

## LPS-stimulated Macrophage Activation Affects Endothelial Dysfunction

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Intestinal microbiota is involved in the atherosclerotic process by development of an atheromatous core with foam cells in carotid arteries. It has reported that lipopolysaccharide (LPS) from *Escherichia coli* localizes in human atherosclerotic plaque and causes inflammation via interaction with toll like receptor 4. However, there is no evidence that whether LPS-activated macrophages regulate endothelial cell (EC) function. We evaluated whether LPS-activated macrophage acts as one of the stimulants activating EC and its underlying signaling pathways. Using Western blotting and quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we confirmed that intraperitoneal injection with LPS increases iNOS protein and inflammatory cytokine, TNF- $\alpha$  and IL-6 mRNA expressions. To determine whether LPS-mediated macrophage inflammatory condition affects EC activation and inflammation, human umbilical vein endothelial cells (HUVECs) were incubated with isolated peritoneal macrophages from LPS-injected mice. Interestingly, p90RSK Serine 380 phosphorylation and protein expression were significantly increased by macrophage treatment in EC. Messenger RNA levels of vascular cell adhesion molecule 1 and p90RSK was increased, but endothelial nitric oxide synthase was decreased. In addition, NF- $\kappa$ B promoter activity, which plays an important role in the pathogenesis of inflammation, was strongly enhanced by the macrophage treatment in EC. We further evaluated the effects of LPS on EC function in the mouse aorta using *en face* staining. In agreement with *in vitro* result, p90RSK expression was strongly increased in the steady laminar flow region of the mouse aorta in mice injected with LPS. Together, our study demonstrates that p90RSK might be a one of the major therapeutic candidates for the prevention of vascular diseases mediated by LPS.

**Key Words:** Atherosclerosis, EC inflammation, Lipopolysaccharide, p90RSK

## INTRODUCTION

The endothelium modulates a lot of biological processes within the blood vessel to regulate vascular tone and blood pressure through stimulation of nitric oxide and endothelin (1, 2) and to stimulate cell proliferation and angiogenesis through secretion of various growth factors and vasoactive substances. Therefore, endothelium plays a key role in the initiation and development of inflammatory atherosclerosis (3).

Endothelial dysfunction is characterized by atherosclerosis with early events including vasoregulation, activation of inflammatory processes, and damaged barrier function due to endothelial cell (EC) death (4, 5). Hemodynamic shear stress affects vascular

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pathophysiologic conditions such as progression of atherosclerosis, vascular dilation, and aneurysms (4, 5).

p90 ribosomal S6 kinase (p90RSK) is one of the downstream regulators of ERK1/2 (6). p90RSK is involved in activation of nuclear factor- $\kappa$ B by phosphorylation of I $\kappa$ B (7) or phosphorylation of transcription factors such as c-Fos (8), Nur77 (9), and cAMP-response element-binding protein (10). Furthermore, we recently reported that p90RSK was highly expressed in atherosclerotic lesion and was associated with EC inflammation and apoptosis via regulating ERK5 and p53-mediated signaling pathway (5, 11).

There is a report that intestinal microbiota is involved in the atherosclerotic process (12). The patients with atheroma showed higher levels of circulating LPS and the presence of an atheromatous core with foam cells in carotid arteries compared with the control patients (12). Mechanistically, it demonstrated that lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) localizes in human atherosclerotic plaque and causes inflammation via interaction with toll like receptor 4. However, there is no evidence that weather LPS-activated macrophages affect EC function.

Here, we have attempted to verify whether LPS-activated macrophage acts as one of the agonists activating EC and its underlying signaling pathways. We found that LPS-induced macrophages increase inducible nitric oxide synthase (iNOS) and inflammatory cytokines, including TNF- $\alpha$  and IL-6. When these macrophages cocultured with EC, they up-regulates p90RSK, VCAM-1, and NF- $\kappa$ B, whereas down-regulates endothelial nitric oxide synthase (eNOS), resulting in EC activation and inflammation. Finally, EC dysfunction was assessed after exposure of LPS in mouse aorta. Consistent with *in vitro* experiment data, p90RSK expression was up-regulated in the LPS-exposed aorta in mice. Together, our finding demonstrates that p90RSK might be a one of the key candidates for treating vascular diseases mediated by LPS.

## MATERIALS AND METHODS

### Cell cultures

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA) and cultured in M200 medium supplemented with low serum growth supplement (LSGS; Cascade biologic, Portland, OR, USA) and 5% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). HUVECs were cultured on 0.2% gelatin pre-coated dishes. Peritoneal neutrophils and macrophages were obtained after intraperitoneal (i.p.) injection of 2 ml autoclaved 3% (w/v) thioglycolate in H<sub>2</sub>O (Sigma-Aldrich, St. Louis, MO, USA). After 3 days, peritoneal cells were harvested by injecting 10 ml DMEM containing 10% FBS into the peritoneal cavity, the abdomen gently massaged to dislodge cells, and cell suspension was collected. The mouse monocyte/macrophage cell line, RAW264.7 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA; #TIB-71) and was propagated in DMEM containing 10% FBS at 37°C and 5% CO<sub>2</sub> in air.

### Plasmids and luciferase assay

The NF- $\kappa$ B-*luc* reporter plasmid was obtained from JD Li (13) lab and the construct was verified by sequencing. Peritoneal macrophages were plated on 12-well plates at  $5 \times 10^4$  cells/well. For a promoter assay, cells were transfected in Opti-MEM (#31985-088; Gibco) with lipofectamine (#15338100, Invitrogen, Waltham, MA) mixture containing the NF- $\kappa$ B-*luc* reporter plasmid along with pRL-*tk* for 4 hrs. Opti-MEM was replaced by fresh complete culture medium 4 hrs post transfection. The cells were collected 36 hrs after transfection, and the luciferase activity was assayed with the dual luciferase kit (#E1960, Promega, Madison, WI, USA) using a TD-20/20 luminometer (Turner Designs, San Jose, CA, USA). Since pRL-*tk* contains the Renilla luciferase gene, the expression and transfection efficiencies were normalized with the Renilla luciferase activity. Transfections were performed in triplicate, and each experiment was repeated at least three times.

## Antibodies and reagents

Rabbit anti-p90RSK S380 (#11989), rabbit anti-p90RSK1 (#9333), eNOS (#32027) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-VCAM-1 (#sc-8304) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TNF- $\alpha$  (#KMC3011) and IL-6 (#KMC0061) mouse ELISA kit were purchased from Invitrogen. LPS from *E. coli* 0127:B8 was purchased from Sigma-Aldrich (#L-3137).

## Animals

C57BL/6 mice were purchased from Samtako Animals (Osan, Korea). All mice were maintained under pathogen-free conditions at the Chungnam National University. All animal procedures were performed with the approval from the University Committee on Animal Resources at the Chungnam National University and approved protocol number was CNU-00826.

## Western blot analysis

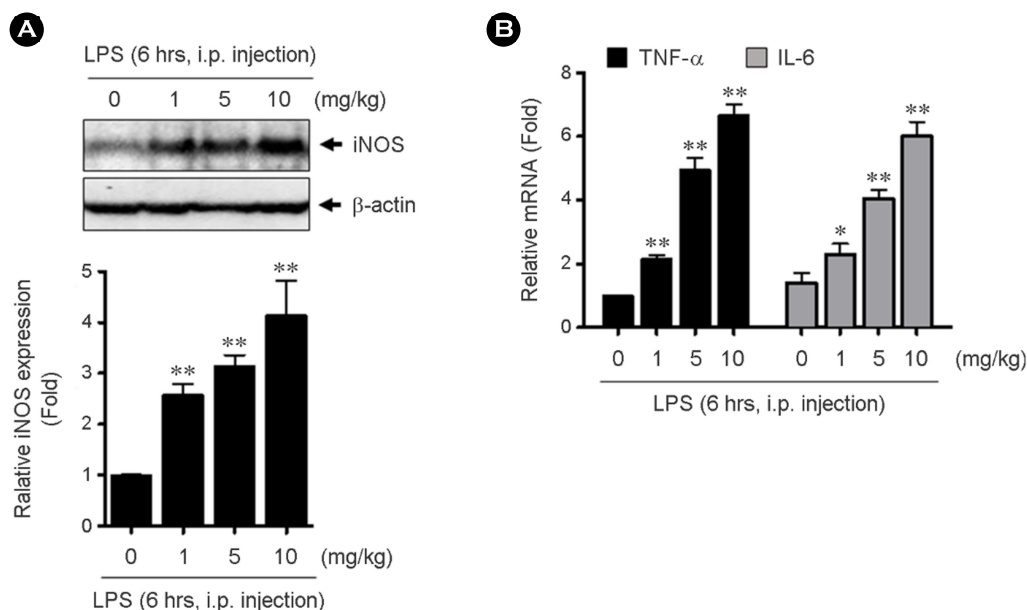
Cell extracts were prepared in modified radioimmunoprecipitation assay (RIPA) buffer including 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1 mM dithiothreitol, 1:200-diluted protease inhibitor cocktail (Sigma), 1 mM PMSF and 10 mM NEM, and 0.1 mM iodoacetamide. The protein extracts were resolved by SDS-PAGE, electro transferred onto a Hybond enhanced chemiluminescence nitrocellulose membrane, and visualized by using the enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. Each protein expression was detected by Western blotting with each corresponding antibody.

## Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA was extracted using the TRIzol reagent according to manufacturer's instructions. The cDNA synthesis was performed in 20  $\mu$ l mixtures containing 1  $\mu$ g of total RNA using an iScript cDNA synthesis kit according to the manufacturer's protocol (#170-8890, Bio-Rad, Hercules, CA, USA). For qRT-PCR, specific mouse primers including TNF- $\alpha$  and IL-6, and human VCAM-1, eNOS, and p90RSK were designed using Primer Express 3.0 software. The primer sequences used were: (**Mouse**)  $\beta$ -actin forward 5'-GGCGCTTTTGACTCAGGATT,  $\beta$ -actin reverse 3'-GGGATGTTTGCTCCAACCAA; TNF- $\alpha$  forward 5'-ATCCGCGACGTGGAAGT, TNF- $\alpha$  reverse 3'-ACCGCCTGGAGTTCTGGAA. IL-6 forward 5'-CCTCGGTCTTCTGGAGTACC, IL-6 reverse 3'-ACTCTTCTGTGACTCCAGC; (**Human**) GAPDH forward 5'-CCTGCACCACCAACTGCTTA, GAPDH reverse 3'-GGCCATCCACAGTCTTCTGAG, VCAM-1 forward 5'-GAACCACTATTTCTCATCAGACA, VCAM-1 reverse 3'-TTTAAAAGCTTGAGAAGCTGCAAAC, eNOS forward 5'-TGGCTTTCCTTCCAGTTC, eNOS reverse 3'-AGAGGCGTTTGCTCCTTC.

## En Face immunostaining

We performed *en face* staining as described in our previous report (14, 15). Briefly, animals of 6~8 weeks of age were euthanized by CO<sub>2</sub> inhalation. The arterial tree was perfused via the left ventricle first with saline containing heparin (40 USPU/ml) and then with 4% paraformaldehyde in PBS for 10 min. Fixed aorta was dissected out and the adipose tissue around the vessel was removed. It was cut open longitudinally and permeabilized with PBS containing 0.1% Triton X-100 and blocked by TBS containing 10% goat serum and 2.5% Tween-20 for 30 min. Aortas were incubated with serum-free protein blocking buffer (X0909, DAKO, Carpinteria, CA, USA) for 30 min followed by incubation with rabbit anti-p90RSK and anti-rabbit IgG as a control (10  $\mu$ g/ml) in the presence of rat anti-VE-cadherin (7  $\mu$ g/ml, an endothelial cell marker) in the antibody diluent buffer (#S0809, DAKO) for overnight. After a PBS rinse, anti-rabbit IgG and anti-rat IgG antibodies (1:1,000 dilution, Alexa Fluor 546 and 488, respectively, Molecular Probes, Eugene, OR, USA) were applied for 1 h at room temperature. The samples were analyzed using a K1-Fluo laser scanning confocal microscope equipped with a PLAPON 60X (NA 1.42) oil objective lens (Nanoscope Systems, Daejeon, Korea).



**Figure 1.** The expression of iNOS and inflammatory cytokines in LPS-activated peritoneal macrophages. LPS was *i.p.* injected with indicated doses in C57BL/6 mice. After 6 hrs, peritoneal macrophages were isolated and then assessed iNOS protein expression by western blotting using specific antibody (A) and TNF- $\alpha$  and IL-6 mRNAs by qRT-PCR using specific primers (B). Bar graph represents the quantification of relative fold changes of iNOS protein expression compared to the control in three independent experiments (0, A, lower panel). Data present mean  $\pm$  SEM. \* $p$  < 0.05 and \*\* $p$  < 0.01, compared with control (0, PBS treated) by 1-way ANOVA followed by Bonferroni's post hoc test.

## Statistical Analysis

Data are presented as mean  $\pm$  SEM. Comparison between 2 independent groups were subjected to the Wilcoxon rank sum test. For multiple comparisons of >2 groups, the Kruskal-Wallis test followed by Dunn post hoc analysis was performed with the use of GraphPad Prism (GraphPad Software, San Diego, CA, USA). A  $p$  value < 0.05 was considered statistically significant.

## RESULTS

### LPS treatment induces inflammatory environment *in vivo*

To determine whether LPS treatment induces inflammation in mice, various concentrations of LPS (1, 5, and 10 mg/kg) were *i.p.* injected in mice. Six hours later, peritoneal macrophage was isolated and evaluated iNOS expression, which produces toxic oxygen species, nitric oxide (NO). As shown in Fig. 1A, iNOS expression was significantly increased at 1, 5, and 10 mg/kg compared with control (PBS treatment). Since induction of nitric oxide is associated with increased inflammation (16), key inflammatory cytokines such as TNF $\alpha$  and IL-6 were evaluated in isolated peritoneal macrophages using qRT-PCR (Fig. 1B). As expected, LPS treatment significantly increases both TNF $\alpha$  and IL-6 mRNA levels in a concentration-dependent manner. Based on this data on LPS-induced inflammatory conditions, we set up a 5 mg/kg LPS treatment for 6 h for further experiments.

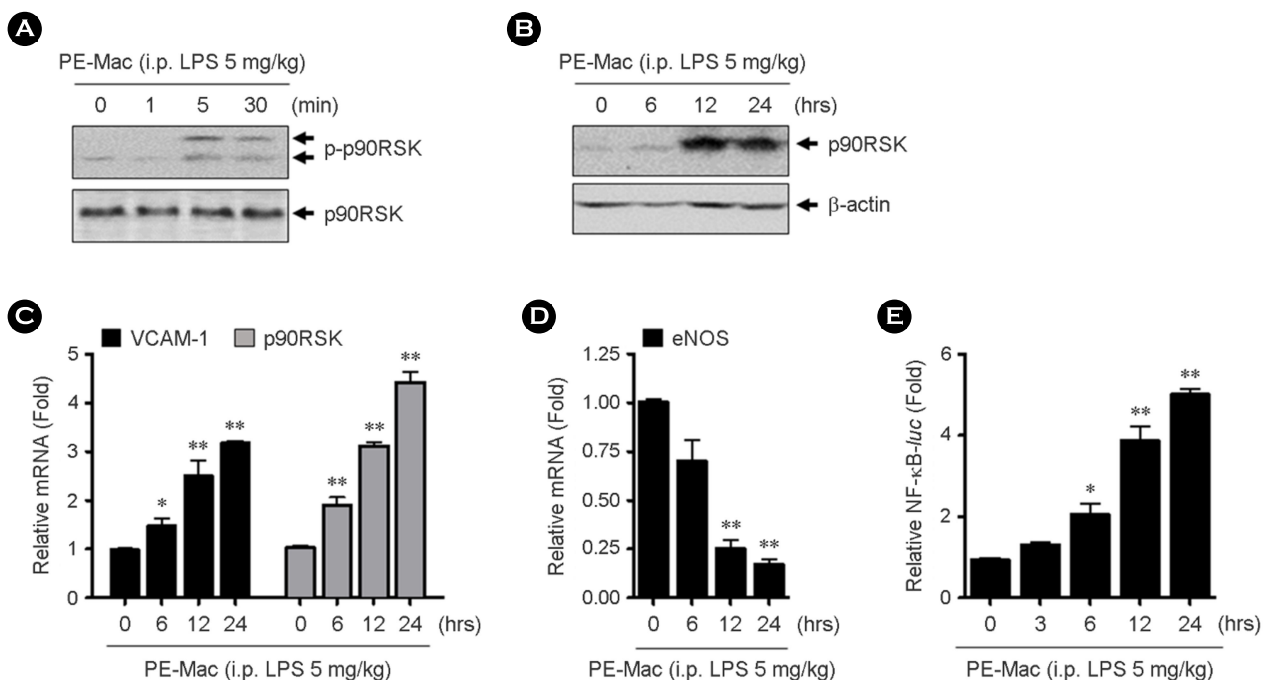
### Co-culture of HUVECs with LPS-treated peritoneal macrophages leads to EC inflammation

To evaluate whether LPS treatment induces macrophage activation-mediated EC inflammation, macrophages were prepared from peritoneum of 5 mg/kg LPS *i.p.* injected mice. Then, HUVECs were co-cultured with isolated macrophages for various

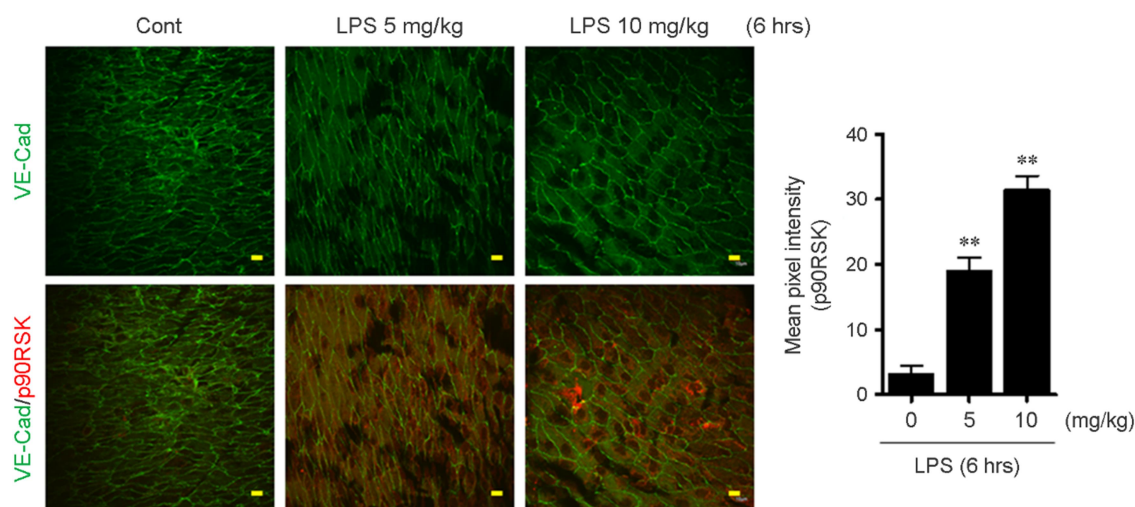
times. Since we recently reported that p90RSK was highly expressed in endothelium of atherosclerotic lesions and increased EC dysfunction via up-regulation of inflammatory signaling pathway (5, 11), we evaluated whether LPS-activated macrophages regulate p90RSK phosphorylation. Interestingly, LPS-activated macrophages increase p90RSK S380 phosphorylation and p90RSK expression at time-dependent manner (Fig. 2A, B). We further evaluated whether these macrophages regulate inflammatory molecule such as vascular adhesion molecule (VCAM)-1. VCAM-1 mRNA expression was significantly increased as well as p90RSK mRNA levels in RNA of co-cultured cells (Fig. 2C). eNOS is a key regulator of cellular function that is important to maintain EC homeostasis (17). When eNOS mRNA level was determined under the same conditions, eNOS mRNA levels significantly reduced in a LPS-activated macrophage treatment time-dependent manner (Fig. 2D). Since nuclear factor  $\kappa$ B (NF- $\kappa$ B) transcription factor is involved in the activation of many genes in response to inflammatory stimuli (18), NF- $\kappa$ B transcriptional activity was determined in co-cultured cells. Consistent with other inflammatory molecule expression, NF- $\kappa$ B transcriptional activities were increased by HUVECs co-cultured with LPS-activated macrophages after 6 hrs incubation (Fig. 2E).

### LPS treatment induces inflammatory environment *in vivo*

To confirm the previous result *in vivo*, male C57BL/6 mice (7-week old) were i.p. injected with 0 (PBS), 5, and 10 mg/kg LPS and the aortas were isolated from the mice. After perfusion with Saline including heparin, it was fixed with 4% paraformaldehyde, and then *en face* preparations were made. The fixed aorta was double-stained with anti-VE-Cad (as an EC marker) or anti-p90RSK. Consistent with the *in vitro* data, increased level of p90RSK was appeared in regions where steady laminar flow area of the aorta of 5 and 10 mg/kg LPS-treated mice compared with control (0, PBS-treated mice) (Fig. 3).



**Figure 2.** The effect of LPS-activated macrophages on EC functions. The HUVECs were co-cultured with macrophages isolated from 5 mg/kg LPS-injected mice for indicated times. (A-B) Phospho-p90RSK and total-p90RSK were determined by western blotting using specific antibody, respectively. (C-D) Total RNAs were isolated and qPT-PCR for VCAM-1, p90RSK, and eNOS mRNA levels were performed. (E) HUVECs were transfected with NF- $\kappa$ B-luc and pRT-tk using Lipofectamine 2000. The cells were co-cultured with the LPS-activated macrophages for indicated times and then promoter assay was performed. Data present mean  $\pm$  SEM. \* $p < 0.05$  and \*\* $p < 0.01$ , compared with control (0, PBS treated) by 1-way ANOVA followed by Bonferroni's post hoc test.



**Figure 3.** LPS stimulation increases p90RSK expression in aortic arch. After stimulation with 0 (PBS), 5, and 10 mg/kg LPS for 6 hrs, *en face* preparations of the aortic arch of 7-week C57BL/6 mice were processed. The samples were double-stained with anti-VE-Cad (as an EC marker) and anti-p90RSK. Images were recorded using a confocal microscope equipped with a Planapo x60 1.42 NA oil objective lens. Scale bars: 10  $\mu$ m. Bar graph indicates quantification of percentage of p90RSK fluorescence intensity. Data present mean  $\pm$  SEM. \*\* $p < 0.01$ , compared with control (PBS treated) by 1-way ANOVA followed by Bonferroni's post hoc test.

## DISCUSSION

ECs are major determinant of vascular tone, leukocyte adhesion, and SMC proliferation (19). In its intact state, the endothelium maintains vasodilator, anti-thrombotic and anti-inflammatory status (20). However, when inflammatory conditions occur, ECs are a major target of inflammatory molecules such as cytokine signals from various immune cells and can cause EC dysfunction and inflammation (20). Thus, endothelial dysfunction is an early event in atherosclerotic disease prior to clinical manifestations and complications. A recent report indicates that intestinal microorganisms are involved in the atherosclerotic process, but there is still lacking informational weather the microorganism infection affects EC inflammation and its underlying signaling pathway. Here, we clearly demonstrate that LPS-induced macrophage activation leads to EC inflammation via p90RSK activation. To determine the role of LPS-induced macrophage activation, we first assessed the effect of LPS on iNOS and cytokine expressions in peritoneal macrophages by treatment with LPS in mice. The enzyme responsible for the NO production by macrophages is the iNOS that catalyzes arginine to form NO and citrulline (21). Compared with the neuronal and endothelial isoforms of NOS (nNOS and eNOS, respectively), iNOS has drawn considerable attention for its important functions in inflammatory diseases (22). Fig. 1 showed that iNOS expressions were dramatically increased by treatment with low dose of LPS for 6 hrs compared to the control (PBS treated) in mice. Consistent with this result, cytokine expression was also greatly increased by LPS treatment in mice (Fig. 1B).

To prove our hypothesis that LPS-activated macrophages affect EC function, peritoneal macrophages was isolated from mice after LPS injection, and then HUVECs were co-cultured various times with these activated macrophages. Interestingly, EC co-cultured with LPS-activated macrophages increases p90RSK S380 phosphorylation at 5 mins as well as p90RSK protein expression after 12 hrs of stimulation (Figs. 2A and B). Previously, we reported that EC transduced with adenovirus containing dominant negative p90RSK (K94A/K447A, Ad-DN-RSK), but in those transduced with Ad-LacZ, abolished atherogenic flow-induced proinflammatory adhesion molecule expression (5). It suggests that p90RSK kinase activation plays a crucial role in regulating EC inflammation. In particular, mRNA levels of VCAM-1 and p90RSK and NF- $\kappa$ B promoter activity were increased by LPS-activated macrophage treatment in HUVECs, whereas mRNA level if eNOS was decreased (Fig. 2).



Finally, we determined whether LPS injection regulates p90RSK expression *in vivo*. It has been reported that mechanical shear stress in the plasma membrane participates in the maintenance of vascular homeostasis, including the control of inflammatory responses that contribute to the production of atheroma (14). The endothelium in the aortic arch is susceptible to two different flows, which are steady laminar flow (anti-atherogenic flow) and disturbed flow (atherogenic flow) (15). Especially laminar shear stress plays a protective role by inhibiting activation of adhesion molecule and inflammatory cytokines (23). It has been reported that p90RSK overexpression was observed in athero-prone area, where is disturbed flow area and p90RSK expression was colocalized with VCAM-1 expression in endothelium of mouse aorta suggesting that p90RSK plays an inflammatory regulator (5, 11, 24). To investigate the effect of LPS on endothelial cells-exposed by laminar shear stress, LPS-injected mouse aorta was co-stained with VE-Cad as EC marker and p90RSK in Fig. 3. Interestingly, LPS stimulation disrupted the laminar shear stress vascular protective function by increasing expression of p90RSK in laminar shear stress-exposed legion. It suggests that LPS-mediated inflammatory environment affects p90RSK expression in EC and is thought be critical for the regulation of atherosclerosis. Therefore, targeting the p90RSK in LPS-induced inflammatory condition is a one of the key therapeutic approaches for the prevention of vascular diseases.

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