

Sustained Activation of p38 Mitogen-Activated Protein Kinase Contributes to the Vascular Response to Injury

HAISONG JU, SANDHYA NERURKAR, CHARLES F. SAUERMECH, ALAN R. OLZINSKI, ROSANNA MIRABILE, DAWN ZIMMERMAN, JOHN C. LEE, JERRY ADAMS, JOSEPH SSKO, MARINELLA BEROVA, and ROBERT N. WILLETTE

Department of Cardiovascular Pharmacology, GlaxoSmithKline, King of Prussia, Pennsylvania

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ABSTRACT

The vascular response to mechanical injury involves inflammatory and fibroproliferative processes that result in the formation of neointima and vascular remodeling. The complex cellular interactions initiated by vascular injury are coordinated and modulated by the elaboration of cytokines and growth factors. The production and transduction of many of these mediators require phosphorylation of p38 mitogen-activated protein kinase (MAPK). In the present investigation, we examined the pattern and localization of p38 MAPK activation following balloon vascular injury. The effects of long-term and selective inhibition of p38 MAPK with SB 239063 (*trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-[2-methoxy]pyrimidin-4-yl]imidazole) were also investigated in a model of vascular injury. Western blotting and immunohistochemical staining demonstrated that phospho-p38 MAPK was increased following balloon injury of the rabbit iliofemoral artery. The p38 MAPK activation was noted as early as 15 min after balloon injury and

remained elevated for at least 28 days. Phospho-p38 MAPK immunoreactivity (IR) was localized primarily in regions of dedifferentiated, smooth muscle α -actin-positive cells in all lamina of the vessel wall. Phospho-p38 MAPK IR was not correlated with the localization of macrophage or proliferating cells (proliferating cell nuclear antigen; PCNA +). Long-term treatment (4 weeks) with SB 239063 (50 mg/kg/day, p.o.) reduced the vascular response to injury in the hypercholesterolemic rabbit. SB 239063 had no effect on platelet-derived growth factor (PDGF)-stimulated migration or proliferation of rabbit vascular smooth muscle cells (VSMCs) in culture. However, SB 239063 produced a concentration-dependent inhibition of transforming growth factor (TGF)- β -stimulated fibronectin production in VSMCs. In conclusion, sustained activation of p38 MAPK plays an important role in the vascular response to injury and inhibition of p38 MAPK may represent a novel therapeutic approach to limit this response.

The vascular response to mechanical injury involves inflammatory and fibroproliferative processes that result in vascular remodeling and the development of neointima (Ferns and Avades, 2000). Under the best circumstances, the complex cellular interactions are highly coordinated and result in a stable/benign vascular lesion complete with compensatory remodeling of the thickened vessel wall and preservation of lumen (Ross, 1993; Owens, 1995). However, factors such as severe mechanical injury and hypercholesterolemia may adversely influence the magnitude and temporal pattern of cellular events, as well as the gross morphology of the lesion.

Numerous cell types are activated during this wound healing response according to a well defined progression of events. Cells activated following vascular injury include platelets, neutrophils, monocytes/macrophages, T-lymphocytes, endothelial cells, VSMCs, and fibroblasts (Ross, 1993; Ferns and Avades, 2000). The p38 mitogen-activated protein

kinase (MAPK) pathway has been shown to play an important role in the chemical and/or mechanical stress response of all these cell types (Junger et al., 1998; Hackeng et al., 1999; Herlaar and Brown, 1999; Li et al., 2000). In particular, the activation of the p38 MAPK pathway plays an important role in the generation of numerous proinflammatory cytokines including interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor- α (TNF- α) (Lee et al., 1994, 2000). In addition, the p38 MAPK pathway also plays an important role in mediating cellular signal transduction of proinflammatory cytokines, growth factors, and hormones acting at G α q-protein-coupled receptors (Widmann et al., 1999; Wang et al., 2000). However, the majority of current studies have been limited to the study of transient activation of p38 MAPK in cultured cells. Information regarding the activation and inhibition of p38 MAPK in the intact animal model is limited.

We hypothesize that p38 MAPK is activated in the vascu-

ABBREVIATIONS: VSMCs, vascular smooth muscle cells; MAPK, mitogen-activated protein kinase; IR, immunoreactivity; IL, interleukin; TNF, tumor necrosis factor; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; FBS, fetal bovine serum; TGF, transforming growth factor; SB 239063, *trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-[2-methoxy]pyrimidin-4-yl]imidazole; FR167653, 1[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-c][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate; RAM-11, rabbit anti-macrophage.

lar wall by mechanical injury and the subsequent response to injury, and the actions mediated by p38 MAPK play an important role in the development of the vascular lesion. Recent evidence suggests that p38 MAPK inhibitors reduce neointimal formation following endothelial denudation in the rat carotid artery (Ohashi et al., 2000). In the present study, we examined the temporal profile and localization of activated p38 MAPK in injured blood vessels in rabbit. Long-term treatment with a selective p38 MAPK inhibitor, SB 239063, was also examined in a hypercholesterolemic rabbit model of vascular injury. Mechanistic studies were carried out to investigate the role of p38 MAPK in VSMCs proliferation, migration, and fibronectin production.

Materials and Methods

Rabbit Balloon Angioplasty. A total of 94 male New Zealand White rabbits were used in the current study. In the initial study, the temporal pattern of phospho-p38 MAPK was examined in blood vessels obtained following balloon injury in rabbits on a normal chow diet ($n = 21$). Balloon injury was performed in the left or right iliofemoral artery, and sham operations were performed in the opposing artery. Briefly, a 3.0 French Fogarty balloon catheter (Baxter, Deerfield, IL) was introduced into the caudal femoral artery and advanced to the iliac bifurcation. The balloon was inflated from 0.09 to 0.11 ml while being pulled with a twisting motion through the iliofemoral artery. This procedure was repeated three times. In the second study, balloon injury was performed similarly in rabbits (3 kg, 3–4 months old) 1 week after introduction of a high fat/cholesterol diet containing 2.5% peanut oil and 0.5% cholesterol (TD 98263; Harlan Teklad, Madison, WI). Rabbits in the group were maintained on a high fat/cholesterol diet until the end of the study. A total of 39 hypercholesterolemic rabbits were used for a time course analysis of p38 MAPK activation by Western blotting ($n = 3$ per time point) and immunohistochemical staining ($n = 3$ per time point). Immunolocalization of phospho-p38, smooth muscle α -actin, proliferating cell nuclear antigen (PCNA) and macrophage RAM-11 were performed in this group. In the third (treatment) study, a total of 34 rabbits received a selective p38 MAPK inhibitor (SB 239063, 50 mg/kg/day, p.o., $n = 18$) or vehicle group (5% hydroxypropyl- β -cyclodextrin, $n = 16$) for 30 days. The chemical structure of SB 239063 has been published (Underwood et al., 2000). The treatment was started 2 days prior to the angioplasty procedure. The dosing regimen, based on previous exposure studies (not shown), was designed to achieve SB 239063 plasma levels >400 ng/ml. All animals were euthanized at specified time points with Fatal Plus (0.33 ml/kg; Vortech Pharmaceuticals, Dearborn, MI) and arteries were fixed in situ by perfusion fixation at 90 mm Hg. All experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Publication 85-23).

Tissue Extraction and Western Blot Analysis. Protein extracts of the iliofemoral artery were prepared for the examination phospho-p38 MAPK expression. The iliofemoral arteries were excised at various times after sham or balloon injury ($n = 3$ per time point). Immediately after dissection, the tissues were snap frozen in liquid nitrogen and stored at -80°C until all the samples from the time course were collected. For extraction, iliofemoral arteries were weighed and pulverized with a mortar and pestle. The tissues were then homogenized and incubated in an extraction buffer consisting of 20 mM HEPES (pH = 7.4), 75 mM NaCl, 20 mM α -glycerophosphate, 100 mM Na_3VO_4 , 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2.5 mM MgCl_2 , 0.1% Triton X-100, and complete protease inhibitors while gently rotating at 4°C for 15 min. The concentration of the initial extraction mixture for each tissue sample was normalized to 400 mg/ml. After the extraction was complete, the samples were centrifuged at 14,000g for 20 min at 4°C and

the supernatants were collected. To check the quality and uniformity of each extraction throughout the study, samples of each extract were analyzed by running on SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and staining with 0.25% Coomassie Brilliant Blue 250 (Sigma-Aldrich, St. Louis, MO). Protein concentration for each sample was determined with the DC Protein Assay (Bio-Rad). For Western blotting, 30 μg of protein was resolved in 10% SDS-polyacrylamide gel electrophoresis precasted gel and transferred to polyvinylidene difluoride membrane. After blocking, the membrane was incubated with primary antibodies in Tris-buffered saline/Tween 20 buffer overnight at 4°C followed by incubating with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Primary antibodies included phospho-p38 (1:1000; Cell Signaling Technology Inc., Beverly, MA), p38 MAPK (1:1000; Cell Signaling Technology Inc.) and glyceraldehyde-3-phosphate dehydrogenase (1:200; Chemicon International, Temecula, CA). Immunoreactive bands were detected using chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Immunohistochemical Staining. Slides were deparaffinized, rehydrated, and placed in phosphate-buffered saline with 0.1% Tween 20. Sections were stained using streptavidin-horseradish peroxidase labeling system on the DAKO Autostainer (Carpenteria, CA). Briefly, the slides were exposed to 3% hydrogen peroxide for 15 min. Normal serum at 1:50 was used to block nonspecific binding. Sections were incubated with primary antibodies for 45 min followed by a 30-min incubation with biotinylated secondary antibodies. Slides were incubated in 1:200 dilution of streptavidin-horseradish peroxidase (DAKO) for 15 min followed by incubation with substrate 3',3'-diaminobenzidine for 5 min. The slides were counterstained with hematoxylin, dehydrated, and coverslipped. All primary antibodies used were monoclonal antibody and included phospho-p38 MAPK at 1:10 dilution (Sigma-Aldrich), macrophage RAM-11 at 1:50 dilution (DAKO), α -actin at a concentration of 2.5 $\mu\text{g}/\text{ml}$ (Roche Diagnostics GmbH, Mannheim, Germany), and PCNA at 1:50 dilution (Chemicon International).

Elastin Van Gieson Staining and Morphometric Analysis. For histological evaluation of the iliofemoral arteries, a 1-cm section of iliofemoral artery was fixed in 10% neutral buffered formalin (Sigma-Aldrich) and then embedded in paraffin. Slides were deparaffinized, rehydrated, and placed in phosphate-buffered saline. Sections were stained in a solution containing hematoxylin, ferric chloride, and Lugol's iodine for 15 min. Slides were differentiated in 2% ferric chloride and treated with 5% sodium thiosulfate followed by staining in Van Gieson solution for 5 min. Slides were dehydrated, cleared, and coverslipped. The morphometric analysis of lesions was performed using Image Pro Plus image analysis software (MediaCybernetics, Inc., Silver Spring, MD). Measurements from four non-overlapping fields from each of four vessels were obtained using 20 \times magnification on an Olympus microscope (Olympus, Tokyo, Japan).

Primary Culture of VSMCs from Rabbit Iliofermal Arteries. An explant method was used to generate primary culture of VSMCs (Simari et al., 1996). Briefly, the iliofermal artery was isolated from male New Zealand White rabbit, and adventitial connective tissue was removed under a microscope. The artery was cut open longitudinally, and endothelial cells were removed by gently scraping the lumen side with a scalpel blade. The iliofermal artery then was cut into 1- to 2-mm sections and plated in a culture dish supplied with DMEM (Invitrogen, Carlsbad, CA) containing 20% FBS and 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B. The explants were incubated in a humidified incubator at 37°C with 5% CO_2 . Initial migration (first phase of migration) of VSMCs was observed between 5 and 6 days. To minimize the contamination of fibroblast, only those cells from the second to fourth phase of migration were used. VSMCs were maintained in DMEM with 10% FBS, and cells from passages 2 to 4 were used in the current study. VSMCs were confirmed by positive staining of α -actin.

Proliferation Assay. VSMCs from passages 2 to 4 were used in the experiments. Cells were seeded at 5000 cells/well in a 96-well

plate in DMEM containing 10% FBS. Cells were allowed to adhere and grow until about 90% confluent. After being serum starved for 48 h in serum-free DMEM, the quiescent cells were stimulated with platelet-derived growth factor (PDGF) or FBS in the absence or presence of a specific p38 inhibitor, SB 239063, at 3 or 10 μ M concentrations for 48 h. Medium was replaced with new DMEM containing PDGF, SB 239063, or vehicle after the first 24 h. Ten microliters of cell proliferation reagent WST-1 was added to each well according to the manufacturer's instruction (Roche Diagnostics GmbH). The absorbance of the samples against background control was read at 440 nm in a plate reader (SPECTRA MAX 250; Molecular Devices, Menlo Park, CA) after 1 to 2 h of incubation at 37°C.

Migration Assay. The migration of VSMCs was quantified using a 96-well transwell culture chamber (Neuro Probe, Gaithersburg, MD). The lower wells were loaded with 32 μ l of DMEM containing 60 ng/ml PDGF or vehicle as control to give a positive meniscus. VSMCs migrated from the upper to the lower chamber through a polycarbonate filter with 8- μ m pores. VSMCs were labeled with a fluorescent dye calcein-acetoxymethyl ester before it was trypsinized and counted. A total of 10,000 cells in 52 μ l were plated in the upper chamber. SB 239063 or vehicle was added into upper and lower chambers to reach a final concentration of 3 and 10 μ M. After a 4-h migration at 37°C, the filter was removed, and cells on the upper side of the filter were removed and those in the lower side of the filter were quantified with a Fluoroskan Ascent (Labsystems, Helsinki, Finland).

Fibronectin Synthesis in Rabbit Vascular Smooth Muscle Cells. VSMCs were cultured in 100-mm dishes and allowed to reach 100% confluence. The cells were serum-starved for 48 h followed by stimulation with transforming growth factor β 1 (TGF- β 1) 10 ng/ml for 48 h with or without SB 239063 (0.3, 1, 3, and 10 μ M). Cells were harvested, and the proteins were extracted (in cell lysis buffer containing 20 mM HEPES (pH = 7.4), 50 mM β -glycerophosphate, 2 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 50 mM NaF, 0.4 mM phenylmethylsulfonyl fluoride, and 1 mM Na_2VO_4). Western blot analysis was performed using a monoclonal anticellular fibronectin antibody (Chemicon International) in a 1:1000 dilution as primary antibody; a horseradish peroxidase-conjugated goat-anti-mouse IgG antibody in 1:5000 dilution was used as secondary antibody. A cell lysate from human foreskin fibroblasts (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control.

Statistical Analysis. All data are expressed as the mean \pm S.E.M.. Student's *t* test was used for comparison between the SB 239063-treated and vehicle-treated groups. Multiple comparisons were made using an analysis of variance for unpaired data followed by post hoc analysis with the Bonferroni correction for multiple comparisons. A probability level of $P \leq 0.05$ was considered to be statistically significant. All statistical analyses were done using In-Stat (GraphPad Software, San Diego, CA).

Results

Activation of p38 MAPK after Balloon Injury. Phospho-p38 was examined by Western blot analysis to determine activation of p38 MAPK after injury of the rabbit iliofemoral artery. A general pattern of p38 MAPK activation was noted in injured versus contralateral sham arteries ($n = 3$ per time point) throughout the 28-day study (Fig. 1). The increase in phospho-p38 MAPK was first noted at 15 min and remained elevated through 28 days in injured arteries. The total p38 MAPK protein was also elevated only at later time points following balloon injury. Phospho-p38 MAPK, normalized to the total p38 MAPK, remained elevated when compared with sham-injured vessels. This pattern of persistent p38 MAPK activation was confirmed by examining phospho-p38 MAPK immunoreactivity (IR) in sham and injured arterial sections. Prominent nuclear staining of phospho-p38 MAPK IR was

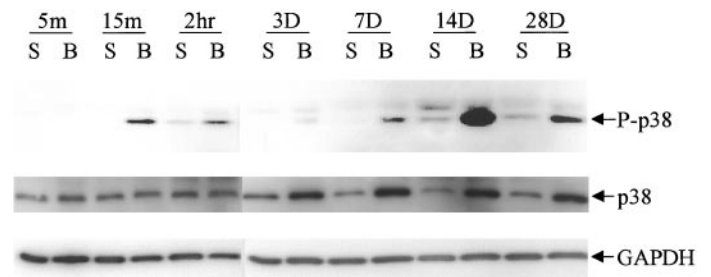


Fig. 1. Temporal activation of p38 in iliofemoral artery following balloon injury in rabbit fed with normal diet. A representative Western blot showing the specific band detected using antibodies against phospho-p38 and p38 MAPK at different time points from 5 min to 28 days following balloon injury as indicated. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. A similar result was obtained in three independent experiments. m, minutes; hr, hours; D, days; S, sham; B, balloon injury.

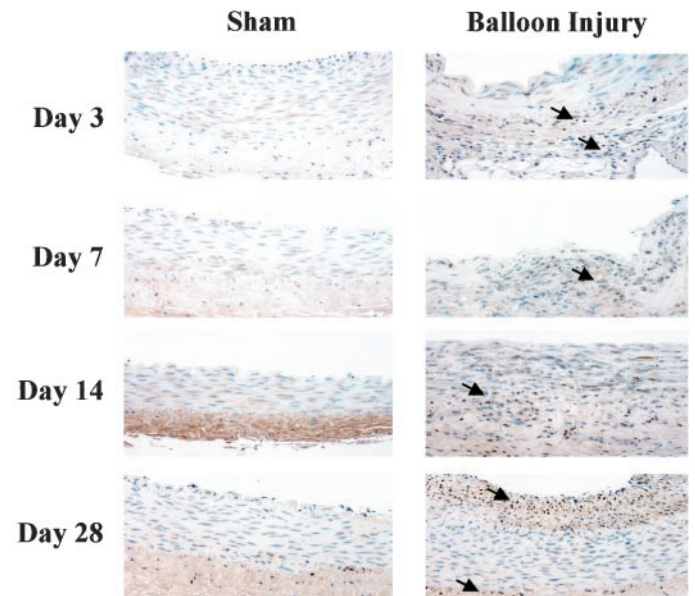


Fig. 2. Immunohistochemical staining showing temporal (3–28 days) alteration of phospho-p38 in iliofemoral artery from balloon-injured and sham-operated animals. Phospho-p38 positive cells appears as dark brown staining and are indicated by arrows. Similar result was obtained in three independent experiments.

noted only in injured arteries. Phospho-p38 MAPK IR cells were first noted in the medium and adventitia (Fig. 2). The phospho-p38 MAPK IR was predominant in the developing neointima throughout 28 days. Phospho-p38 MAPK IR was restricted to what appeared to be rounded dedifferentiated VSMCs, whereas elongated differentiated (contractile) VSMCs in the medium or neointima showed little or no phospho-p38 MAPK IR (Fig. 3). The temporal profile of phospho-p38 MAPK IR did not correlate with the abbreviated course of cellular proliferation (PCNA IR), which reached a maximum between 3 and 7 days (Fig. 4). Phospho-p38 MAPK IR also did not correlate with the localization of macrophage/foam (RAM-11 IR) cells (Fig. 4).

Effects of p38 MAPK Inhibition on the Vascular Response to Balloon Injury. SB 239063 is a potent and selective p38 MAPK inhibitor (Table 1), and preliminary studies indicated that plasma levels of SB 239063 >400 ng/ml are sufficient to abolish TNF- α generation in whole blood stimulated with lipopolysaccharide (0.1 μ g/ml). The plasma concentration of SB 239063 (50 mg/kg/day) in the present study

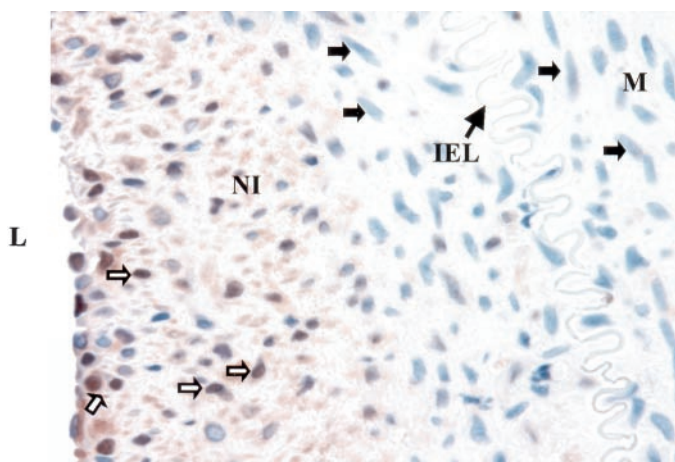


Fig. 3. A representative staining showing nuclear translocation of phospho-p38 at 28 days after balloon injury under high power. Phospho-p38 positive cells are indicated by open arrows and phospho-p38 negative cells are indicated by solid arrows. L, lumen; NI, neointima; M, medium; IEL, internal elastic lamina.

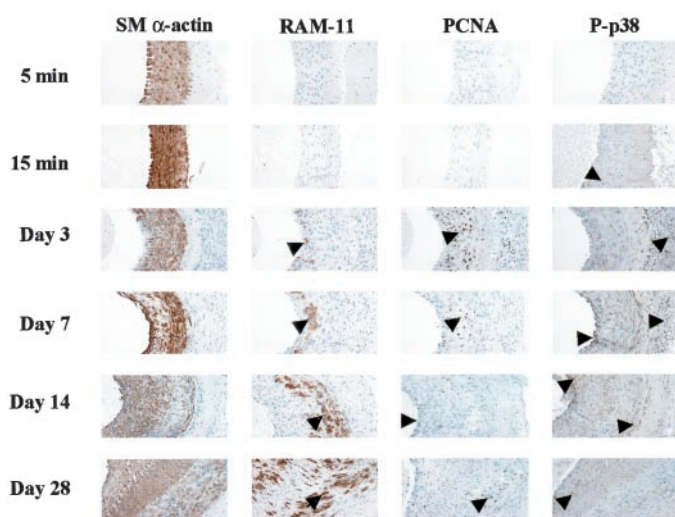


Fig. 4. Immunohistochemical staining showing a temporal localization of smooth muscle α -actin, macrophage RAM-11, PCNA, and phospho-p38 in iliofemoral artery from 5 min to 28 days after balloon injury in rabbits fed with high fat/cholesterol diet. The specific staining for each marker is indicated by arrowheads.

was 470 ± 84 ng/ml (Fig. 5). Plasma levels of SB 239063 did not differ significantly on study days 0, 7, 14, or 28.

Long-term treatment (30 days) with SB 239063 significantly inhibited the vascular lesion at 28 days following balloon injury of the iliofemoral artery in hypercholesterolemic rabbits (Fig. 6). A detailed analysis of the effects of SB 239063 on vascular remodeling indicate a significant reduction of wall thickness, neointima thickness, neointima/medium ratio, and wall/lumen ratio (Table 2). The lumen circumference (in millimeters) was increased from 3.4 ± 0.14 in vehicle-treated animals to 3.88 ± 0.16 in SB 239063-treated rabbits. The RAM-11 staining (normalized for wall size) was not significantly reduced in SB 239063-treated animals (0.4 ± 0.045) compared with vehicle group (0.48 ± 0.033).

Effect of p38 MAPK Inhibition on Rabbit VSMCs Migration, Proliferation, and Fibronectin Synthesis. In an effort to examine p38 MAPK-dependent mechanisms of altering

TABLE 1
Selectivity profile of SB 239063 on protein kinase inhibition

Kinase	SB 239063 IC ₅₀ (nM)
p38 α	34
p38 β	1,000
p38 γ and δ	>50,000
ERK2	>50,000
JNK1	>50,000
JNK2 β 2	5,000
cdc2	>50,000
PKC α	83,000
cRaf	>50,000
EGFR	83,000
p56 Lck	18,000

PKC α , protein kinase C α ; ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; JNK, c-Jun NH₂-terminal kinase.

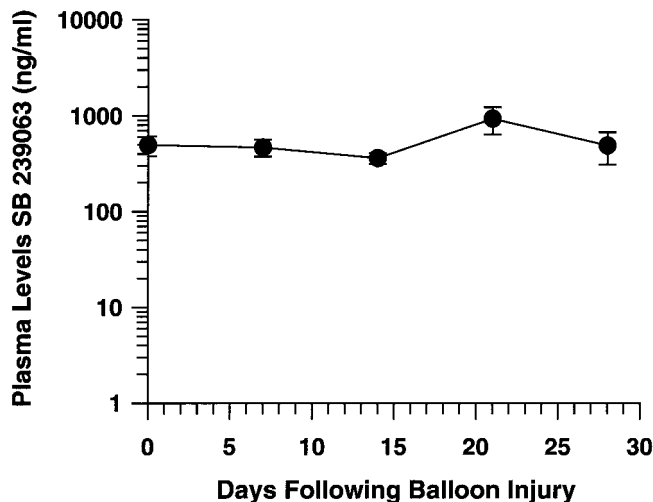


Fig. 5. Trough plasma concentration of SB 239063 after initiation of treatment until the end of the study. Data are mean \pm S.E.M. for 17 to 18 rabbits for each time point.

the vascular lesion, we examined the effects of SB 239063 on rabbit VSMCs migration, proliferation, and fibronectin synthesis. Based on our pilot proliferation and migration assays, an ED₅₀ concentration of PDGF was employed in the current study. SB 239063 (0.1–10 μ M) had no significant effect on PDGF-induced migration or proliferation of cultured VSMCs derived from the rabbit iliofemoral artery (Fig. 7, A and B). In addition, SB 239063 did not have any significant effect on VSMCs proliferation and migration induced by 1 and 3% FBS (data not shown). The concentration of SB 239063 that we used in the current experiment has been shown to significantly inhibit cytokine production induced by lipopolysaccharide in cellular assays (Underwood et al., 2000).

In contrast, SB 239063 produced a concentration-dependent inhibition of rabbit VSMCs cellular fibronectin production induced by TGF- β stimulation (Fig. 8). The IC₅₀ of SB239063 was in the range of 1 to 3 μ M, and the maximum efficacy of SB 239063 produced 40 to 50% inhibition of the TGF- β -induced fibronectin production at 3 μ M.

Discussion

Despite advances in interventional coronary techniques, late luminal loss continues to limit the efficacy of balloon angioplasty/stent procedures. In the present study, we have evalu-

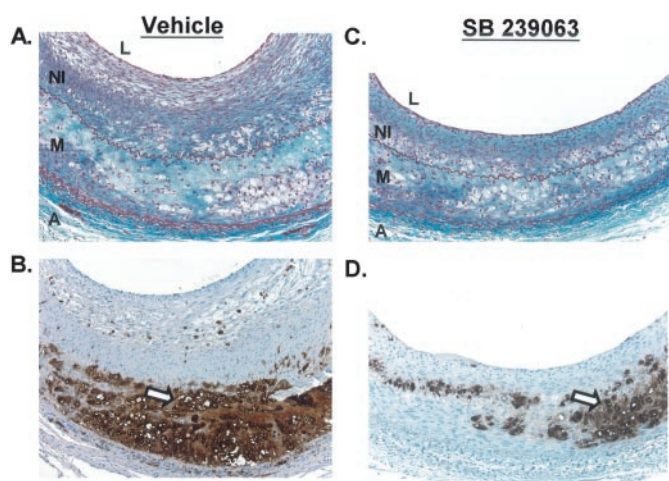


Fig. 6. Representative lesions in iliofemoral arteries obtained at 28 days from vehicle-treated (A and B) and SB 239063-treated (C and D) hypercholesterolemic rabbits. Sections in A and C were stained with Mason's trichrome. RAM-11 immunohistochemical staining demonstrate macrophage (brown) in sections B and D as indicated by arrows. L, lumen; NI, neointima; M, medium; A, adventitia.

TABLE 2

Morphometric analysis of iliofemoral artery lesions obtained 28 days following balloon injury in hypercholesterolemic rabbits
All values are the mean \pm S.E.M. The vehicle was 5% 2-hydroxypropyl- β -cyclodextrin.

Morphometric Parameter	Vehicle (n = 16)	SB 239063 (n = 18)
	5% β -cyclodextrin	50 mg/kg/day, p.o.
EEL circumference (mm)	5.70 \pm 0.10	5.67 \pm 0.6
Lumen circumference (mm)	3.40 \pm 0.14	3.88 \pm 0.16*
Avg. wall thickness (mm)	0.37 \pm 0.02	0.30 \pm 0.02*
Avg. NI thickness (mm)	0.23 \pm 0.20	0.16 \pm 0.01**
NI area (mm ²)	0.89 \pm 0.08	0.63 \pm 0.06*
Media area (mm ²)	0.75 \pm 0.03	0.73 \pm 0.06
NI/medium	1.23 \pm 0.11	0.92 \pm 0.08*
Wall area (mm ²)	1.63 \pm 0.10	1.36 \pm 0.12*
Wall/lumen	2.57 \pm 0.51	1.38 \pm 0.14*

EEL, external elastic lamina; NI, neointima.
* $p < 0.05$; ** $p < 0.01$.

ated the role of p38 MAPK in the vascular response to balloon injury. Evidence suggests that p38 MAPK is involved in a variety of cellular responses including cytokine production, proliferation, and migration. Activation of p38 MAPK (phosphorylation), mediated by "upstream" signaling kinases MKK6 or 3, participates in signal transduction initiated by a variety of cytokines, growth factors, and G-protein-coupled receptors, as well as chemical and mechanical cellular stress (Widmann et al., 1999). For example, growth factors including PDGF, TGF- β 1, basic fibroblast growth factor, and inflammatory cytokines, such as TNF- α and IL-1, all activate p38 MAPK and are also increased in balloon-injured blood vessels (Ferns and Avades, 2000; Chamberlain et al., 2001; Chin et al., 2001). In addition, p38 MAPK mediates the production of a variety of cytokines via transcriptional and post-transcriptional mechanisms. Thus, p38 MAPK appears to play a critical role in the production and transduction of cytokines that are believed to coordinate the vascular response to injury.

Using immunoblotting and immunohistochemical techniques, we have identified a sustained activation of p38 MAPK (phosphorylation) in balloon-injured blood vessels at

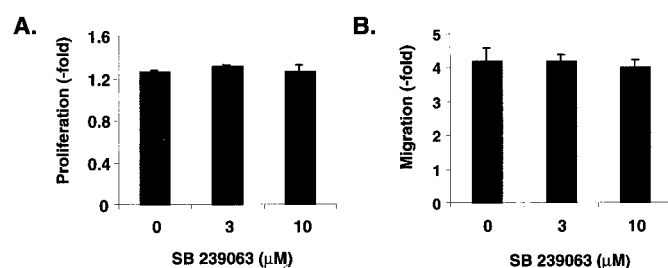


Fig. 7. A, effect of SB 239063 on VSMCs proliferation induced by PDGF (10 ng/ml). After being serum-starved for 48 h in serum-free DMEM, the quiescent cells were stimulated with PDGF in the absence or presence of a specific p38 inhibitor SB 239063 at 3 or 10 μ M concentrations for 48 h. Results are fold increase over basal level (unstimulated) and are expressed as mean \pm S.E.M. (n = 12). B, effect of SB 239063 on VSMCs migration induced by PDGF (60 ng/ml). SB 239063 or vehicle was added into the upper and lower chambers to reach a final concentration of 3 and 10 μ M. Results are fold increase over basal level (unstimulated) and are expressed as mean \pm S.E.M. (n = 12–18).

all time points examined (up to 28 days). In addition, total p38 MAPK was also up-regulated at later time points after balloon injury. The increase in phospho-p38 MAPK and total p38 MAPK is perhaps not surprising given the attendant changes in cellularity and cellular phenotype observed in the progressing neointima. Thus, the up-regulation of phospho-p38 MAPK in the early phase of post-balloon injury is mainly due to the increase in phosphorylation of p38 MAPK. The combination of up-regulation of p38 protein and increased phosphorylation of p38 is the cause of overall up-regulation of phospho-p38 in the later phase.

The regional localization of phospho-p38 MAPK in the injured vessel wall did not parallel cellular proliferation, macrophage invasion, or foam cell accumulation. In addition, phosphorylated p38 MAPK was not observed in fusiform VSMCs (differentiated/contractile phenotype). Rather, phosphorylated p38 MAPK was localized to the neointima and medium in regions of rounded synthetic VSMCs (Thyberg, 1998). The dense nuclear staining observed was consistent with nuclear translocation of phospho-p38 MAPK.

Long-term treatment (4 weeks) with an orally active and selective p38 MAPK inhibitor (SB 239063) reduced the complex vascular lesion induced by balloon injury in the hypercholesterolemic rabbit. SB 239063 treatment preserved the lumen area neointimal/medium ratio in the injured iliofemoral arteries. Our results are consistent with a very recent report showing that another p38 MAPK inhibitor, FR167653, reduced neointima formation induced by balloon injury in rat carotid arteries (Ohashi et al., 2000). These data strongly suggested that activation of p38 MAPK plays an important role in the vascular response to balloon injury.

The mechanisms by which p38 MAPK inhibitors attenuate neointimal hyperplasia appear to be complex. For example, PDGF is increased at the site of vascular injury, and it is believed to play an important role in neointimal development by mediating cellular proliferation and migration. However, selective inhibition of p38 MAPK did not inhibit PDGF-induced VSMCs proliferation and migration in vitro. Recent studies performed in the rat suggest that inhibitors of p38 MAPK may act to reduce neointimal hyperplasia by inhibiting interleukin-1 β production (Ohashi et al., 2000). In the present study, p38 MAPK inhibition also produced a concentration-related reduction in fibronectin production induced by TGF- β in VSMCs. Similar effects on fibronectin gene

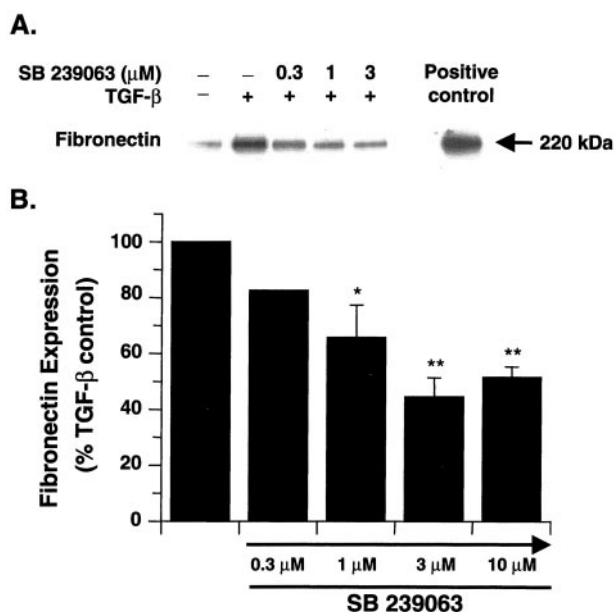


Fig. 8. The effect of the p38 MAPK inhibitor, SB 239063, on TGF- β -induced fibronectin expression in rabbit iliofemoral VSMCs. **A**, representative Western blot showing a specific band detected with antibody against fibronectin. The positive control was prepared from cultured human foreskin fibroblasts. The effects of SB 239063 on fibronectin synthesis are summarized in **B** (*, $P < 0.05$; **, $P < 0.01$ versus control; $n = 4-5$ per lane). The data demonstrated that TGF- β (10 ng/ml) stimulates cellular fibronectin synthesis and SB 239063 inhibits fibronectin in a concentration-dependent manner.

expression have been observed in A498 renal epithelial cells where p38 MAPK inhibitors selectively reduced TGF- β induction of fibronectin expression, but not collagen expression (N. J. Laping, personal communication). The decrease in fibronectin expression correlated well with inhibition of p38 MAPK activity as evidenced by inhibition of ATF-2 phosphorylation in the A498 cells (N. J. Laping, personal communication). Thus, the p38 MAPK signaling pathway appears to play a critical role in TGF- β stimulated fibronectin expression as well as cytokine production.

Fibronectin plays an important role in development and wound healing by mediating a variety of cellular processes, i.e., differentiation, migration, and proliferation (Potts and Campbell, 1996). Following balloon injury in blood vessels, an early and sustained fibronectin gene and protein expression are observed (Kim et al., 1995; Thyberg et al., 1997). A similar expression profile has been observed for the fibronectin integrin receptor, $\alpha_5\beta_1$, and the fibronectin stimulant, TGF- β , and all are localized to the developing neointima (Kim et al., 1995; Pickering et al., 2000). Fibronectin, like phospho-p38 MAPK, is associated with synthetic VSMCs in the neointima (Thyberg, 1998), where it is believed to regulate the VSMCs phenotype (Hedin and Thyberg, 1987). Fibronectin interactions with $\alpha_5\beta_1$ -mediated migration, via focal adhesion kinase and proliferation by a Ras-MAPK-mediated disinhibition of retinoblastoma protein (Danen et al., 2000; Sieg et al., 2000; Davenpeck et al., 2001). Thus, the inhibition of fibronectin production by p38 MAPK inhibitors would be expected to indirectly limit VSMCs differentiation, proliferation, and migration associated with vascular injury.

In conclusion, we have demonstrated that the sustained activation of the p38 MAPK pathway plays an important role

in neointima formation associated with vascular injury, perhaps by mediating TGF- β -induced fibronectin production. The present study also suggests that p38 MAPK inhibitors represent a novel therapeutic approach to the treatment of restenosis associated with coronary angioplasty.

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Address correspondence to: Dr. Robert N. Willette, Department of Cardiovascular Pharmacology, UW2510, GlaxoSmithKline, 709 Swedeland Road, Box 1539, King of Prussia, PA 19406-0939. E-mail: robert_n_willette@gsk.com