. The parameters used for PCR were an initial denaturation at 95°C for 480 s followed by 40 cycles of 95°C for 10 s, 50°C for 10 s, and 72°C for 22 s. Real-time PCR monitoring was achieved by measuring the fluorescent signal at the end of the annealing phase for each cycle. Levels of COX-1 and COX-2 mRNA were normalized to GAPDH mRNA levels.

Western Blot Analysis of Cell Lysates

Western blot analyses were performed as described previously [Chen et al., 2002]. Briefly, a cell lysate was prepared by lysing cells for 1 h at 4°C in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, pH 7.4, then centrifuging the lysate at 4,000*g* for 30 min at 4°C and taking the supernatant. An aliquot of cell lysate (20 μg total protein) was subjected to 12% SDS–PAGE electrophoresis and transferred onto PVDF membranes, which were then blocked for 1 h at room temperature with 2% skimmed milk in PBS-0.05% Tween 20.

To measure levels of COX-1 or COX-2, the membranes were incubated with mouse polyclonal antibody against human COX-1 or COX-2 (1:1,000, Cayman), then with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:3,000, Chemicon), bound antibody being detected using Chemiluminescence

Reagent Plus (NEN). The intensity of each band was quantified using a densitometer. Anti-β-actin antibodies (1:1,000, Oncogen) were used to quantify β-actin, used as the internal control. In other studies, the antibodies used were rabbit anti-human phospho-JNK, mouse anti-human phopho-ERK1/2, rabbit anti-human phospho-p38, rabbit anti-human total JNK, rabbit anti-human total ERK1/2, and goat anti-human total p38 (1:1,000, Cell Signaling).

Measurement of PGE_2 Levels

HASMCs were subcultured into 10 cm dishes and cultured as indicated in the figure legends. At the selected time points, the culture supernatant was centrifuged at 1,000*g* for 5 min, then 100 μl of the supernatant was assayed for PGE_2 by enzyme immunoassay (R&D Systems, Inc.).

Plasma Membrane Preparation, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Assay, and Western Blot Analysis of $p47^{phox}$

For NADPH oxidase assay, plasma membrane fractions were prepared by lysing the cells by three cycles of freeze-thaw (liquid nitrogen for 5 min, 37°C for 5 min) in lysis buffer containing 1 mM EDTA, 20 mM potassium phosphate, pH 7.0, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 10 μg/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuging the lysate at 29,000*g* at 4°C for 20 min. The pellet was suspended in oxidase assay buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 150 mM sucrose, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.5 mM PMSF) and assayed for protein (Bio-Rad) and NADPH oxidase activity measured in a lucigenin chemiluminescence assay using 5 μM lucigenin (Sigma) and 100 μM NADPH (Sigma) as described previously [Lin et al., 2005]. Chemiluminescence as relative light units was measured in a microtiter luminometer (Wallac 1420 Multilabel counter, Perkin Elmer) as an indicator of enzyme activity. Western blot analysis for $p47^{phox}$ was performed on the cytosolic and plasma membrane fractions as described above using a monoclonal mouse antibody against $p47^{phox}$ (BD, Biosciences Pharmingen).

Animal Care and Experimental Procedures

The apoE-deficient mice were purchased from Jackson Laboratory (Bar Harbor, ME). After 6 months on a commercial mouse chow diet, 20 mice were randomly allocated to one of two groups; both of which received a 0.15% cholesterol diet (Purina Mills, Inc., USA), supplemented in one group with 0.3% Sal B, a dose previously shown to reduce lesion formation in hypercholesterolemic rabbits [Chen et al., 2001b]. Water was available *ad libitum*. C57BL/6 mice were used as the control. Mice were allowed free access to the diets for 3 months during the experiment and were deprived of food overnight at the end of the experiment. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). Blood samples were taken periodically for assessment of liver and renal function. After 3 months on the diet, the mice were euthanized by intraperitoneal injection of 35–40 mg/kg of sodium pentobarbital and the thoracic aortas were gently dissected free of adherent tissue, rinsed with ice-cold PBS, immersion-fixed in 4% buffered paraformaldehyde, paraffin-embedded, then cross-sectioned for immunohistochemisty. To examine the expression of COX-1 and COX-2 protein, immunohistochemisty was performed on serial sections of the aorta, which were deparaffinised, rehydrated, and washed with PBS, then non-specific binding was blocked by preincubation for 1 h at room temperature with PBS containing 5 mg/ml of bovine serum albumin. The sections were then incubated for 1 h at 37°C with mouse anti-human COX-1 or COX-2 antibody (1:15 dilution in PBS) and for 1 h at room temperature with biotinylated rabbit anti-mouse IgG antibody, bound antibody being detected by incubation for 1.5 h at room temperature with avidin-biotin-horseradish peroxidase complex, followed by 0.5 mg/ml of 3,3′-diaminobenzidine/0.01% hydrogen peroxide in 0.1 M Tris-HCl buffer, pH 7.2, as chromogen (Vector Lab, USA). Negative controls were performed by omitting the primary antibodies. Semiquantification of antigen expression was evaluated under the light microscope at magnification 200×. Each specimen was read by three investigators who were blinded as to the type of immuno-stain and they were asked to assign arbitrarily an immuno-score of 0, no; 1, weak; 2, moderate; 3, strong; and 4, very strong staining. A score of 0 was given to areas where staining was absent or as weak as the same area on the control section

without primary antibodies incubation. A score of 4 was given to the darkest staining intensity observed. When staining intensities ranged between 2 levels, for example 1 and 2, a score of 1.5 was assigned. To avoid inter-assay variability, all sections used for quantitation were from the same staining batch.

Statistical Analysis

All data were expressed as mean ± SEM. The difference in mean values among different groups was analyzed by one-way ANOVA and subsequent post-hoc Dunnett test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Sal B Reduces COX-2 mRNA and Protein Expression in LPS-Treated HASMCs

When the cytoxicity of LPS or Sal B for HASMCs was assessed by MTT assay after 24 h of incubation, cell viability was unaffected by the presence of 1 μg/ml of LPS or 20 μM Sal B (data not shown). Concentrations of 10–100 ng/ml of LPS and 2.5–10 μM Sal B were therefore used in subsequent experiments.

To determine whether LPS or Sal B affected levels of COX-1 and COX-2 mRNA and protein, RT-PCR, real-time PCR, and Western blotting were performed. Control cells expressed COX-1 mRNA and the amount was not affected by treatment with 100 ng/ml of LPS alone or by prior treatment for 24 h with 10 μM Sal B (Fig. 1A). After 24 h of incubation in serum-free medium, the unstimulated HASMCs produced small amount of COX-2 mRNA. However, for HASMCs treated with 100 ng/ml of LPS, the amount of COX-2 mRNA first increased to a maximum after 2 h of treatment, then decreased gradually. This increase was markedly inhibited by 24 h preincubation with 10 μM Sal B (Fig. 1B). In cells incubated with Sal B alone, COX-2 mRNA levels were similar to those in unstimulated samples (data not shown).

Western blot analysis showed that treatment with LPS induced COX-2 protein expression in a time- and dose-dependent manner (data not shown), but had no effect on COX-1 protein expression (Fig. 1C). After treatment with 100 ng/ml of LPS for 8 h, COX-2 protein expression increased by about four-fold and this effect was significantly decreased by 24 h pretreatment with 10 μM Sal B (Fig. 1D), which had no effect on COX-1 protein expression (Fig. 1C). In cells incubated with Sal B alone, COX-2 protein levels were similar to those in the unstimulated samples (data not shown).

Sal B Attenuates PGE_2 Production in LPS-Treated HASMCs

Unstimulated HASMCs produced low amounts of PGE_2 (13.0 ± 0.4 pg/ml) and this was not affected by incubation with Sal B alone (data not shown). When the cells were incubated with 100 ng/ml of LPS for various times, the concentration of PGE_2 in the medium increased, reaching levels of 34.0 ± 0.2 and 33.2 ± 0.5 pg/ml at 6 and 8 h, respectively (data not shown). When cells were precultured in medium containing 2.5–10 μM Sal B for 24 h, then incubated with 100 ng/ml of LPS for 8 h, only background levels of PGE_2 were released (16.9 ± 2.2 pg/ml of cell protein at 10 μM Sal B) (Fig. 2).

Sal B Reduces ICAM-1 Expression in LPS-Treated HASMCs via COX-2

COX-2 is suggested to modulate the expression of adhesion molecules involved in the interaction between leukocytes and vascular cells during endotoxemia [Kyrkanides et al., 2002; Katagiri et al., 2004]. Time-dependency experiments showed that ICAM-1 expression increased in an almost linear fashion in HASMCs in response to incubation with 100 ng/ml of LPS and that 10 μM Sal B effectively suppressed the induction of ICAM-1 by LPS (Fig. 3A). The amount of β-actin protein as an internal control remained unchanged. Cotreatment of cultures with 3–30 μM NS-398, a COX-2 inhibitor [Futaki et al., 1993], markedly inhibited LPS-induced ICAM-1 expression (Fig. 3B).

Sal B Reduces LPS-Induced Phosphorylation of ERK and JNK

Previous studies have shown that LPS can activate MAPKs in the signaling pathway leading to inflammation [Lee and Young, 1996; Guha and Mackman, 2001]. In the next set of experiments, the effects of LPS on the activation of the MAPK pathway (ERK1/2, JNK, p38), a signaling cascade contributing to COX-2 expression, and the effects of Sal B or MAPK inhibitors on LPS-stimulated COX-1 and COX-2 expression were studied. As shown in Figure 4A–C, phosphorylation of JNK1/2 and ERK was increased to 36.2- and 4.9-fold of

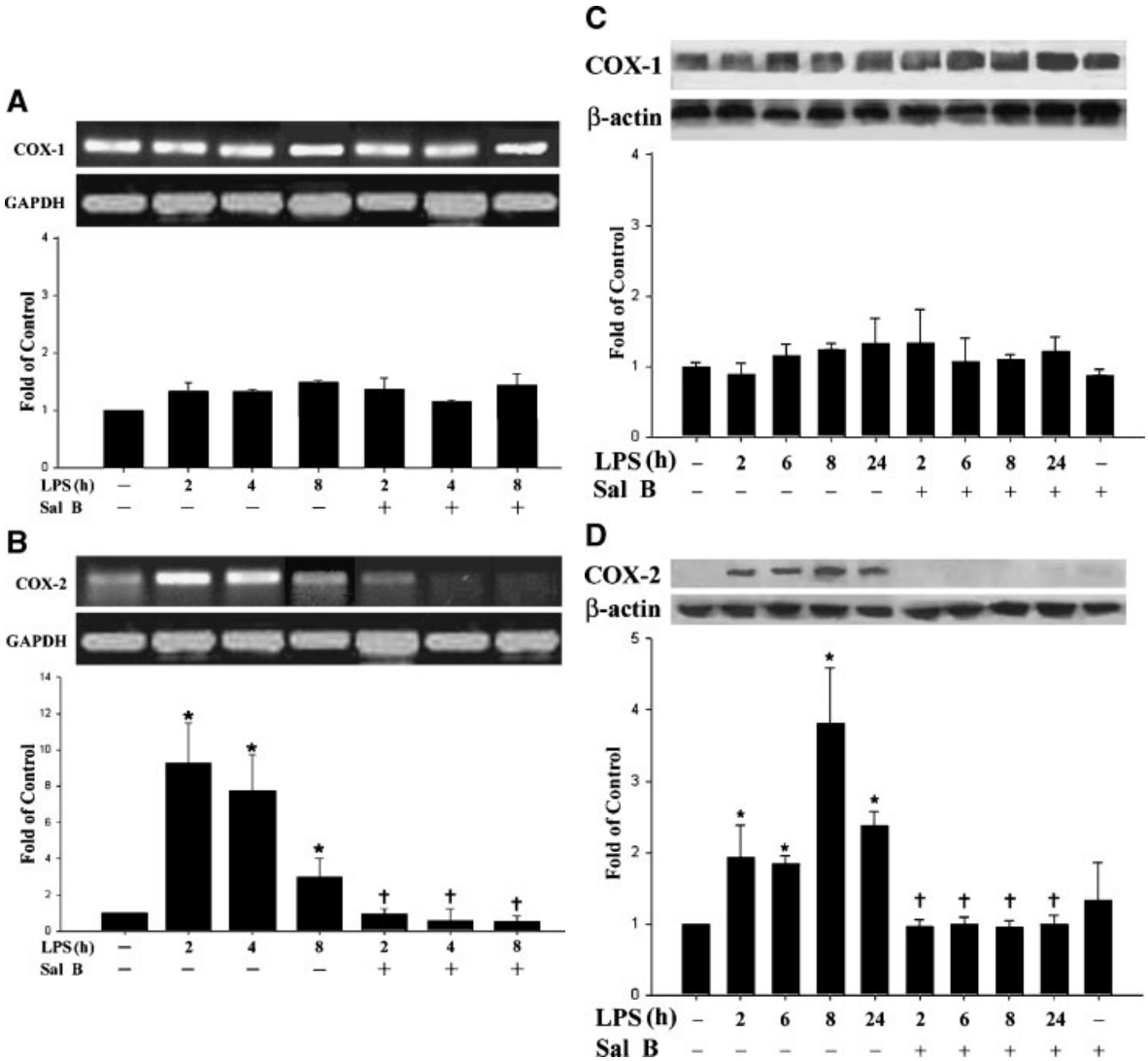


Fig. 1. Sal B inhibits the LPS-induced increase in COX-2 mRNA and protein levels in HASMCs. HASMCs were pretreated with 10 μM Sal B for 24 h before addition of 100 ng/ml of LPS in the continued presence of Sal B for the indicated time, then levels of COX-1 mRNA **(A)** or COX-2 mRNA **(B)** were assessed by RT-PCR and real-time PCR as well as COX-1 **(C)** or COX-2 **(D)** protein levels were examined by Western blots. The data are the mean ± SEM for three separate experiments. $^{*}P < 0.05$ compared to the control cells; $^{\dagger}P < 0.05$ compared to HASMCs treated with LPS alone for the same time.

control levels, respectively, at 5 min after addition of 100 ng/ml of LPS, whereas p38 phosphorylation was less affected (increased to 1.4-fold of control levels). Interestingly, pretreatment with 10 μM Sal B decreased LPS-induced JNK and ERK phosphorylation, but increased p38 phosphorylation. As shown in Figure 4D, LPS-induced COX-2 expression was inhibited by SP600125 (a JNK inhibitor), PD98059 (an ERK inhibitor), and SB20350 (a p38 inhibitor). These results suggest that Sal B inhibits COX-2 expression by preventing LPS-induced phosphorylation of JNK and ERK.

Sal B Inhibits the Increase in NADPH Oxidase Activity and p47^{phox} Translocation Seen in LPS-Treated Hasmcs

Activation of vascular NADPH oxidase and the production of reactive oxygen species by this enzyme system contribute to cardiovascular diseases, including atherosclerosis and hypertension [Cai et al., 2003]. To examine whether LPS and Sal B affected NADPH oxidase activity, HASMCs were treated with 100 ng/ml of LPS for 40 min, then the plasma membrane fraction was prepared to measure NADPH oxidase activity. As shown in Figure 5A, LPS

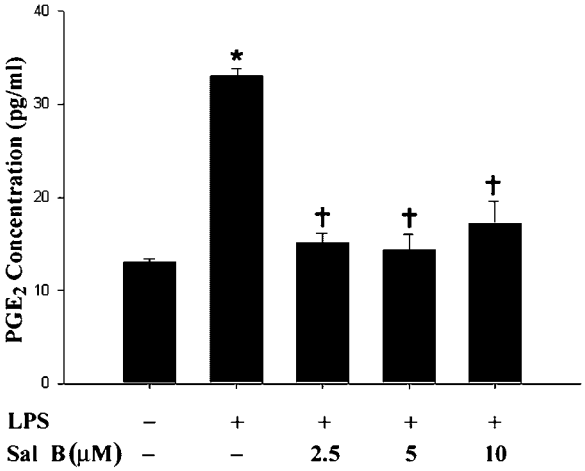


Fig. 2. Sal B inhibits the increase in PGE_2 levels in LPS-stimulated HASMCs. Cells grown to confluency in 10 cm dishes were incubated for 24 h with 2.5, 5, or 10 μM Sal B, then stimulated for 8 h with 100 ng/ml of LPS. The medium was then collected and the PGE_2 concentration measured by ELISA. The data are the mean ± SEM for three separate experiments. $^*P < 0.05$ compared to control cells. $^\dagger P < 0.05$ compared to HASMCs treated with LPS alone.

addition resulted in a significant increase in enzymatic activity which was inhibited by Sal B pretreatment. We then determined whether this effect of Sal B was associated with translocation of $p47^{phox}$ in HASMCs, as this translocation mechanism has been reported to play an important role in the activation of NADPH oxidase [Mortensen and Zhong, 2000]. HASMCs were treated with 100 ng/ml of LPS for 20, 40, or 60 min, then cytosolic and plasma membrane fractions were prepared and immunoblotted for $p47^{phox}$. As shown in Figure 5B and C, stimulation of HASMCs with 100 ng/ml of LPS for different times induced $p47^{phox}$ translocation to the membrane. Using an LPS incubation time of 40 min, 24 h pretreatment with Sal B resulted in a decrease in the $p47^{phox}$ content of both the cytosolic and membrane fractions in LPS-treated HASMCs (Fig. 5D and E), suggesting an effect on both expression and translocation. In contrast, using a LPS incubation time of

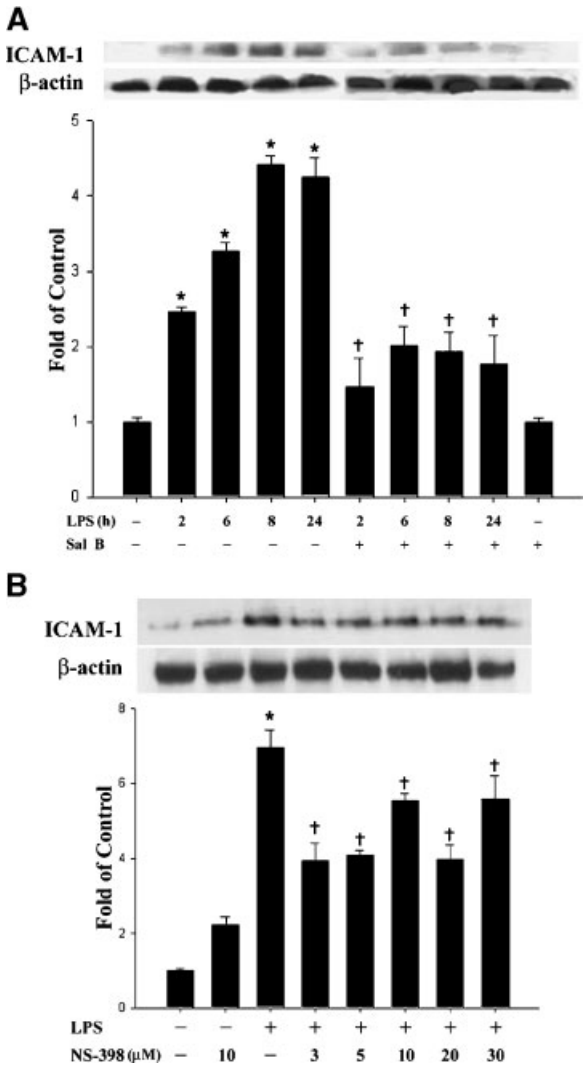
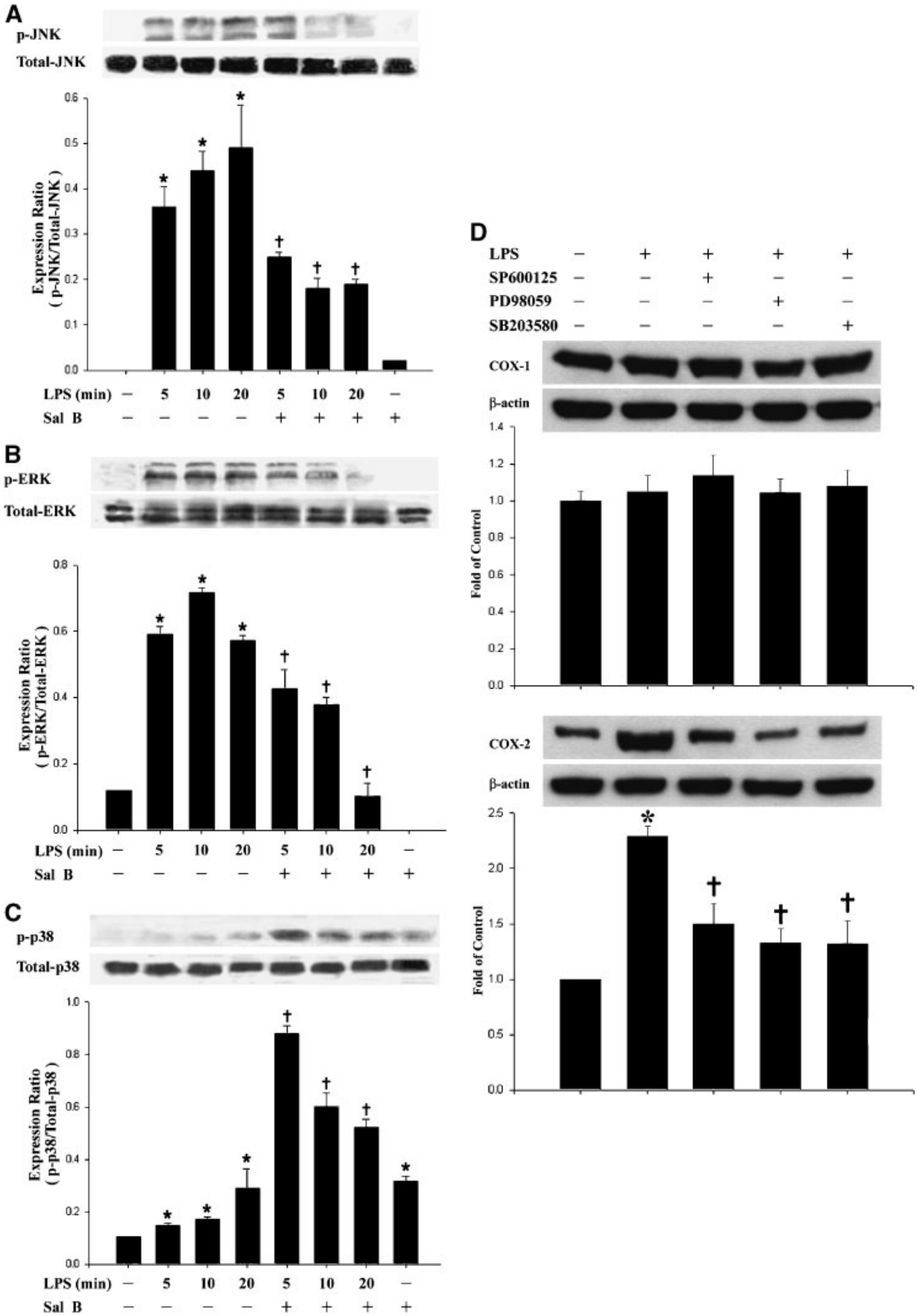


Fig. 3. Western blots of HASMC cell lysates showing that Sal B inhibits the LPS-induced increase in ICAM-1 levels via the COX-2 pathway. **A**: ICAM-1 expression in HASMCs treated with 100 ng/ml of LPS for the indicated time, with and without preincubation for 24 h with 10 μM Sal B, was assessed by Western blotting. **B**: Cultures were incubated for 24 h with medium alone, for 1 h with medium containing 10 μM NS-398 (a COX-2 inhibitor), for 8 h with 100 ng/ml of LPS, or for 1 h with 3–30 μM NS-398, then for 8 h with 100 ng/ml of LPS. $^*P < 0.05$ compared to control cells. $^\dagger P < 0.05$ compared to LPS-treated HASMCs.

40 min, 1 h pretreatment with Sal B had no effects on the $p47^{phox}$ content of both the cytosolic and membrane fractions compared with LPS-treated HASMCs (Fig. 6A and B).

Fig. 4. Western blots of HASMC cell lysates showing that Sal B inhibits the LPS-induced increase in ERK and JNK phosphorylation. **A–C**: Control cells or HASMCs pretreated with 10 μM Sal B for 24 h were incubated with 100 ng/ml of LPS for the indicated time, then equal amounts of cell lysate protein were subjected to immunoblotting using antibodies against JNK (A), ERK (B), or p38 (C). **D**: COX-1 and COX-2 expression in cells incubated for 1 h with medium or 30 μM SP600125 (a JNK inhibitor), 30 μM PD98059 (an ERK inhibitor), or 30 μM SB20350 (a p38 inhibitor), followed by addition of medium or 100 ng/ml of LPS and incubation for a further 8 h. $^*P < 0.05$ compared to control cells. $^\dagger P < 0.05$ compared to LPS-treated HASMCs.


A
p-JNK
Total-JNK
Expression Ratio (p-JNK/Total-JNK)
LPS (min) – 5 10 20 5 10 20 –
Sal B – – – – + + + +
B
p-ERK
Total-ERK
Expression Ratio (p-ERK/Total-ERK)
LPS (min) – 5 10 20 5 10 20 –
Sal B – – – – + + + +
C
p-p38
Total-p38
Expression Ratio (p-p38/Total-p38)
LPS (min) – 5 10 20 5 10 20 –
Sal B – – – – + + + +
D
LPS – + + + +
SP600125 – – + – –
PD98059 – – – + –
SB203580 – – – – +
COX-1
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COX-2
β-actin
Fold of Control

Fig. 4.

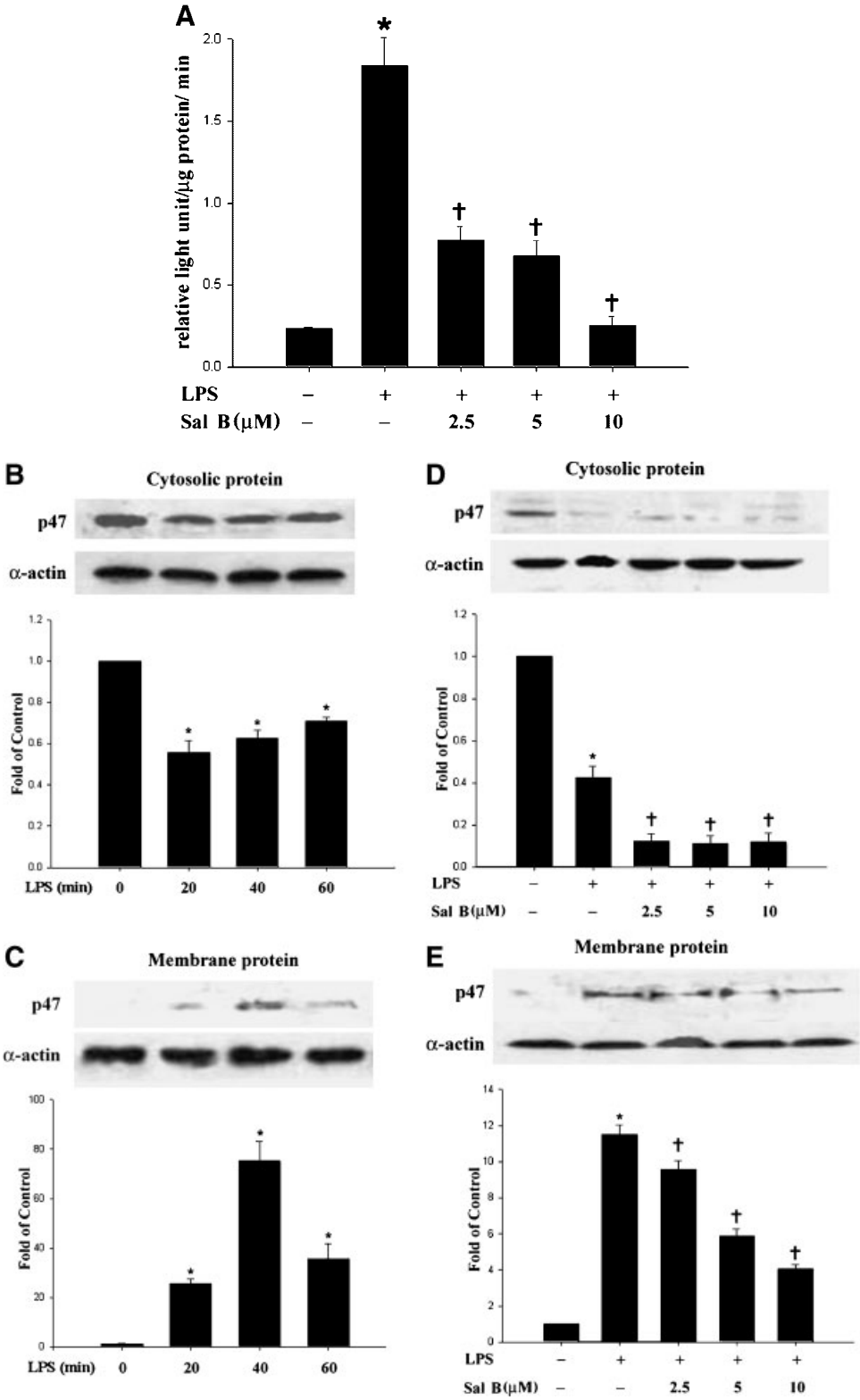

Fig. 5. Sal B attenuates the increase in NADPH oxidase activity and reduces the translocation of the NADPH oxidase subunit, $p47^{phox}$, to the cell membrane in LPS-treated HASMCs. Control cells or cells pretreated for 24 h with the indicated concentration of Sal B were incubated with 100 ng/ml of LPS for 40 min, then 50 μg of plasma membrane protein was assayed for oxidase activity **(A)**. **B**, **C**: $p47^{phox}$ levels in the cytosolic (B) and plasma membrane (C) fractions of HASMCs treated with 100 ng/ml LPS for the indicated time. **D**, **E**: $p47^{phox}$ levels in the cytosolic (D) and membrane (E) fractions of HASMCs pretreated for 24 h with the indicated concentration of Sal B, then stimulated with 100 ng/ml of LPS for 40 min. The data are the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared to untreated controls. $^{\dagger}P < 0.05$ compared to LPS-treated HASMCs.

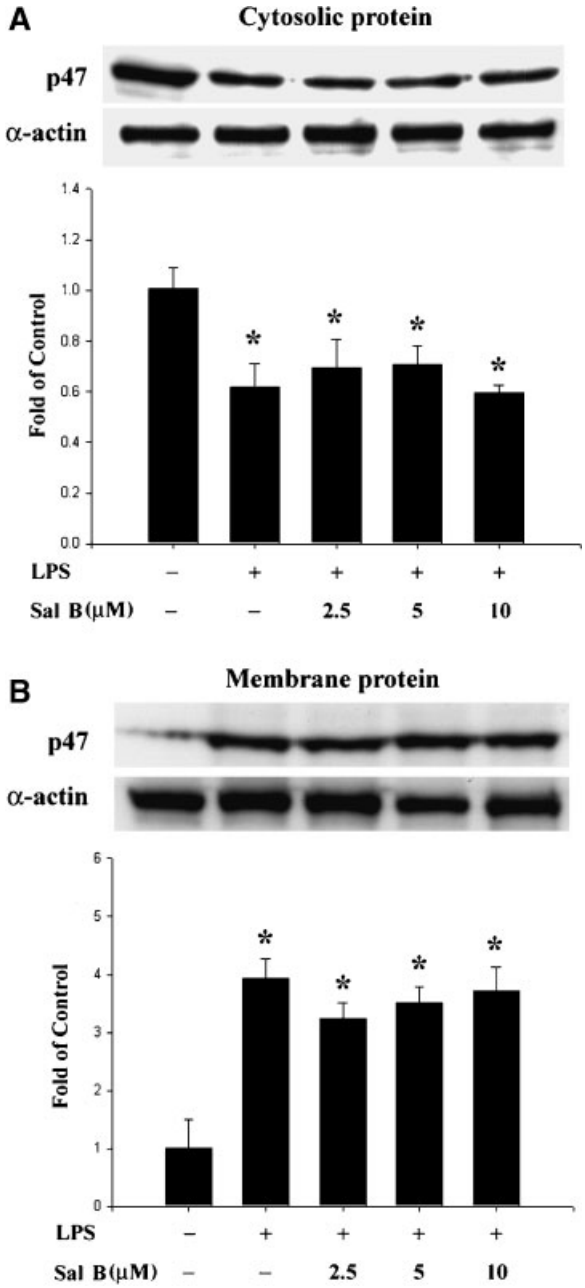

Fig. 6. Sal B pretreatment for 1 h did not affect LPS-induced the translocation of the NADPH oxidase subunit, $p47^{phox}$ in HASMCs. $p47^{phox}$ levels in the cytosolic **(A)** and membrane **(B)** fractions were examined in HASMCs pretreated for 1 h with the indicated concentration of Sal B, then stimulated with 100 ng/ml of LPS for 40 min. The data are the mean ± SEM of three independent experiments. $^*P < 0.05$ compared to untreated controls.

Sal B Decreases COX-2 Protein Expression in Thoracic Aortas of apoE-Deficient Mice

Over the experimental period, there was no difference in weight gain and final weight of the C57BL/6, cholesterol-fed apoE-deficient mice, and Sal B-treated cholesterol-fed apoE-deficient mice. Morphometric analysis showed that the intima/media area ratio in the Sal B-treated cholesterol-fed apoE-deficient mice ($0.010 \pm 0.009\%$) was significantly less than that in the cholesterol-fed apoE-deficient mice ($0.114 \pm 0.043\%$). In the C57BL/6 control group, the intima was very thin. To study the effect of Sal B on COX-1 and COX-2 expression in apo E-deficient mice, immunohistochemical staining with antibodies against COX-1, COX-2 or anti-α-actin antibody (staining smooth muscle cells) was carried out on serial sections. In the cholesterol-fed apoE-deficient mice, α-actin-positive staining was seen on the thickened intima of the thoracic aortas (Fig. 7) and strong COX-2 staining was seen on the thickened intima. In Sal B-treated cholesterol-fed apoE-deficient mice, the intimal area was reduced. Semiquantitative analysis showed that the expression of COX-2 in Sal B-treated group was significantly reduced from 3.8 ± 0.3 to 1.5 ± 0.2. In contrast, there was no significant difference in the expression of COX-1 between the Sal B-treated group and the cholesterol-fed apo-E deficient group (1.6 ± 0.3 vs. 1.8 ± 0.2). In the control group, there were a little COX-1 and COX-2 staining of the vascular walls.

DISCUSSION

In this study, we found that LPS treatment of cultured HASMCs induced COX-2 mRNA and protein expression in a time- and dose-dependent manner, but did not affect constitutive COX-1 mRNA and protein expression. Sal B treatment effectively blocked COX-2 expression in LPS-stimulated HASMCs and apoE-deficient mice, but did not affect COX-1 expression. Our data demonstrate that the inhibitory effect of Sal B on COX-2 expression was mediated by downregulation of JNK and ERK phosphorylation.

Increased expression of COX-2 is associated with inflammatory responses and also with serious disorders, such as cardiovascular failure [Hocherl et al., 2002]. Eicosanoids, including PGE_2, which contribute to the pathogenesis of cardiovascular diseases through proinflammatory and proaggregatory activities [Murakami et al., 2002], are formed from arachidonic acid in membrane phospholipids by the action of COX [Smith et al., 1994]. COX activity is the rate-limiting step in the production of PGE_2 and

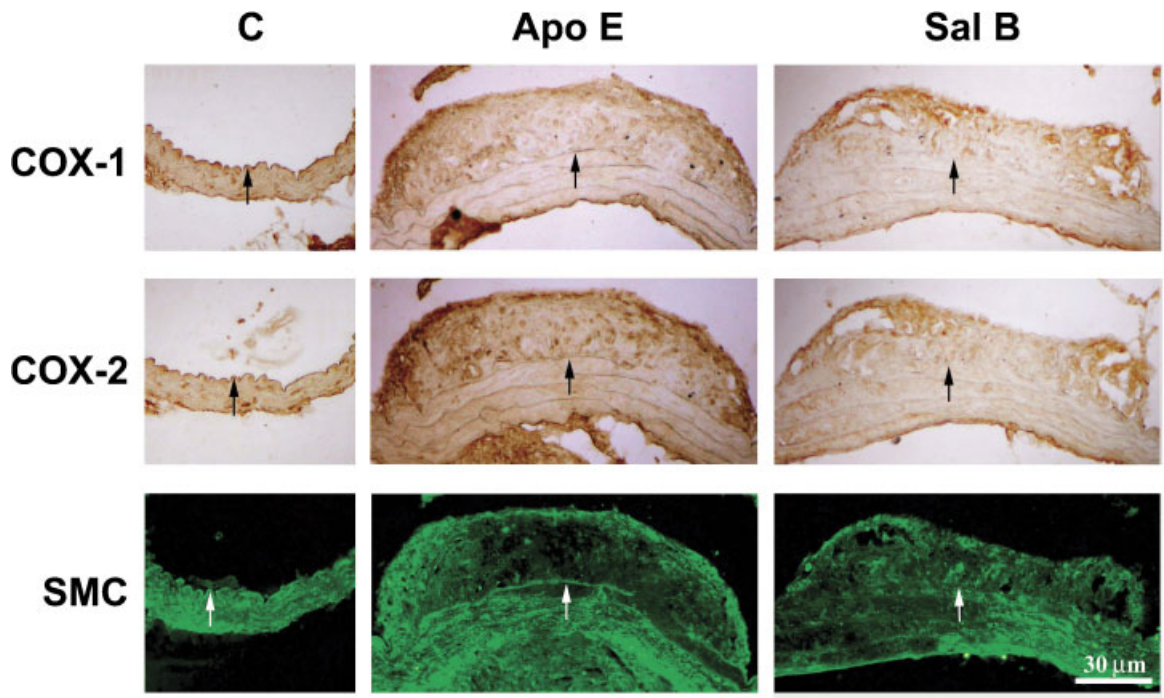

Fig. 7. Immunohistochemical staining for COX-1, COX-2, or α-actin in serial sections of thoracic aortas from C57BL/6 mice (C), cholesterol-fed apoE-deficient mice (Apo E), and Sal B-treated cholesterol-fed apoE-deficient mice (Sal B). The lumen is uppermost in all sections. The internal elastic membrane is indicated by an arrow. Strong COX-2 staining was seen in the markedly thickened intima of the cholesterol-fed apoE-deficient mice, while weak COX-2 staining was seen in the less thickened intima of the Sal B-treated cholesterol-fed apoE-deficient mice. The intensity of COX-1 staining was similar among three groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

is determined by the level of the enzyme and the presence of the activating oxidant, hydroperoxide [Smith and Langenbach, 2001; Nakatani and Kudo, 2002]. Two forms of COX exist. COX-1 is constitutively expressed and is involved mainly in the control of normal physiologic functions, whereas COX-2 expression is regulated by growth factors, tumor promoters, cytokines, glucocorticoids, and LPS [Masferrer et al., 1994; Lo et al., 1998]. Rimarachin et al. [1994] showed that mechanical injury increases COX-2 expression in vascular smooth muscle cells during the development of proliferative lesions in the injured vessels. In the present study, we demonstrated that HASMCs treated with LPS showed a marked increase in COX-2 expression and PGE_2 production. However, during induction of COX-2 mRNA and protein expression, expression of the constitutive COX-1 remained unchanged, as shown by real-time PCR and Western blot analyses. These findings are consistent with the hypothesis that COX-2 has a pathophysiological role in the early modulation of vascular responses to inflammatory stimuli.

We decided to test the effects of Sal B, as it is derived from a Chinese herb, *Salvia miltiorrhiza*, commonly used in traditional Chinese medicine for the treatment of blood stasis, a cardiovascular-related disorder [Lei and Chiou, 1986]. Nonpolar extracts of the plant contain tanshinones, which inhibit platelet aggregation [Onitsuka et al., 1983] and protect the myocardium against ischemia-induced derangement [Yagi et al., 1989]. *Salvia mitiorrhiza* extract (SME), an aqueous ethanolic extract of *Salvia miltiorrhiza*, is rich in polyphenolic compounds that protect liver microsomes, hepatocytes, and erythrocytes against oxidative damage [Liu et al., 1992]. SME scavenges DPPH radicals, inhibits Cu^{2+}-induced LDL oxidation, and reduces endothelial damage and the severity of atherosclerosis in cholesterol-fed rabbits [Wu et al., 1998] and inhibits intimal hyperplasia and monocyte chemotactic protein-1 expression after balloon injury in cholesterol-fed rabbits [Chen et al., 2001b]. Sal B, one of these compounds in SME, is a potent hepatoprotective agent and water-soluble antioxidant and attenuates ischemia-reperfusion injury-indu-

ced skin flap necrosis [Lay et al., 2003]. In our previous study, we demonstrated that Sal B markedly attenuates the tumor necrosis factor-α-induced expression of VCAM-1 and reduces the binding of human monocytes to human aortic endothelial cells [Chen et al., 2001a]. In the present study, we found that pretreatment with Sal B significantly attenuated LPS-induced COX-2 expression in HASMCs and inhibited PGE_2 production. Collectively, these data suggest that Sal B treatment decreases COX-2 expression and that its arachidonic acid-derived products produced by COX-2 action may participate in the prevention of inflammation and pathogenesis of atherosclerosis.

Mitogen-activated protein kinases (MAPKs) play an important role in regulating the expression of proinflammatory molecules in many cells [Tibbles and Woodgett, 1999; Lin et al., 2005]. Recent studies have indicated a possible role for MAPKs in the COX-2 gene expression induced by LPS, growth factors, and cytokines [Lo, 2003; Lin et al., 2004]. For example, LPS has been shown to activate p42/44 MAPKs (ERK) and p38 MAPK and induce COX-2 gene expression in macrophages/monocytes [Niiro et al., 1998]. Consistent with these findings, the present study showed that phosphorylation of JNK1/2 and ERK was significantly increased at 5 min after LPS addition, whereas phosphorylation of p38 was less affected. Interestingly, Sal B administration decreased LPS-induced JNK and ERK phosphorylation, but increased p38 phosphorylation. COX-2 expression in response to LPS was partially inhibited by SP600125 (a JNK inhibitor), PD98059 (an ERK inhibitor), or SB20350 (a p38 inhibitor). Our results indicate that Sal B attenuates LPS-stimulated COX-2 expression and PGE_2 production via early inactivation of ERK and JNK phosphorylation, but not via p38 phosphorylation. It appears that the COX-2 pathway is very complex and may involve cross-talk between MAPKs. Future studies should be focused on how these MAPKs interact with each another, leading to the ultimate cell activation.

Accumulating evidence suggests that cardiovascular diseases are associated with increased oxidative stress in blood vessels. Reactive oxygen species (ROS), such as superoxide and H_2O_2, cause blood vessels to thicken and produce inflammation in the vessel wall, and are therefore regarded as "risk factors" for vascular disease, and also act as signaling molecules in many aspects of inflammatory cytokine-mediated physiological responses. Activation of vascular NADPH oxidase and the production of ROS by these enzyme systems are common in cardiovascular disease [Touyz et al., 2003]. Recently, efforts have been devoted to developing NADPH oxidase inhibitors that will provide useful experimental tools and might have therapeutic potential in the treatment of human diseases. NADPH oxidase is localized on the cell surface and generates superoxide extracellularly. After synthesis, the components of this oxidase are transported to the cell membrane where the functional NADPH oxidase complex is assembled. In the present study, LPS treatment of HASMCs resulted in a significant increase in enzymatic activity of NADPH oxidase. The amount of $p47^{phox}$, a key component of NADPH oxidase, was decreased in the cytosolic fraction of LPS-treated cells compared to untreated controls, while $p47^{phox}$ levels in the cell membrane increased after 40 min of treatment. These data suggest that LPS induces $p47^{phox}$ translocation from the cytoplasm to the membrane. Sal B pretreatment for 24 h inhibited the LPS-induced expression and activation of NADPH oxidase and resulted in a decrease in the $p47^{phox}$ content of both the cytoplasmic and membrane fractions in LPS-treated HASMCs. These results suggest that Sal B may serve as an inhibitor of NADPH oxidase and have therapeutic potential in the treatment of inflammation and cardiovascular diseases.

COX-2 has been detected in fatty streaks in both humans and mice [Hong et al., 2000; Burleigh et al., 2002], strongly suggesting that it is as an important determinant in the formation of atherosclerotic lesions. In our previous study [Chen et al., 2001a], we used cholesterol-fed endothelium-denuded rabbits as an animal model and found that SME significantly reduce areas of atheroma and MCP-1 expression. In the present study, we used apoE-deficient mice as an animal model with severe hypercholesterolemia and extensive atherosclerosis to test the effects of Sal B, a pure compound, on the generation of atherosclerotic lesions and inflammatory cytokine expression. The intima/media area ratio in thoracic aortas in cholesterol-fed apoE deficient mice was significantly decreased by Sal B treatment. Immunohistochemical studies using anti-COX-2 antibody showed that COX-2 was localized to smooth muscle cells in atherosclerotic lesions of

apoE-deficient mice and that COX-2 expression was significantly reduced in apoE-deficient mice pretreated with Sal B, whereas COX-1 expression was not affected. Although the previous study showed that neither selective COX-2 inhibitor (tricyclic) nor non-selective COX inhibitor (sulindac) influenced the development of advanced atherosclerosis in apoE-deficient mice [Olesen et al., 2002], our findings suggest an additional mechanism by which Sal B can prevent the progress of atherosclerosis, namely by inhibiting COX-2 expression. The difference between these findings may be related to differences of the dosage and duration of drug treatment and the drugs' properties.

In conclusion, this study shows that LPS induces COX-2 expression in HASMCs. This is the first study to show that Sal B, a water-soluble antioxidant, reduces COX-2 expression and consequently decreases PGE_2 production by HASMCs. Sal B inhibits LPS-inducible COX-2 expression in HASMCs by suppression of JNK and ERK phosphorylation. Since PGE_2 production by smooth muscle cells after COX-2 overexpression is a crucial step in the pathogenesis of atherosclerosis and restenosis, our study implies that antioxidants may have a therapeutic potential in the prevention of cardiovascular disease. Water-soluble polyphenolic antioxidants, such as Sal B, may have an additional beneficial effect in multiple pathological events involving inhibition of COX-2 expression, including inflammation and atherosclerosis.

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