

*Full Paper***p38 Mitogen-Activated Protein Kinase Mediates Sidestream Cigarette Smoke-Induced Endothelial Permeability**Brad Low<sup>1</sup>, Mei Liang<sup>1</sup>, and Jian Fu<sup>1,\*</sup><sup>1</sup>Center for Biomedical Research, University of Texas Health Center at Tyler, Tyler, Texas 75708, USA

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**Abstract.** Second-hand smoke is associated with increased risk of cardiovascular diseases. So far, little is known about the signaling mechanisms of second-hand smoke-induced vascular dysfunction. Endothelial junctions are fundamental structures important for maintaining endothelial barrier function. Our study showed that sidestream cigarette smoke (SCS), a major component of second-hand smoke, was able to disrupt endothelial junctions and increase endothelial permeability. Sidestream cigarette smoke stimulated the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and myosin light chain (MLC). A selective inhibitor of p38 MAPK (SB203580) prevented SCS-induced loss of endothelial barrier integrity as evidenced by transendothelial resistance measurements. Resveratrol, an antioxidant that was able to inhibit SCS-induced p38 MAPK and MLC phosphorylation, also protected endothelial cells from the damage. Thus, p38 MAPK mediates SCS-induced endothelial permeability. Inhibition of p38 MAPK may have therapeutic potential for second-hand smoke-induced vascular injury.

**Keywords:** myosin light chain (MLC), p38 mitogen-activated protein kinase (MAPK), Rho kinase, endothelial permeability, cigarette smoke

**Introduction**

Second-hand smoke is associated with heart disease deaths in adult nonsmokers in the United States (1, 2). Nonsmokers exposed to second-hand smoke are more likely to develop cardiovascular diseases compared with nonsmokers not exposed to smoke (3), but at present little is known about the mechanisms by which second-hand smoke contributes to vascular dysfunction.

The endothelium of the vascular system forms a barrier between the blood and extravascular compartments to regulate the trans-endothelial flux of liquid, plasma proteins, and white blood cells. Endothelial cells establish the selective barrier function through cell-cell junctions (4). Tight junctions and adherens junctions are two basic structures important for maintaining endothelial barrier integrity. Here, we propose that sidestream cigarette smoke (SCS), a major component of second-hand smoke, disrupts endothelial barrier by activating signals controlling cell contraction and cell-

cell junctions.

Actin and myosin interaction plays a critical role in the development of contractile force in endothelial cells (5), and their interaction is regulated by the phosphorylation status of myosin light chain (MLC) (6, 7). Endothelial junctions are mechanically coupled to the actin cytoskeleton (4, 5). Thus, the generation of mechanical forces through MLC phosphorylation may lead to disruption of the cell-cell junctions, which then results in an efflux of macromolecules from the bloodstream and tissue edema. Vascular injuries caused by inflammatory mediators are often coupled with vessel wall leakage and edema formation (8–10). Recent studies suggest that pro-inflammatory stimuli such as thrombin, VEGF, and TGF- $\beta$ 1 increase endothelial permeability by boosting MLC phosphorylation (11–13).

MLC phosphorylation is regulated by MLC kinase (MLCK) and Rho kinase (14). While MLCK directly phosphorylates MLC, Rho kinase regulates MLC phosphorylation indirectly through inactivation of MLC phosphatase (15, 16). p38 mitogen-activated protein kinase (MAPK) is another important mediator of vascular permeability (10, 17). p38 MAPK activation

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has been shown to increase endothelial permeability through both MLC-dependent and independent pathways (18, 19). In the present study, we examined the signaling pathways regulating SCS-induced endothelial permeability. The extract from sidestream cigarette smoke was prepared and used in the experiments. Our studies indicate that p38 MAPK and MLC mediate SCS-induced endothelial barrier dysfunction.

## Materials and Methods

### *Antibodies and reagents*

Rabbit anti-MLC, anti-phospho-MLC, rabbit anti-p38 MAPK, and anti-phospho-p38 MAPK antibodies were purchased from Cell Signaling (Beverly, MA, USA). Goat anti-VE-cadherin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Resveratrol, Rho-kinase inhibitor Y27632, p38 MAPK inhibitor SB203580, and an inactive control SB202474 were purchased from Calbiochem (San Diego, CA, USA).

### *Cell culture*

Human pulmonary artery endothelial cells (HPAECs) were obtained from Cambrex (East Rutherford, NJ, USA) at passage 3, cultured in complete EGM-2 media (Cambrex) supplemented with 10% fetal bovine serum, and used at passages 5–8. The endothelial cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>–95% air in T-75 tissue culture flasks, plates, or dishes coated with 0.2% gelatin and grown to confluent monolayers. Cells were seeded into 6-well plates for immunoblotting analysis or into eight-well gold microelectrode chambers (Applied Biophysics, Troy, NY, USA) for measurement of transendothelial electrical resistance (TER).

### *Preparation of SCS solutions*

SCS extract was prepared by combusting research filtered 2R4F cigarettes (University of Kentucky Tobacco Research and Development Center, Lexington, KY, USA) in a 4-liter side-arm flask. The SCS was bubbled into phosphate-buffered saline (PBS) using an automatic peristaltic pump at a rate of 200 ml/min. SCS extract was filtered through a 0.1- $\mu$ m filter unit (Millipore, Bedford, MA, USA) and used the same day. SCS concentration was determined spectrophotometrically (230 nm). For all experiments, an absorbance of 1 at 230 nm was considered 100%. Final concentrations of these solutions are expressed as percent values. Final concentrations from 5%–20% were used in the studies. To study signal transduction pathways affected by SCS, the Rho-kinase inhibitor Y27632 (5  $\mu$ M), p38 MAPK

inhibitor SB203580 (2.5  $\mu$ M), and an inactive control SB202474 (2.5  $\mu$ M) were used. HPAECs were pretreated with the inhibitors for 15 min and then exposed to SCS.

### *Immunoblotting*

After treatments, cells were washed with PBS (Invitrogen, Carlsbad, CA, USA) and lysed with cell lysis buffer containing PBS, 1% Triton X-100, and protease inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Total cell lysates were cleared by centrifugation and boiled in SDS sample buffer for 5 min. Protein extracts were separated by SDS-PAGE on 10% or 12% gels for detection of p38 and MLC. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The blots were subsequently blocked with 5% blocking buffer (1  $\times$  PBS, 5% milk, 0.1% Tween-20) at room temperature for 1 h and then incubated with primary specific antibodies at room temperature for 1 h or overnight at 4°C. After 6 washes for 5 min each with PBST (1  $\times$  PBS, 0.1% Tween-20), the membrane was incubated with horseradish peroxidase-conjugated IgG secondary antibody at room temperature for 1 h, followed by six washes for 5 min each with PBST. Immunoreactive proteins were detected using an enhanced chemiluminescent detection system according to the manufacturer's protocol (Pierce, Rockford, IL, USA). Densitometry analysis was carried out using the Quantity One program (Biorad, Hercules, CA, USA).

### *Immunofluorescence*

Endothelial cells grown on gelatin-coated coverslips were washed 3 times in ice-cold PBS and fixed in pre-chilled (–20°C) methanol after treatment with SCS. They were then blocked in 3% BSA in PBS for 1 h at room temperature and incubated with antibody to VE-Cadherin. After incubation, the cells were washed with PBS and incubated with Alexa-594-labeled secondary antibodies (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After three washes in PBS, the coverslips were mounted on slides using Prolong antifade mounting medium (Molecular Probes) and examined under an epifluorescence microscope Nikon U2000 with a 40 $\times$  oil objective lens (Nikon, Tokyo).

### *Measurement of endothelial permeability*

TER, an indicator of endothelial cell barrier integrity, was measured in real time using an electric cell-substrate impedance sensing (ECIS) system (Applied BioPhysics, Troy, NY, USA) as described previously (20). For resistance experiments, HPAEC cells were plated on sterile eight-well gold-plated electrode arrays (8W10E) pre-coated with 0.2% gelatin at 1  $\times$  10<sup>5</sup>/well one day

prior to the experiment. Fresh complete medium was added to the cells prior to the experiment. The arrays were then mounted on the ECIS system within an incubator (37°C, 5% CO<sub>2</sub>) and connected to its recorder device. Monolayer resistance was measured at continuous intervals and normalized at time 0.

### Statistics

All values are expressed as means  $\pm$  S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni test. A *P* value <0.05 was considered significant.

## Results

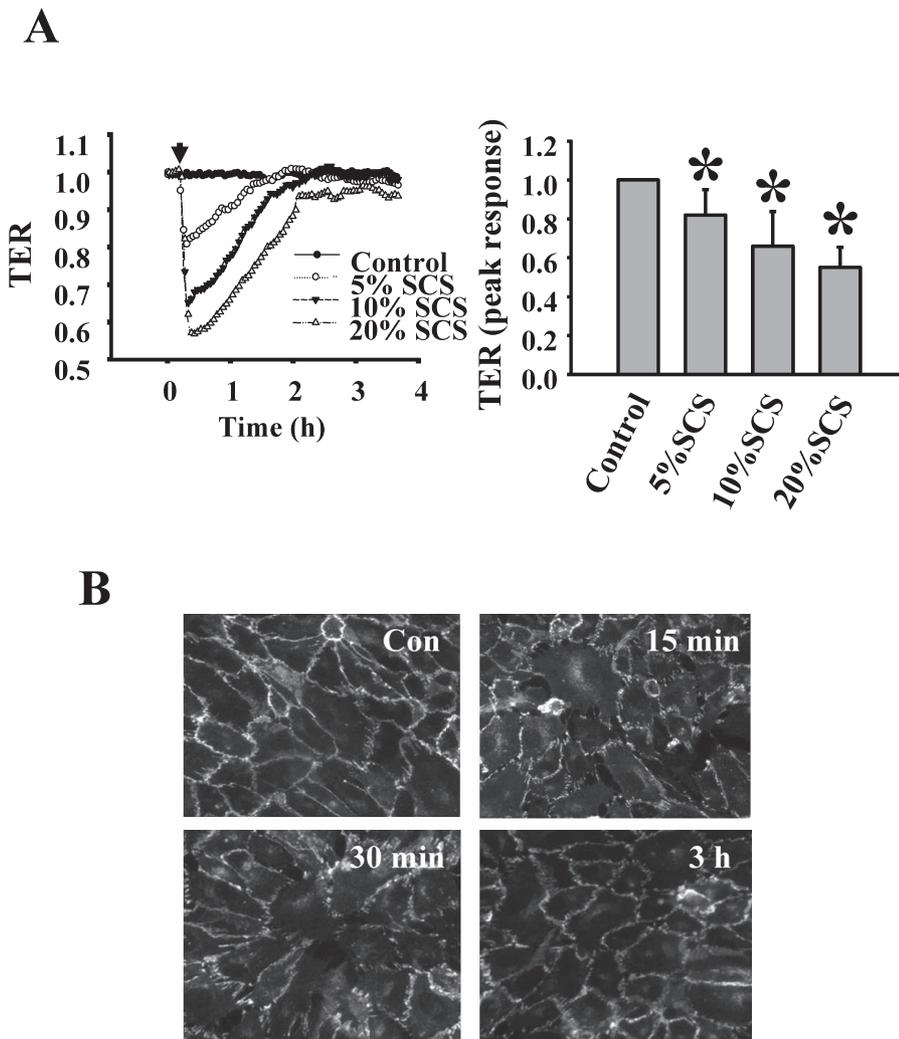
### Disruption of endothelial barrier function and dynamics of adherens junctions following SCS challenge

We first examined the effect of side stream cigarette smoke on endothelial barrier function. Human pulmonary artery endothelial cells cultured on electrode

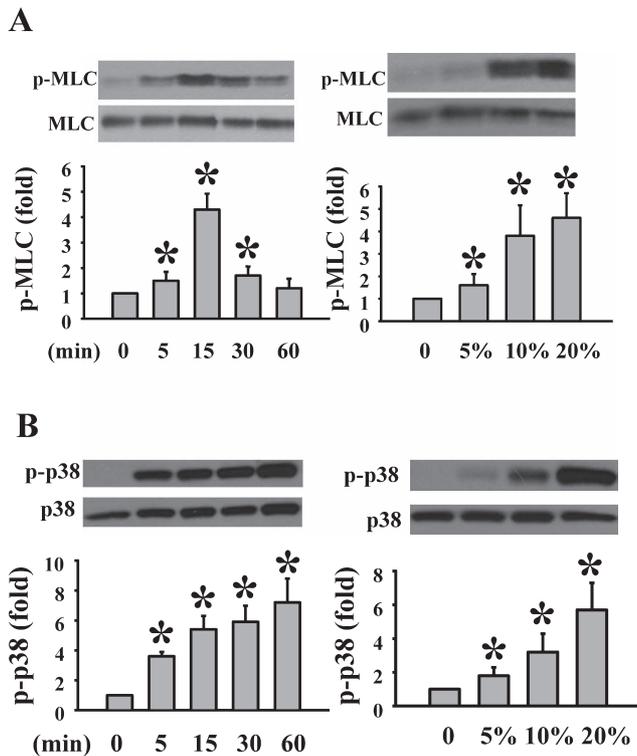
culturewares were treated with different concentrations of SCS. SCS treatment decreased TER within minutes (Fig. 1A). TER was able to return to basal level gradually within 2–3 h. The immunostaining pattern of anti-VE-cadherin along the cell-cell contacts exhibited the presence of intact adherens junctions in confluent HPAE cells (Fig. 1B, Con). SCS treatment induced disassembly of adherens junctions within minutes, as seen by the disrupted perimeter VE-cadherin staining (Fig. 1B, 15 and 30 min). By 3 h after continuous SCS challenge, the cell perimeter staining of VE-cadherin was restored (Fig. 1B, 3 h), which was consistent with TER recovery shown in Fig. 1A.

### SCS challenge induces MLC and p38 MAPK phosphorylation

HPAEs were incubated either with an increasing dose (0, 5%, 10%, and 20%) of SCS for 15 min or one dose (20%) in time course experiments (0, 5, 15, 30, and 60 min). SCS challenge induced MLC (Fig. 2A)



**Fig. 1.** Increase of endothelial permeability and dynamics of adherens junctions following sidestream cigarette smoke (SCS) challenge. **A:** Human pulmonary artery endothelial (HPAE) cells were seeded onto gold electrodes and cultured for 24 h, and transendothelial electrical resistance (TER, normalized to baseline) was monitored after SCS challenge. Representative traces (normalized to baseline) and bar graphs (peak responses to SCS treatment) show the change of TER. Results are representative of 6 independent experiments. Data are each presented as a mean  $\pm$  S.E.M. Asterisk indicates a value significantly different from that of control, *P*<0.05. The large arrowhead indicates the time point at which 5%, 10%, or 20% SCS was added. PBS was used for the Control. **B:** Stainings of VE-cadherin in confluent HPAEs treated with SCS (10%) for 0 min (Con), 15 min, 30 min, and 3 h. Stainings were examined under an epifluorescence microscope with a 40 $\times$  oil objective lens. Results are representative of 3 independent experiments.



**Fig. 2.** SCS stimulates MLC and p38 MAPK phosphorylation in human pulmonary artery endothelial cells. Confluent HPAEs were challenged with SCS (20%) in time course experiments or varying doses (0–20%) for 15 min. Cells were then lysed and subjected to immunoblotting analysis by using anti-phospho-MLC and anti-phospho p38 antibody; Membranes were stripped and re-probed with either anti-MLC or p38 antibody to confirm equal loading. Representative blots and bar graphs show the change of MLC (A) or p38 (B) phosphorylation. Results are representative of 3 independent experiments. Data are presented as the mean  $\pm$  S.E.M. Asterisk indicates a value significantly different from that of cells without SCS treatment,  $P < 0.05$ .

and p38 MAPK (Fig. 2B) phosphorylation in a time- and dose-dependent manner.

#### *Blockade of SCS-induced endothelial permeability by Rho-kinase and p38 MAPK inhibitors*

To address the roles of Rho kinase and p38 MAPK signaling in SCS-induced endothelial permeability, we examined the effects of Rho-kinase and p38 MAPK inhibitors on SCS-induced MLC phosphorylation. Both Rho kinase and p38 MAPK inhibitors decreased SCS-induced MLC phosphorylation (Fig. 3A). We then examined the effects of the selective Rho kinase and p38 MAPK inhibitors on preserving endothelial barrier function during the challenge with SCS extract. HPAEs cultured on electrode culturewares were pre-treated with either Rho kinase or p38 MAPK inhibitors and then challenged with 10% SCS. Both inhibitors significantly reduced the decrease of transendothelial

resistance by SCS challenge (Fig. 3B).

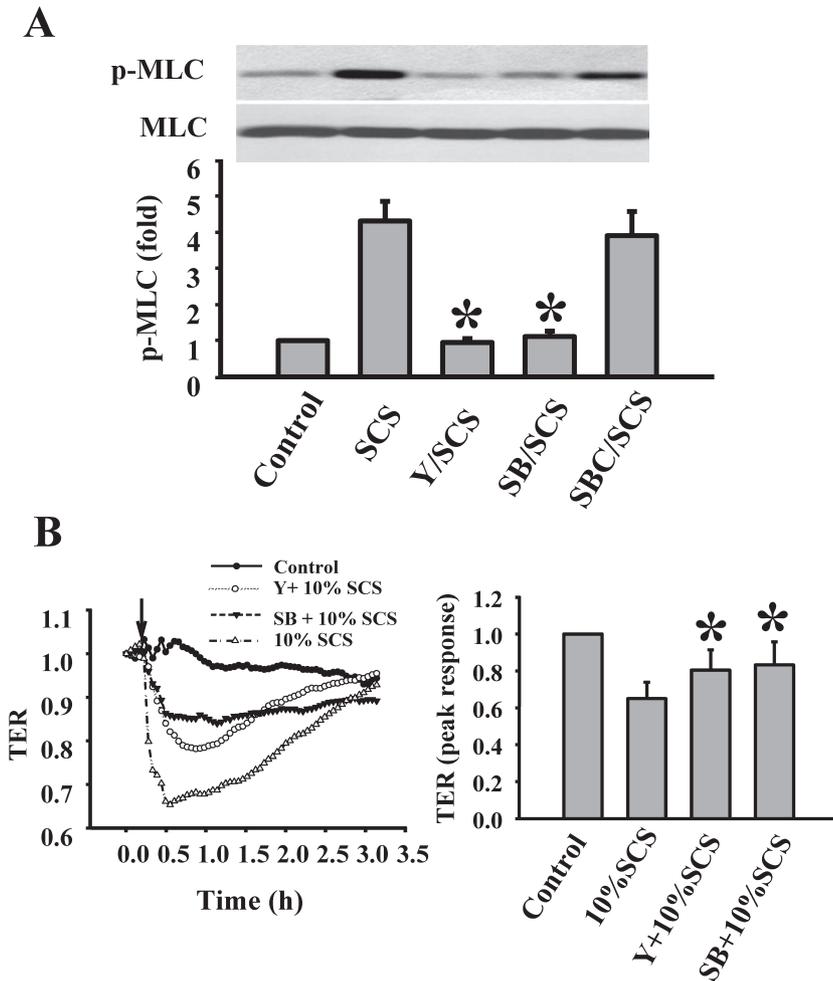
#### *Resveratrol inhibits SCS-induced MLC and p38 MAPK phosphorylation and prevents SCS-induced endothelial permeability*

Resveratrol is an antioxidant that has been shown to have protective effects against cardiovascular damage (21). Here, we tested its effects on SCS-induced cell signaling and endothelial permeability. Resveratrol was used as an antioxidant in this particular study because it alone had no effect on transendothelial resistance but consistently inhibited SCS-induced decrease of transendothelial resistance. Pre-treatment of the cells with resveratrol for 30 min inhibited the phosphorylation of both p38 MAPK and MLC (Fig. 4A) and reduced the decrease of transendothelial resistance by SCS challenge (Fig. 4B).

#### **Discussion**

There is increasing evidence suggesting that cigarette smoke not only affects those who smoke, non-smokers who are exposed to second-hand smoke environment are also adversely affected by the toxic substances of cigarette smoke (1–3). Second-hand cigarette smoke has two components: sidestream smoke released from the burning end of a cigarette and mainstream smoke inhaled and then exhaled by an active smoker. Sidestream smoke accounts for about 85% of the total amount of second-hand smoke (2, 22). Importantly, when compared with mainstream smoke, sidestream smoke contains more concentrated toxic chemicals (23). Although it has been reported by others that cigarette smoke can induce endothelial dysfunction (24, 25), the molecular mechanisms of the second-hand smoke induced-vascular injury are not well understood. In this study, we determined the effects of sidestream cigarette smoke extract on endothelial permeability and used this in vitro model to explore the underlying mechanisms and potential treatments.

We first tested the effects of sidestream cigarette smoke on endothelial junctional integrity. We observed the decrease in transendothelial electrical resistance and disruption of cell-cell junctions when HPAEs were exposed to SCS. The change of endothelial permeability is transient and reversible, and therefore permanent damage to the cells may not occur. Similar changes were also observed when HPAEs were treated with mainstream cigarette smoke extract (J Fu et al., unpublished data), suggesting that SCS could be as harmful as mainstream cigarette smoke. We then examined the signaling pathways involved in regulating sidestream cigarette smoke-induced endothelial permeability. After

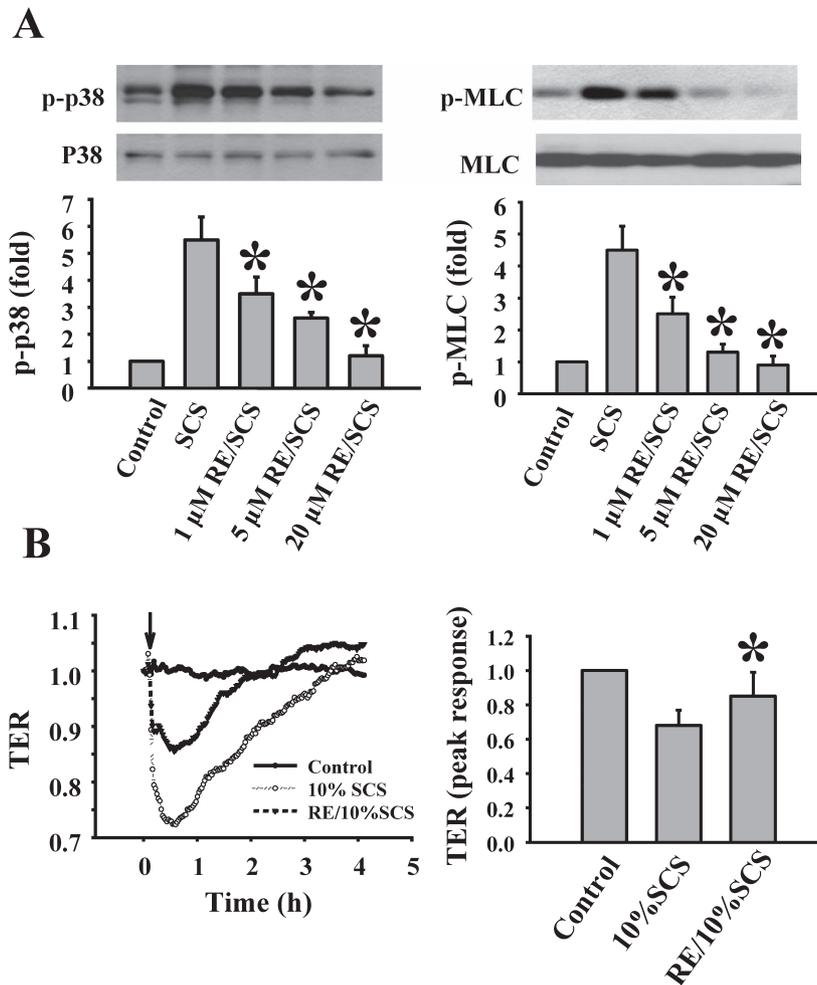


**Fig. 3.** Inhibition of MLC phosphorylation by Rho kinase and p38 MAPK inhibitors reduces SCS-induced endothelial permeability. **A:** HPAEs were pre-treated with inhibitors of Rho kinase Y27632 (Y, 5  $\mu$ M), p38 MAPK SB203580 (SB, 2.5  $\mu$ M), or an inactive control SB202474 (SBC, 2.5  $\mu$ M) for 15 min and then challenged with 20% SCS. SCS-induced phosphorylation of MLC was detected by immunoblotting. Representative blots and bar graphs show the change of MLC phosphorylation. Results are representative of 3 independent experiments. Data are each presented as a mean  $\pm$  S.E.M. Asterisk indicates a value significantly different from that of cells treated with SCS alone,  $P < 0.05$ . **B:** HPAEs were seeded onto gold electrodes and measured for changes in endothelial permeability via TER measurement. Cells were pre-treated with Rho kinase inhibitor Y27632 (Y, 5  $\mu$ M) or p38 MAPK inhibitor SB203580 (SB, 2.5  $\mu$ M) for 15 min, and then challenged with 10% SCS. Representative traces (normalized to baseline) and bar graphs (peak responses to SCS treatment) show the change of TER. The arrow indicates the time point at which 10% SCS was added. Results are representative of 4 independent experiments. Data are each presented as a mean  $\pm$  S.E.M. Asterisk indicates a value significantly different from that of cells treated with 10% SCS alone,  $P < 0.05$ .

exposing human pulmonary artery endothelial cells to sidestream cigarette smoke extract, we observed the activation of cell signaling pathways (e.g., MLC phosphorylation and p38 MAPK activation) that have been shown to promote vascular injury (5, 10). Even though SCS induced persistent P38 MAPK phosphorylation, MLC phosphorylation was transient, which could be due to dephosphorylation of MLC by MLC phosphatase. Inhibition of Rho kinase, which is known to stimulate MLC phosphorylation indirectly through inactivation of MLC phosphatase (15, 16), blocked MLC phosphorylation induced by SCS treatment. Importantly, inhibition of p38 MAPK protected endothelial cells from SCS-induced damage by inhibiting the phosphorylation of MLC, suggesting that p38 MAPK activation is necessary for MLC phosphorylation. Thus, we provide new evidence supporting the link between second-hand smoke and vascular injury and disclose the signaling mechanisms that may lead to endothelial dysfunction and acute lung injury by second hand smoke exposure.

Cigarette smoke, which contains thousands of active compounds, for example, nicotine, carbon monoxide,

and aromatic amines such as the carcinogenic compound *o*-toluidine (23), has been shown to decrease nitric oxide production, increase oxidative stress, and stimulate cytokine production (25–29). Thus, the chemicals in sidestream smoke extract may induce endothelial permeability through diverse signaling pathways. Exposure to second-hand smoke induces free radical generation (25, 29), which could be a major cause of second-hand smoke-induced vascular injury. The oxidants can come directly from cigarette smoke and indirectly through activation of endogenous sources of free radicals, for example, NADPH oxidase (25, 29). Studies from Yamaguchi et al. indicate that aqueous extract of cigarette smoke may contain stable oxidants with peroxynitrite-like activity (30–32). Interestingly, peroxynitrite-like species in cigarette smoke extract have been reported to oxidize plasma low-density lipoprotein (LDL) (31). Oxidized LDL is known to play important roles in the early development of atherosclerosis (33). Association of oxidized LDL with endothelial dysfunction is well established (34, 35). Thus, oxidative modification of LDL could be an early



**Fig. 4.** Resveratrol inhibits SCS-induced MLC and p38 MAPK phosphorylation and endothelial permeability. **A:** HPAEs were pre-treated with resveratrol (RE) for 30 min, and then challenged with 20% SCS. SCS-induced phosphorylation of MLC and p38 MAPK was detected by immunoblotting. Representative blots and bar graphs show the change of p38 MAPK and MLC phosphorylation. Results are representative of 3 independent experiments. Data are each presented as a mean  $\pm$  S.E.M. Asterisk indicates a value significantly different from that of cells treated with SCS alone,  $P < 0.05$ . **B:** HPAEs were seeded onto gold electrodes and measured for changes in endothelial permeability via TER measurement. HPAEs were pre-treated with resveratrol (RE, 20  $\mu$ M) for 30 min and then stimulated with 10% SCS. Representative traces (normalized to baseline) and bar graphs (peak responses to SCS treatment) show the change of TER. The arrow indicates the time point at which 10% SCS was added. Results are representative of 4 independent experiments. Data are each presented as a mean  $\pm$  S.E.M. Asterisk indicates a value significantly different from that of cells treated with 10% SCS alone,  $P < 0.05$ .

cause of vascular endothelial dysfunction induced by secondhand smoke exposure and may facilitate the further development of cardiovascular diseases. Oxidant stress-mediated acute endothelial dysfunction has also been well studied (36). Reactive oxygen species are able to activate phospholipases and their downstream signaling or alter the structure and function of cytoskeleton and adhesion molecules through oxidative modification (36, 37). All those pathways may mediate second-hand smoke-induced endothelial permeability. Our studies pointed out the involvement of p38 MAPK. Resveratrol, a compound with antioxidant activity, was able to reduce SCS-induced endothelial permeability, which was correlated with its inhibitory effects on SCS-induced p38 MAPK and MLC phosphorylation.

Our studies allowed a better understanding of second-hand smoke-induced endothelial dysfunction, which may help us to design a strategy to prevent vascular injury from the damaging effects of second-hand cigarette smoke. Indeed, inhibition of p38 MAPK is showing promise as a therapeutic approach. SB203580,

a selective p38 MAPK inhibitor, is now applied in the treatment of pulmonary diseases without significant side effects (38). Thus, inhibition of p38 MAPK may provide protective effects against second-hand smoke-induced vascular injury in clinical settings.

## References

- 1 He J, Whelton PK. Passive cigarette smoking increases risk of coronary heart disease. *Eur Heart J.* 1999;20:1764–1765.
- 2 Raupach T, Schafer K, Konstantinides S, Andreas S. Second-hand smoke as an acute threat for the cardiovascular system: a change in paradigm. *Eur Heart J.* 2006;27:386–392.
- 3 Steenland K. Passive smoking and the risk of heart disease. *JAMA.* 1992;267:94–99.
- 4 Bazzoni G, Dejana E. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev.* 2004;84:869–901.
- 5 Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol.* 2001;91:1487–1500.
- 6 Sheldon R, Moy A, Lindsley K, Shasby S, Shasby DM. Role of myosin light-chain phosphorylation in endothelial cell retraction.

- Am J Physiol. 1993;265:L606–L612.
- 7 Goeckeler ZM, Wysolmerski RB. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. *J Cell Biol.* 1995;130:613–627.
  - 8 Siflinger-Birnboim A, Johnson A. Protein kinase C modulates pulmonary endothelial permeability: a paradigm for acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* 2003;284:L435–L451.
  - 9 Roviezzo F, Tsigkos S, Kotanidou A, Bucci M, Brancaleone V, Cirino G, et al. Angiopoietin-2 causes inflammation in vivo by promoting vascular leakage. *J Pharmacol Exp Ther.* 2005;314:738–744.
  - 10 Issbrucker K, Marti HH, Hippenstiel S, Springmann G, Voswinkel R, Gaumann A, et al. p38 MAP kinase – a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *Faseb J.* 2003;17:262–264.
  - 11 Satpathy M, Gallagher P, Lizotte-Waniewski M, Srinivas SP. Thrombin-induced phosphorylation of the regulatory light chain of myosin II in cultured bovine corneal endothelial cells. *Exp Eye Res.* 2004;79:477–486.
  - 12 Birukova AA, Birukov KG, Adyshev D, Usatyuk P, Natarajan V, Garcia JG, et al. Involvement of microtubules and Rho pathway in TGF-beta1-induced lung vascular barrier dysfunction. *J Cell Physiol.* 2005;204:934–947.
  - 13 Sun H, Breslin JW, Zhu J, Yuan SY, Wu MH. Rho and ROCK signaling in VEGF-induced microvascular endothelial hyperpermeability. *Microcirculation.* 2006;13:237–247.
  - 14 Somlyo AP, Somlyo AV. Ca<sup>2+</sup> sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev.* 2003;83:1325–1358.
  - 15 Ikebe M, Hartshorne DJ. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem.* 1985;260:10027–10031.
  - 16 Totsukawa G, Yamakita Y, Yamashiro S, Hartshorne DJ, Sasaki Y, Matsumura F. Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J Cell Biol.* 2000;150:797–806.
  - 17 Garcia JG, Wang P, Schaphorst KL, Becker PM, Borbiev T, Liu F, et al. Critical involvement of p38 MAP kinase in pertussis toxin-induced cytoskeletal reorganization and lung permeability. *Faseb J.* 2002;16:1064–1076.
  - 18 Goldberg PL, MacNaughton DE, Clements RT, Minnear FL, Vincent PA. p38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. *Am J Physiol Lung Cell Mol Physiol.* 2002;282:L146–L154.
  - 19 Borbiev T, Birukova A, Liu F, Nurmukhambetova S, Gerthoffer WT, Garcia JG, et al. p38 MAP kinase-dependent regulation of endothelial cell permeability. *Am J Physiol Lung Cell Mol Physiol.* 2004;287:L911–L918.
  - 20 Giaever I, Keese CR. Micromotion of mammalian cells measured electrically. *Proc Natl Acad Sci U S A.* 1991;88:7896–7900.
  - 21 Hung LM, Su MJ, Chu WK, Chiao CW, Chan WF, Chen JK. The protective effect of resveratrols on ischaemia-reperfusion injuries of rat hearts is correlated with antioxidant efficacy. *Br J Pharmacol.* 2002;135:1627–1633.
  - 22 Taylor AE, Johnson DC, Kazemi H. Environmental tobacco smoke and cardiovascular disease. A position paper from the Council on Cardiopulmonary and Critical Care, American Heart Association. *Circulation.* 1992;86:699–702.
  - 23 Lofroth G. Environmental tobacco smoke: overview of chemical composition and genotoxic components. *Mutat Res.* 1989;222:73–80.
  - 24 Holden WE, Maier JM, Malinow MR. Cigarette smoke extract increases albumin flux across pulmonary endothelium in vitro. *J Appl Physiol.* 1989;66:443–449.
  - 25 Jaimes EA, DeMaster EG, Tian RX, Raji L. Stable compounds of cigarette smoke induce endothelial superoxide anion production via NADPH oxidase activation. *Arterioscler Thromb Vasc Biol.* 2004;24:1031–1036.
  - 26 Barua RS, Ambrose JA, Eales-Reynolds LJ, DeVoe MC, Zervas JG, Saha DC. Dysfunctional endothelial nitric oxide biosynthesis in healthy smokers with impaired endothelium-dependent vasodilatation. *Circulation.* 2001;104:1905–1910.
  - 27 Barua RS, Ambrose JA, Srivastava S, DeVoe MC, Eales-Reynolds LJ. Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase: an in vitro demonstration in human coronary artery endothelial cells. *Circulation.* 2003;107:2342–2347.
  - 28 van der Vaart H, Postma DS, Timens W, Hylkema MN, Willemse BW, Boezen HM, et al. Acute effects of cigarette smoking on inflammation in healthy intermittent smokers. *Respir Res.* 2005;6:22.
  - 29 Zhang J, Liu Y, Shi J, Larson DF, Watson RR. Side-stream cigarette smoke induces dose-response in systemic inflammatory cytokine production and oxidative stress. *Exp Biol Med (Maywood).* 2002;227:823–829.
  - 30 Yamaguchi Y, Kagota S, Haginaka J, Kunitomo M. Peroxynitrite-generating species: good candidate oxidants in aqueous extracts of cigarette smoke. *Jpn J Pharmacol.* 2000;82:78–81.
  - 31 Yamaguchi Y, Kagota S, Haginaka J, Kunitomo M. Participation of peroxynitrite in oxidative modification of LDL by aqueous extracts of cigarette smoke. *FEBS Lett.* 2002;512:218–222.
  - 32 Yamaguchi Y, Nasu F, Harada A, Kunitomo M. Oxidants in the gas phase of cigarette smoke pass through the lung alveolar wall and raise systemic oxidative stress. *J Pharmacol Sci.* 2007;103:275–282.
  - 33 Galle J, Hansen-Hagge T, Wanner C, Seibold S. Impact of oxidized low density lipoprotein on vascular cells. *Atherosclerosis.* 2006;185:219–226.
  - 34 Chen J, Mehta JL, Haider N, Zhang X, Narula J, Li D. Role of caspases in Ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells. *Circ Res.* 2004;94:370–376.
  - 35 Kontush A, Chancharme L, Escargueil-Blanc I, Therond P, Salvayre R, Negre-Salvayre A, et al. Mildly oxidized LDL particle subspecies are distinct in their capacity to induce apoptosis in endothelial cells: role of lipid hydroperoxides. *Faseb J.* 2003;17:88–90.
  - 36 Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol.* 2001;280:C719–C741.
  - 37 Kamata H, Hirata H. Redox regulation of cellular signalling. *Cell Signal.* 1999;11:1–14.
  - 38 Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL. Inhibition of p38 MAP kinase as a therapeutic strategy. *Immunopharmacology.* 2000;47:185–201.