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Salvianolic Acid B Attenuates MMP-2 and MMP-9 Expression In Vivo in Apolipoprotein-E-Deficient Mouse Aorta and In Vitro in LPS-Treated Human Aortic Smooth Muscle Cells

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Abstract Salvianolic acid B (Sal B), a water-soluble antioxidant derived from a Chinese medicinal herb, is believed to have multiple therapeutic and preventive against human vascular diseases, including atherosclerosis and restenosis. To elucidate the underlying cellular mechanisms, we produced hypercholesterolemia by feeding apo-E-deficient mice a 0.15% cholesterol diet and inflammation in human aortic smooth muscle cells (HASMCs) with the endotoxin lipopolysaccharide (LPS), focusing on the metallopreteinases MMP-2 and MMP-9, the relevant signal transduction pathways and the effects of Sal B. Immunohistochemical analyses indicated apo-E-deficient mice fed a 0.15% cholesterol diet for 12 weeks exhibited thickened intima and elevated levels of MMP-2 and MMP-9 in aortic sections, both of which were attenuated by 0.3% Sal B dietary supplement. Western blotting and zymography analyses indicated that unstimulated HASMCs exhibited basal levels of protein and activity levels for MMP-2 and barely detectable levels for MMP-9, both of which were markedly upregulated by LPS, which also induced cell migration. Sal B significantly attenuated upregulations of both MMPs as well as the LPS-induced cell migration through the inactivation of MMP-2 and MMP-9 protein synthesis as well as the downregulation of the extracellular-signal-regulated kinase 1/2 (ERK1/2) and c-Jun NH_2-terminal kinase (JNK). These results demonstrate that Sal B has anti-migration properties on smooth muscle cells and may explain its anti-atherosclerotic properties. This novel 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. 100: 372–384, 2007.

Key words: salvianolic acid B; matrix metalloproteinase (MMP); mitogen-activated protein kinases (MAPKs); apo-E-deficient mice; smooth muscle cells

The migration of vascular smooth muscle cells (SMCs) plays an important role in the pathogenesis of atherosclerosis, restenosis, and vascular graft stenosis [Ross, 1993]. In the past several years, a number of studies have demonstrated that matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9, are required for the breaking down of the extracellular matrix to allow the migration of SMCs from the tunica media to the intima, resulting in neointima hyperplasia [Newby and Zaltsman, 2000; Luttun et al., 2004]. In these studies, overexpression of MMP-2 and MMP-9 was

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detected in the injured arteries which invariably developed a pronouncedly thickened neointima [Bendeck et al., 1994]. A recent report by Cho et al. [2000] shows that in vascular SMCs the cytokine tumor necrotic factor alpha (TNF-α) markedly induces the expression of MMP-9 and that this induction is regulated by activation of the ERK pathway. In addition, MMP-2 and MMP-9 levels may reflect the degree of inflammation and provide a measurement for assessing the effect of drugs on the inflammatory process. The development of MMP inhibitors has been a major advancement in the therapy of inflammatory processes and their use includes the prevention or treatment of disorders associated with the induction of these enzymes.

The herb, *Salvia miltiorrhiza* (SM) Bunge, 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 SMCs [Zhou et al., 1996]. *Salvia miltiorrhiza* extract (SME) inhibits LDL oxidation in vitro and ex vivo and reduces atherosclerosis in hypercholesterolemic rabbits [Wu et al., 1998]. Salvianolic acid A (Sal A) inhibits lipid peroxidation in rat liver microsomes [Liu et al., 1992] and scavenges oxygen radicals generated by activated neutrophils [Lin et al., 1996]. Salvianolic acid B (Sal B), another water-soluble polyphenolic antioxidant found in 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 the adhesion molecules VCAM-1 and ICAM-1 in TNF-α-stimulated human aortic endothelial cells by partial blockage of NF-κB expression and also significantly inhibits the adhesion of U937, a human monocytic cell line, to human aortic endothelial cells [Chen et al., 2001a]. Sal B attenuates ischemia-reperfusion injury of skin flaps [Lay et al., 2003] and improves regional cerebral blood flow in the ischemic cerebral hemisphere in rats [Tang et al., 2002]. However, the molecular mechanisms underlying such actions are not known with certainty. To elucidate the molecular mechanisms and identify the potential loci for therapeutic intervention, we employed in the present study a two-pronged approach with an in vivo system of simulating hypercholesterolemia by feeding apo-E-deficient mice a high cholesterol diet and a more simplistic in vitro approach of eliciting inflammation, a key step in atherosclerosis, in cultured human aortic smooth muscle cells (HASMCs) by the bacterial endotoxin lipopolysaccaride (LPS) and the effects of Sal B treatment on these systems, focusing on MMP-2 and MMP-9 as well as the modulating cyclooxygenase 2 (COX-2) and relevant signal transduction pathways. Our study shows that Sal B attenuates the expression of MMP-2 and MMP-9 both in vitro and in vivo. Results of the present study clearly reveal that Sal B reduces HASMC migration via the inhibition of MMP-2 and MMP-9 as well as the down-regulation of ERK and JNK 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 (v/v) 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, as 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. When used, it was dissolved as a 14 mM stock solution in phosphate-buffered saline (PBS), pH 7.4. Unless otherwise specified, all reagents were from Sigma (MO).

Animal Care and Experimental Procedures

We chose cholesterol-fed apo-E-deficient mouse model to study the effects of Sal B on the expression of MMP-2 and MMP-9 expression because it offers an accepted model for studying atherosclerosis and inflammation. The apo-E-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 were fed a 0.15% cholesterol diet (Purina Mills, Inc.), 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 study and were deprived of food overnight prior to being used for experiments. All procedures involving animals were in compliance with the guidelines set down in 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 the assessment of liver and renal functions. After 3 months on the 0.15% cholesterol 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 tissues, rinsed with ice-cold PBS, immersion-fixed in 4% buffered paraformaldehyde, paraffin-embedded, then cross-sectioned for immunohistochemistry. To examine the cellular expression and localization of the MMP-2 and MMP-9 proteins, immunohistochemistry was performed on the first and second serial sections of the aorta, which were deparaffinized, rehydrated, and washed with PBS. 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 MMP-2 and MMP-9 antibodies (1:20 dilution) and for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody. Bound antibodies were detected by incubation in 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). The third section was incubated with mouse anti-α-smooth muscle actin (IA4, Sigma) for the positive localization and identification of SMCs, and then incubated with FITC-conjugated goat anti-mouse secondary antibody (Sigma) and observed by fluorescent microscopy. Negative controls were performed by omitting the primary antibodies.

Culture of HASMCs

HASMCs, purchased as cryopreserved tertiary cultures from Cascade Biologics (OR), were grown in culture flasks in SMC growth medium, M231 (Cascade Biologics, Inc.) supplemented with fetal bovine serum (FBS, 5%), human epidermal growth factor (10 ng/ml), human basic fibroblast growth factor (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 confluence, when the cells were passaged by division between 4 Petri dishes and again grown to confluence. 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. At confluence, cells were serum-starved for 24 h and then treated with Sal B and/or lipopolysaccharide (LPS), as indicated in each experiment. At the end of treatment, conditioned media were collected for zymography, Western blotting, and cell migration assay. The remaining cells were rinsed once with ice-cold PBS and extracted for MAPKs proteins determinations using Western blot analysis.

MTT assay for 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 different concentrations of LPS or Sal B for various durations following which cell viabilities were assessed 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.

Western Blot Analysis for Levels of MAPKs and MMPs

Western blot analyses were performed as described previously [Chen et al., 2002]. Briefly, a cell lysate was prepared by lysing the 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. The supernatant fractions were harvested. An aliquot of cell lysate or the conditioned medium (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 the levels of MMP-2 or MMP-9, the membranes were incubated with mouse polyclonal antibody against human MMP-2 or MMP-9 (latent and active, 1:1,000 dilution, Calbiochem, CA), then with HRP-conjugated goat anti-mouse secondary antibodies (1:3,000, Chemicon). Bound antibodies were detected using Chemiluminescence Reagent Plus (NEN). The intensity of each band was quantified using a densitometer. In other studies, antibodies employed included 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 dilution, Cell Signaling).

Gelatin Zymography for MMPs Enzymatic Activation

Conditioned media from control, LPS-treated, and Sal B + LPS treated cells were collected and concentrated. Equal amounts (20 μg) of total protein were loaded onto 10% SDS polyacrylamide gels containing 1 mg/ml gelatin for the assessment of MMP activity. After gel electrophoresis, the gels were washed in 2.5% Triton X-100 twice for 15 min at room temperature to remove the SDS and permit partial renaturation of the protein. Gels were incubated subsequently at 37°C overnight in a buffer containing 10 mM $CaCl_2$, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5 and then stained with 0.2% Coomassie blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field. For quantitative analysis of MMP-2 and MMP-9 activation, the resultant bands were scanned and analyzed with a digital image analysis.

Cell Migration Assay

HASMCs were grown to 70–80% confluence, serum-starved for 24 h, trypsinized, washed with PBS, and resuspended in the media (DMEM + 0.2% BSA). Approximately 1×10^5 cells were placed in the upper chamber of a 24-well transwell (Corning) precoated with 0.1% type IV collagen. The media collected from control, LPS-treated, Sal B + LPS treated cells were added to the lower chamber, respectively. Inserts were incubated for 8 h at 37°C with 5% CO_2. HASMCs that did not migrate were scraped off the membrane, whereas cells that migrated into the bottom surface of the transwell membranes were turned face up and fixed with methanol, and then stained with hematoxylin. Migrated cells were counted in five randomly chosen fields at ×200 magnification under a microscope. Each treatment was performed in triplicates.

Statistical Analyses

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 Decreases MMP-2 and MMP-9 Expression in Thoracic Aortas of Cholesterol-Fed apo-E-Deficient Mice

Over the experimental period, there was no difference in weight gains and final weights between the C57BL/6, cholesterol-fed apo-E-deficient, and Sal B-treated, cholesterol-fed apo-E-deficient mice. To study the effect of Sal B on MMP-2 and MMP-9 expression in apo-E-deficient mice, immunohistochemical staining with antibodies against MMP-2 and MMP-9 or anti-α-actin antibody (positive staining for SMCs) was carried out on serial sections. In the cholesterol-fed apo-E-deficient mice, α-actin-positive staining was seen in the markedly thickened intima which also stained strongly for MMP-2 and MMP-9 (Fig. 1). In Sal B-treated cholesterol-fed apo-E-deficient mice, intimal thickening was reduced. Sal B also reduced MMP-2 and MMP-9 staining of the intima. In the control group, the intima was very thin and SMCs were only detected in the tunica media. The vascular wall showed faint staining for MMP-2 and MMP-9.

Sal B Reduces MMP-2 and MMP-9 Protein Expression in LPS-Treated HASMCs

MTT assessment of the cytotoxicity of LPS or Sal B for HASMCs after 24 h of incubation

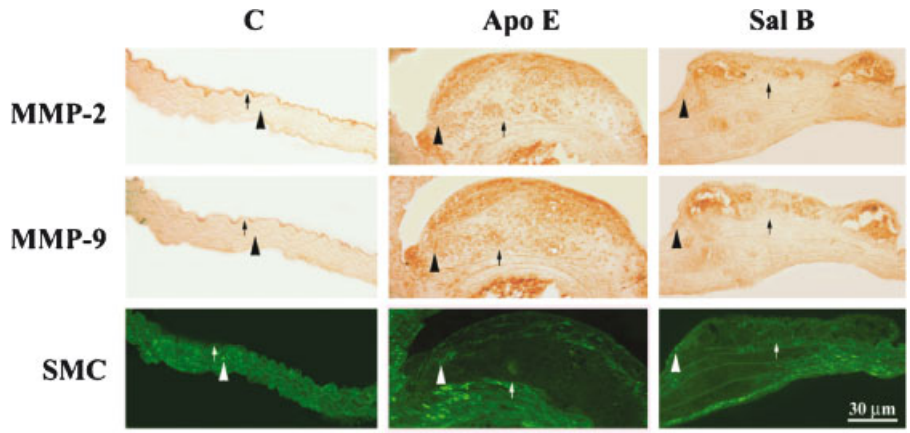


Fig. 1. Immunohistochemical staining for MMP-2, MMP-9, or α-actin in serial sections of thoracic aortas from C57BL/6 mice (C), cholesterol-fed apo-E-deficient mice (Apo E), and Sal B-treated cholesterol-fed apo-E-deficient mice (Sal B). The lumen is uppermost in all sections. The internal elastic membrane is indicated by an arrow. The arrowhead indicates smooth muscle cells overlapping with MMP-2 and MMP-9 expression. Cholesterol-fed apo-E-deficient mice exhibited markedly thickened intima and strong MMP-2 and MMP-9 staining. Sal B treatment attenuated the intimal thickening and staining of MMP-2 and MMP-9. Bar = 30 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

indicated that cell viability was unaffected by 1 μg/ml of LPS or 20 μM Sal B (data not shown). Concentrations of 10–100 ng/ml of LPS and 5–20 μM Sal B were therefore used in subsequent experiments.

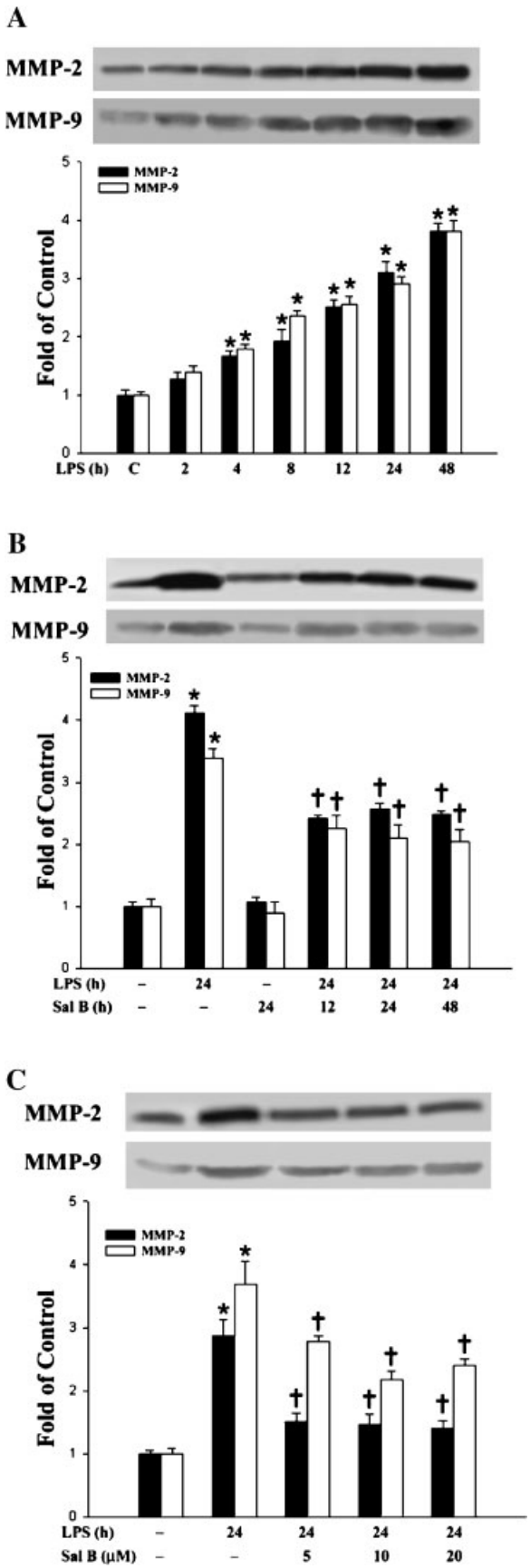
To determine whether LPS or Sal B affected levels of MMP-2 and MMP-9 protein, conditioned media collected from SMCs following the various treatments were analyzed by Western blotting. That from control cells showed low levels of MMP-2, whereas MMP-9 was barely detectable. LPS (100 ng/ml) treatment significantly elevated the levels of MMP-2 and MMP-9 protein expression in a time-dependent manner (Fig. 2A). After treatment with 100 ng/ml of LPS for 48 h, MMP-2 and MMP-9 protein expression increased by about fourfold. These effects were significantly attenuated by 12, 24, and 48 h pretreatments with 10 μM Sal B (Fig. 2B) or by 5, 10, and 20 μM of Sal B for 24 h (Fig. 2C). In cells incubated with Sal B alone, MMP-2 and MMP-9 protein levels were similar to those in the unstimulated samples.

Sal B Inhibits LPS Induced MMP-2 and MMP-9 Activation by Gelatin Zymography

To identify the relationship between LPS-stimulation and MMP-activation, we measured MMP-2 and MMP-9 activity in LPS-treated HASMCs by gelatin zymography. Gelatin zymography of the conditioned medium collected from control HASMCs showed bands of 62, 72, 82, 92 kDa bands, representing, respectively, the proenzyme form of MMP-2 (Pro-MMP-2), the active form of MMP-2 (MMP-2), the proenzyme form of MMP-9 (Pro-MMP-9), and the active form of MMP-9 (MMP-9) (Fig. 3A). Pro-MMP-2 was constitutively expressed in HASMCs, whereas MMP-2, pro-MMP-9, and MMP-9 were rarely detected. Time-dependency experiments showed that expression of the active forms of MMP-2 and MMP-9 increased in an almost linear fashion in HASMCs in response to incubation with 100 ng/ml of LPS for the different incubation times (Fig. 3A). The bands for the active forms of MMP-2 and MMP-9 were increased by 3.1- and 3.2-folds versus control cells, respectively, after treatment with 100 ng/ml of LPS for 24 h. We next investigated the effects of Sal B on LPS-induced MMP-2 and MMP-9 activation. This induction of MMP-2 and MMP-9 activation by LPS was inhibited by 10 μM of Sal B for the different incubation times (Fig. 3B) or for 24 h with different concentrations of Sal B (Fig. 3C).

Sal B Attenuates MMP-2 and MMP-9 Production in LPS-Treated HASMCs Via the COX-2 Pathway

COX-2 has been suggested to modulate the expression of MMPs involved in the migration of vascular cells during endotoxemia [Marcet-Palacios et al., 2003]. Time-dependency experiments showed that COX-2 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

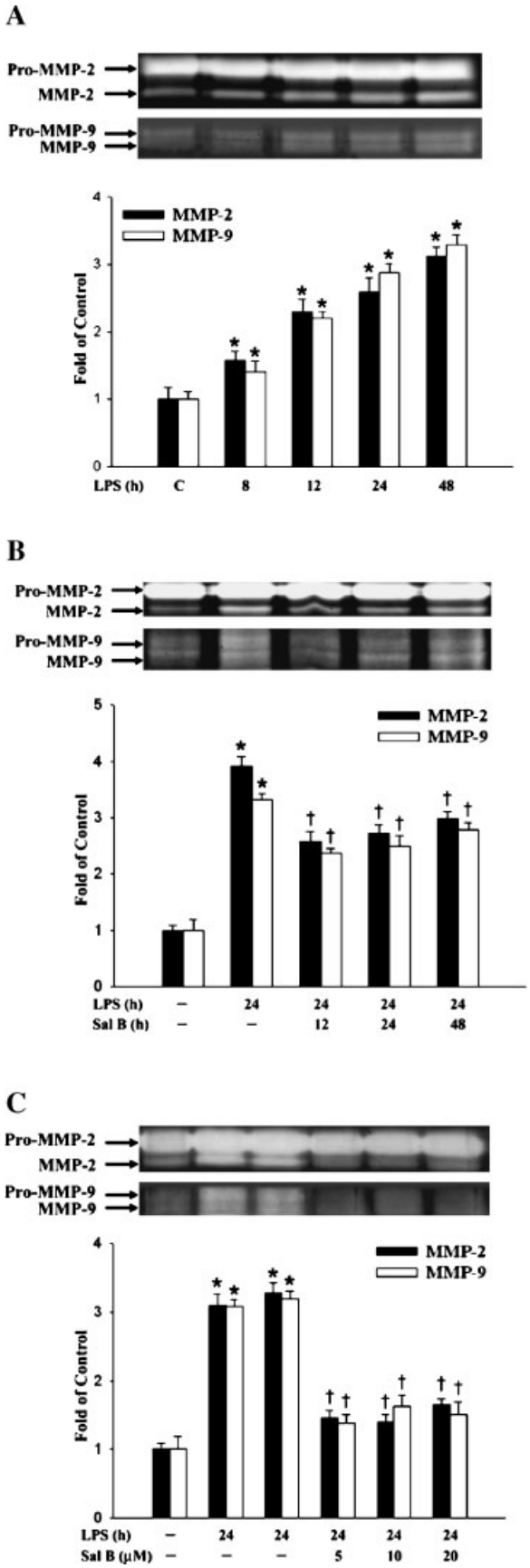


induction of COX-2 by LPS in our previous study [Chen et al., 2006]. Coincubation of the cultures with 10–30 μM NS-398, a COX-2 inhibitor [Futaki et al., 1993], markedly inhibited LPS-induced MMP-2 (Fig. 4A). Furthermore, the levels of MMP-2 expression in LPS and NS-398-treated cells were similar to those in the unstimulated cells and in the NS-398-treated cells. The level of MMP-9 expression under LPS stimulation was also attenuated by NS-398 to $60 \pm 5\%$ of LPS treatment (Fig. 4B).

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 and atherosclerosis [Guha and Mackman, 2001]. In the next set of experiments, we explored the effects of LPS on the activation of the MAPK pathway (ERK1/2, JNK, p38), a signaling cascade contributing to the regulation of MMP-2 and MMP-9 expression. As shown in Fig. 5A–C, phosphorylation of ERK and JNK1/2 was increased to 3.2- and 3.3-folds of control levels, respectively, at 5 min after the addition of 100 ng/ml of LPS. Interestingly, LPS had little effect on the p38 phosphorylation level (Fig. 5C). Pretreatment with 10 μM Sal B moderately decreased LPS-induced JNK and ERK phosphorylation, but increased p38 phosphorylation (Figs. 5A–C). Furthermore, to examine directly whether MAPKs regulate MMP-2 and MMP-9 expression, cells were stimulated in the presence of specific MAPK inhibitors. Western blot analysis of the conditioned media of cells pretreated with SP600125 (a JNK inhibitor) and PD98059 (an ERK inhibitor) reduced LPS-induced MMP-2 and MMP-9 expression, whereas SB203580 (a p38 inhibitor) increased MMP-2 and MMP-9 expression (Fig. 5D). These results suggest that Sal B inhibits MMP-2 and MMP-9 expression by

Fig. 2. Sal B inhibits the LPS-induced increase in MMP-2 and MMP-9 protein levels in HASMCs as analyzed by Western blotting. Western blot analyses were carried out for MMP-2 and MMP-9 protein levels on media collected from HASMCs treated with vehicle (control) or 100 ng/ml of LPS for various durations (**A**). HASMCs treated with vehicle, LPS (100 ng/ml), Sal B (10 μM) for 24 h and LPS for 24 h plus Sal B for times indicated (**B**) and HASMCs treated with vehicle, LPS (100 ng/ml) and various concentrations of Sal B for 24 h (**C**). 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.



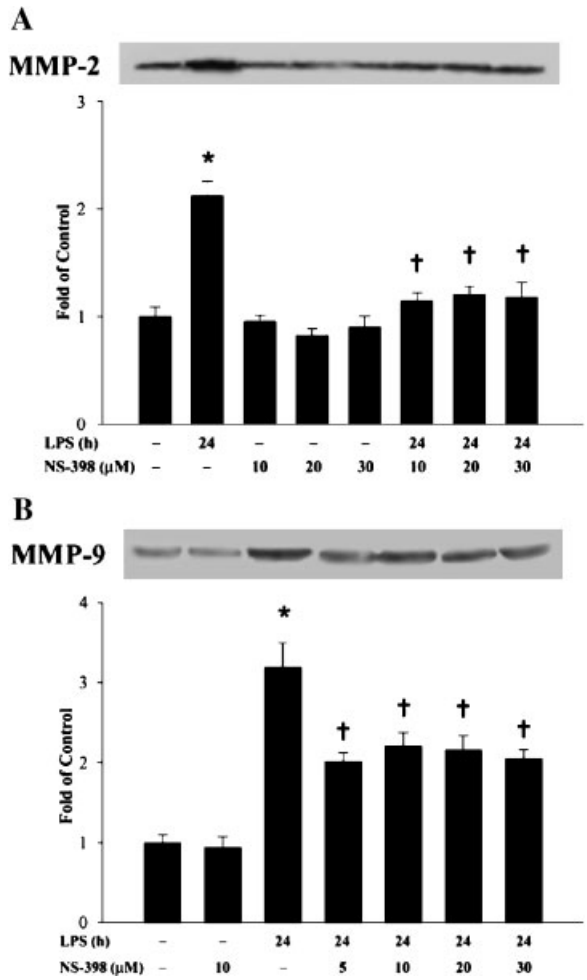


Fig. 4. Western blots of conditioned media from HASMCs showing that Sal B inhibits the LPS-induced increase in MMP-2 and MMP-9 levels via the COX-2 pathway. Cultures were incubated for 24 h with medium alone, for 1 h with medium containing 10 μM NS-398 (a COX-2 inhibitor), 24 h with 100 ng/ml of LPS, or 1 h with 10–30 μM NS-398, then 24 h with 100 ng/ml of LPS. The conditioned media containing equal amounts of proteins were subjected to Western blot. $^{*}P < 0.05$ compared to control cells. $^{\dagger}P < 0.05$ compared to LPS-treated HASMCs.

Fig. 3. Sal B inhibits the LPS-induced increase in MMP-2 and MMP-9 activities as indicated by gelatin zymography. MMP-2 and MMP-9 activities were analyzed by gelatin zymography following incubation for the durations indicated with 100 ng/ml of LPS or the vehicle (control), conditioned media containing equal amounts of proteins were subjected to gelatin zymography (**A**). HASMCs were pretreated with 10 μM Sal B for the indicated times (**B**) or for 24 h with the indicated concentrations (**C**) of Sal B before the addition of 100 ng/ml of LPS in the continued presence of Sal B. 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.

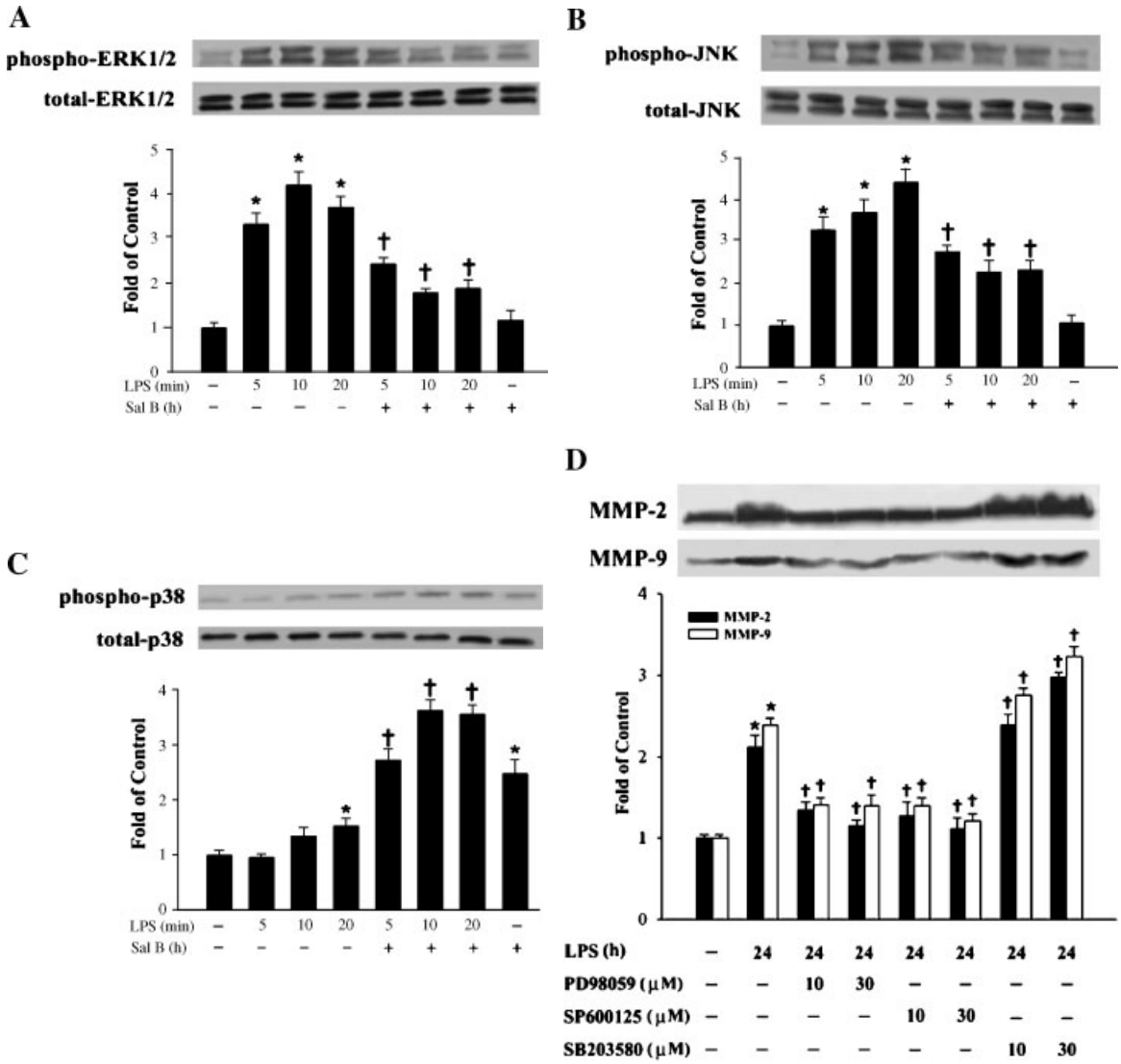


Fig. 5. Sal B inhibits the LPS-induced increase in ERK and JNK phosphorylation by Western blots. **A–C**: Immunoblotting using antibodies against ERK (A), JNK (B), or p38 (C) were carried out on cell lysate proteins from control cells or HASMCs pretreated with 10 μM Sal B for 24 h then incubated with 100 ng/ml of LPS for the indicated times. **D**: MMP-2 and MMP-9 expression in cells incubated for 1 h with medium or 10, 30 μM SP600125 (a JNK inhibitor), 10, 30 μM PD98059 (an ERK inhibitor), or 10, 30 μM SB203580 (a p38 inhibitor), followed by addition of medium or 100 ng/ml of LPS and incubation for a further 8 h. The conditioned media containing equal amounts of proteins were subjected to Western blot. $^{*}P < 0.05$ compared to control cells. $^{\dagger}P < 0.05$ compared to LPS-treated HASMCs.

preventing LPS-induced phosphorylation of JNK and ERK.

Sal B Inhibits HASMC Migration Associated With MMP-2 and MMP-9 Expression

In the past several years, a number of studies have demonstrated that MMP-2 and MMP-9 are important for VSMC migration into the intima [George et al., 1997; Galis et al., 2002]. Therefore, we next explored the efficacy of Sal B in blocking the LPS induced cell migration through the inhibition of MMP-2 and MMP-9 through an in vitro transwell migration assay. HASMCs were seeded to the top compartments of transwells, media collected from control, LPS-treated, Sal B + LPS-treated HASMCs were added to the lower compartments, respectively, and the number of transmigrated cells was quantified after 8 h. The conditioned media from cells only with LPS treatment significantly induced cell migration (Fig. 6A). In contrast, the conditioned media from HASMCs pretreated

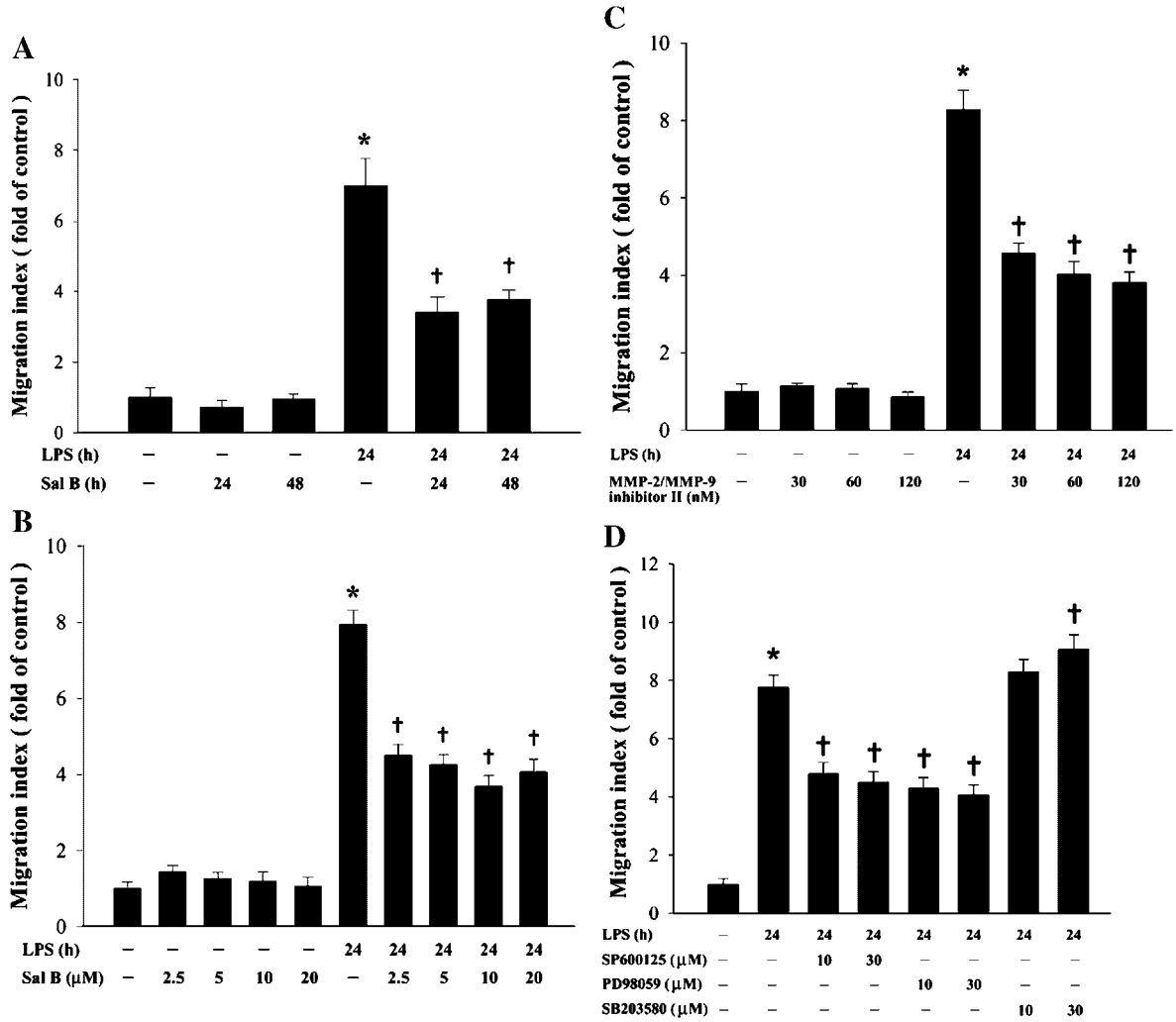


Fig. 6. Sal B inhibits LPS-induced HASMC migration. HASMCs were added to the top compartment of transwells, conditioned media from HASMCs pretreated with 10 μM of Sal B for different incubation times (**A**) or different concentrations (**B**) of Sal B for 24 h, and then 100 ng/ml of LPS for 24 h were added to the lower compartments, and the number of transmigrated cells was quantified after 8 h. **C**,**D**: HASMCs were added to the top compartment of transwells, conditioned media from HASMCs pretreated with different concentrations of MMP-2/MMP-9 inhibitor II (C) or 10, 30 μM of SP600125 (a JNK inhibitor), 10, 30 μM of PD98059 (an ERK inhibitor), or 10, 30 μM of SB203580 (a p38 inhibitor) (D) for 1 h, followed by 100 ng/ml of LPS for a further 24 h was added to the lower compartment. The number of transmigrated cells was quantified after 8 h. D: HASMCs were added to the top compartments of transwells, conditioned media from HASMCs incubated for 1 h with medium or 10, 30 μM of SP600125 (a JNK inhibitor), 10, 30 μM of PD98059 (an ERK inhibitor), or 10, 30 μM of SB203580 (a p38 inhibitor), followed by 100 ng/ml of LPS for a further 24 h, respectively, were added to the lower compartment. The number of transmigrated cells was quantified after 8 h. 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.

with Sal B for different times (Fig. 6A) or for different concentrations (Fig. 6B) and then with LPS treatment significantly decreased cell migration. Further, we examined whether cell migration was due to the expression of MMP-2 and MMP-9. The conditioned media were collected from HASMCs which was first pretreated with MMP-2/MMP-9 inhibitor II ((2R)-[(4-Biphenylylsulfonyl) amino]-N-hydroxy-3-phenylpropionamide (Merck)), an inhibitor of MMP-2 and MMP-9 activities [Tamura et al., 1998], and followed by LPS treatment. The media were added into the lower compartment of transwells. As shown in Fig. 6C, MMP-2/MMP-9 inhibitor II significantly reduced the cell migration from the top compartments of transwells. We further characterized MAPKs signaling pathways involved in the induction of HASMC migration, conditioned media collected from HASMCs pretreated with SP600125 (a

JNK inhibitor), PD98059 (an ERK inhibitor), or SB203580 (a p38 inhibitor), respectively, and then with LPS treatment was added into the lower compartment. As shown in Figure 6D, cell migration was inhibited either with SP600125 or with PD98059, whereas SB203580 increased cell migration.

DISCUSSION

In this study, we found that Sal B treatment effectively reduced MMP-2 and MMP-9 expression in cholesterol-fed apo-E-deficient mice and LPS-stimulated HASMCs. We also showed that LPS activated MMP-2 and MMP-9 expression in HASMCs through JNK and ERK signaling pathways. Finally, we further demonstrated for the first time that Sal B reduced HASMC migration via the inhibition of the activity and expression of MMP-2 and MMP-9 as well as the downregulation of JNK and ERK phosphorylation.

Initial degradation of the extracellular matrix is an inevitable step for vascular cells to hypertrophy, proliferate, and migrate. Vascular cells, including SMCs, secrete MMPs, enzymes that selectively digest the individual components of the ECM. Among MMPs, MMP-2 and MMP-9 regulate SMC migration and proliferation by acting specifically on basement membrane components that modulate the cell–cell communication with surrounding activated cells [Galis et al., 2002]. It has been shown that MMP-2 is constitutively expressed in SMCs in normal artery and in addition to increased MMP-2 expression, MMP-9 expression is induced in SMCs and macrophages in the atherosclerotic artery [Pasterkamp et al., 2000]. Our aim was to investigate the anti-atherogenic mechanism of Sal B in HASMC against LPS treatment as LPS functions as a stimulator in the pathogenesis of vascular lesions such as atherosclerosis [Recalde et al., 2004]. In cultured SMCs, recent studies have shown that LPS stimulates IL-6 and COX-2 expressions and also upregulates the expression of IL-1β [Morris et al., 2005; Chen et al., 2006]. Our present study provided new evidence that in cultured HASMCs, LPS enhances MMP-2 and MMP-9 protein expression and activity. The conditioned media collected from LPS-treated HASMCs significantly induced cell migration. In addition, the study also demonstrated that NS-398, a COX-2 inhibitor, attenuated MMP-2 and MMP-9 expression. Taken together, it is possible that LPS can contribute to the development of atherosclerotic vascular remodeling at multiple steps mediated by MMP-2 and MMP-9: the initial activation of the inflammatory cytokine network, recruitment of inflammatory cells to the plaque, plaque destabilization, and finally plaque rupture associated with clot formation. Therefore, the inhibition of MMP-2 and MMP-9 appears to be the appropriate target for the development of anti-atherogenic agents. Next, we decided to examine whether treatment of Sal B inhibited LPS-stimulated MMP-2 and MMP-9 activity in HASMCs. Of considerable interest in this study was the marked decrease by Sal B the secretion of MMP-2 and MMP-9 from LPS-stimulated VSMC as determined by zymography and Western blotting. These findings suggest that Sal B may have anti-atherogenic and anti-inflammatory effects on HASMCs through the inhibition of MMP-2 and MMP-9 expression, which has been linked to progression of plaque rupture and intimal formation in arterial lesions.

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]. *Salvia mitiorrhiza* extract (SME), an aqueous ethanolic extract of *Salvia miltiorrhiza*, is rich in polyphenolic compounds that inhibits intimal hyperplasia and monocyte chemotactic protein-1 expression after balloon injury in cholesterol-fed rabbits [Chen et al., 2001b]. Sal B, one of the active principles in SME, is a potent hepatoprotective agent and water-soluble antioxidant and attenuates ischemia-reperfusion injury-induced skin flap necrosis [Lay et al., 2003]. Pretreatment with Sal B significantly attenuated the LPS-induced COX-2 expression in HASMCs and inhibited PGE_2 production in our previous study [Chen et al., 2006]. In the present study, Sal B treatment effectively reduced MMP-2 and MMP-9 expression as well as cell migration in LPS-stimulated HASMCs. Collectively, these data suggest that Sal B treatment decreases MMP-2 and MMP-9 expression via the inhibition of COX-2 expression and that LPS-induced cell migration mediated by MMP-2 and MMP-9 action may participate in the prevention of inflammation and pathogenesis of atherosclerosis.

Chronic infection and inflammation are ultimately implicated as important etiologic factors for atherosclerosis. MMPs, inflammatory mediators, are expressed in vascular cells in the course of atherosclerosis [Pasterkamp et al., 2000]. Increased MMP-2 and MMP-9 levels are detected in the plasma of abdominal aortic aneurysm patients and patients with acute coronary syndromes [Zeng et al., 2005]. In addition, aneurysm and atherosclerotic tissues overexpress MMP-2 and MMP-9, strongly suggesting that they are important determinants in the formation of atherosclerotic lesions [Carrell et al., 2002]. Denudation of rat arteries with a balloon catheter leads to an early increase in MMP-9 expression, accompanied by a later increase in MMP-2 activity [Bendeck et al., 1994]. Plaques resulting from feeding a cholesterol-rich diet to apo-$E^{-/-}$:MMP-$9^{-/-}$ mice, a cross between apo-E- and MMP-9 deficient mice, were smaller than those from apo-$E^{-/-}$:MMP-$9^{+/+}$ mice [Luttun et al., 2004]. Deficiency in MMP-9 protects apo-E-deficient mice against atherosclerotic media destruction and ectasia. In the present study, we used apo-E-deficient mice as an animal model for severe hypercholesterolemia and extensive atherosclerosis to test the effects of Sal B on MMP-2 and MMP-9 expression. Immunohistochemical studies using anti-MMP-2 or MMP-9 antibodies in the present study showed that MMP-2 or MMP-9 was localized to SMCs in atherosclerotic lesions of cholesterol-fed apo-E-deficient mice and that MMP-2 and MMP-9 expression were significantly reduced in apo-E-deficient mice treated with Sal B. Our findings suggest an additional mechanism by which Sal B can prevent the progress of atherosclerosis, namely by inhibiting MMP-2 and MMP-9 expression.

Published data show that MAPKs play an important role in regulating the expression of MMP-2 andMMP-9 in SMC culture and injured vessels [Nagata et al., 2003; Sharony et al., 2006]. Therefore, we examined the effect of Sal B on translating extracellular stimuli to intracellular molecular signals, which is thought to regulate cell growth, differentiation, and migration. Recent studies have indicated a possible role for MAPKs in the MMP-2 and MMP-9 gene expression induced by LPS, growth factors, and cytokines [Arai et al., 2003]. For example, LPS has been shown to activate p42/44 MAPKs (ERK) and p38 MAPK and induce MMP-2 and MMP-9 gene expression in macrophages/monocytes [Niiro et al., 1998]. The involvement of ERK1/2 kinase inhibitor U0126 and p38-MAPK inhibitor SB203580 has been demonstrated for the regulation of TNF-α-induced MMP-9 expression in rat and mouse SMC lines [Cho et al., 2000; Khan et al., 2002]. TNF-α activates MMP-9 expression in HASMC through the ERK signaling pathway [Moon et al., 2004]. However, how the activation of JNK results in the induction of MMP-2 and MMP-9 is unclear. Consistent with these findings, the present study shows that phosphorylation of JNK1/2 and ERK was significantly increased at 5 min after LPS addition, whereas phosphorylation of p38 was less affected. MMP-2 and MMP-9 expression in response to LPS was inhibited by SP600125 (a JNK inhibitor) and PD98059 (an ERK inhibitor). These findings may explain the cell type specific differences in MMP-2 and MMP-9 regulation by cytokines or growth factors in vascular SMCs. It appears that the MMP-2 and MMP-9 pathways are very complex and may involve cross-talk between MAPKs. Future studies should focus on how these MAPKs interact with one another, leading to the ultimate cell activation. Interestingly, Sal B decreased LPS-induced JNK and ERK phosphorylation, but increased p38 phosphorylation. Our results indicate that Sal B attenuates LPS-stimulated MMP-2 and MMP-9 expression via early inactivation of ERK and JNK phosphorylation, but not p38 phosphorylation.

SMC migration presumably requires degradation of the basement membrane and extracellular matrix surrounding the cell [Newby and Zaltsman, 2000]. Gelatinases such as MMP-2 and MMP-9 have been implicated in the removal of the first extracellular matrix barrier to migrating SMCs [Kenagy et al., 1997]. Many studies identify increased expression of MMP-2 and MMP-9 coincident with SMC migration after injury in vitro and in vivo. Treatment with MMP-2 antibody can inhibit SMC migration across a synthetic extracellular matrix membrane [Uzui et al., 2000]. Recent data from knock-out studies indicate that MMP-9 is critical for the development of arterial lesions by regulating both VSMC migration and proliferation [Cho and Reidy, 2002; Galis et al., 2002]. In this study, we are the first one to show that MMP-2 and MMP-9 expression as well as the upregulation of JNK and ERK phosphorylation are required for LPS-induced cell migration. The reduction of LPS-induced cell

migration by Sal B may be mediated by the inhibition of the activity and expression of MMP-2 and MMP-9 expression as well as the downregulation of JNK and ERK phosphorylation.

In conclusion, this study shows that Sal B treatment effectively reduced MMP-2 and MMP-9 expression in cholesterol-fed apo-E-deficient mice and LPS-stimulated HASMCs. This is the first study to show that Sal B, a water-soluble antioxidant, reduces MMP-2 and MMP-9 expression and consequently decreases cell migration. Sal B inhibits LPS-inducible MMP-2 and MMP-9 expression in HASMCs by suppression of JNK and ERK phosphorylation. Since degradation of the ECM and basement membrane by HASMCs and the migration of SMCs through MMP-2 and MMP-9 overexpression are crucial steps in the pathogenesis of atherosclerosis and restenosis, our study implies that Sal B, a water-soluble polyphenolic antioxidant, may have therapeutic potentials in the prevention of cardiovascular disease.

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