Role of p38 Mitogen-Activated Protein Kinase in Neointimal Hyperplasia After Vascular Injury

Naohiro Ohashi, Akira Matsumori, Yutaka Furukawa, Koh Ono, Masaharu Okada, Atsushi Iwasaki, Tadashi Miyamoto, Atsushi Nakano, Shigetake Sasayama

Abstract—p38 mitogen-activated protein kinase (MAPK) is involved in intracellular signals that regulate a variety of cellular responses during inflammation. However, the role of p38 MAPK in atherosclerosis, a chronic inflammatory disorder, remains uncertain. The aim of the present study was to examine the role of p38 MAPK in the development of neointimal hyperplasia in balloon-injured rat carotid arteries. Immunohistochemical studies indicated that p38 MAPK was rapidly activated in the majority of medial cells in injured arterial walls. Rats treated with FR167653, a selective inhibitor of p38 MAPK, at a dosage of 10 mg·kg⁻¹·d⁻¹, had a 29.4% lower intima-to-media ratio than the untreated controls at 14 days after balloon injury (P<0.05). The percentage of proliferating nuclear antigen-positive cells in the media at 48 hours was significantly lower in the FR167653-treated group than in the control group. Quantitative competitive reverse transcription–polymerase chain reaction analysis revealed that interleukin-1β mRNA expression in arteries was significantly inhibited by FR167653 (to 18.1% of control, P<0.05) at 8 hours after balloon injury. Moreover, p38 MAPK activation and interleukin-1β production by lipopolysaccharide-stimulated vascular smooth muscle cells were inhibited by FR167653 in a concentration-dependent manner in vitro. These results indicate that p38 MAPK is activated in vascular walls after injury and promotes neointimal formation and suggest that selective inhibition of p38 MAPK may be effective in the prevention of restenosis after percutaneous transluminal coronary angioplasty.

Key Words: p38 mitogen-activated protein kinase vascular smooth muscle cells interleukin-1β angioplasty coronary restenosis

Balloon angioplasty is widely used to treat atherosclerotic coronary artery disease. However, restenosis after angioplasty continues to limit the long-term success of the procedure. This accelerated form of atherosclerosis results from inflammatory and proliferative responses of the vascular wall to mechanical injury and is in part attributable to neointimal hyperplasia. Vascular cells, such as vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), and infiltrating inflammatory cells, including monocytes/macrophages and T-lymphocytes, play important roles in neointimal hyperplasia.

p38 mitogen-activated protein kinase (MAPK) is a new member of the MAPK superfamily that is involved in intracellular signaling pathways in various cell types. p38 MAPK regulates cellular responses to a variety of cellular stresses, such as heat shock, hyperosmolarity, ultraviolet radiation, the endotoxin lipopolysaccharide (LPS), and the proinflammatory cytokines, including interleukin (IL)-1β and tumor necrosis factor (TNF)-α.

The activation of p38 MAPK leads to the activation of monocytes/macrophages, T-lymphocytes, and VSMCs and the production of proinflammatory cytokines, such as IL-1β and TNF-α, by these cells. In vitro observations suggest an important role of p38 MAPK in the pathogenesis of atherosclerosis, although its role in vivo remains uncertain.

We hypothesized that mechanical vascular injury induces the direct activation of p38 MAPK within the vascular wall and eventually leads to the development of neointimal hyperplasia. The results of the present study show that p38 MAPK is rapidly activated in the injured walls of rat carotid arteries and that its inhibition reduces IL-1β gene expression, proliferative response of medial VSMCs, and neointimal hyperplasia.

Methods

Biochemicals
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) was synthesized at Fujisawa Pharmaceutical Co, Ltd. LPS from Escherichia coli 055:B5 was purchased from Difco Laboratories. Phospho-p38 MAPK antibody (Ab), immobilized phospho-p38 MAPK monoclonal Ab (MAB), ATF-2 fusion proteins, and phospho-ATF-2 Ab were obtained from New England BioLabs. Enhanced chemiluminescence (ECL) Western blotting detection system were obtained from Amersham Pharmacia Biotech. Goat anti-p38 MAPK Ab and donkey anti-goat IgG horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology.

Received February 15, 2000; revision accepted August 10, 2000.
From the Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan.
Correspondence to Akira Matsumori, MD, PhD, Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8397, Japan. E-mail amat@kuhp.kyoto-u.ac.jp
© 2000 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://www.atvbaha.org

2521
Balloon Injury of Rat Carotid Arteries

Eleven- to 12-week-old male Sprague-Dawley rats were anesthetized, and the endothelium of the left common carotid artery was denuded with a 2F Fogarty embolectomy catheter (Baxter Healthcare), as previously described.10 In the in vivo experiments with FR167653, actively treated rats received the drug orally, dissolved in drinking water, and control rats received drinking water only. Both regimens were administered every 12 hours, starting 3 days before carotid arterial injury and continuing until death. No apparent toxic effects were observed in rats treated with FR167653 at doses of 3 or 10 mg · kg⁻¹ · d⁻¹. All FR167653-treated animals remained healthy and had an appropriate increase in body weight throughout the period of the in vivo experiments.

Immunohistochemical Staining

Immunohistochemical staining for phosphorylated p38 MAPK was performed to examine the activation of p38 MAPK in injured arterial walls. Ten minutes after balloon injury, the nontreated rats were anesthetized, perfused with saline that had been precooled at 4°C, and perfusion-fixed with 10% neutral buffered formalin at 90 mm Hg. The carotid arteries were gently removed, fixed further, and embedded in paraffin. The tissue was sliced into 4-μm-thick slices and processed for immunohistochemical analysis with the use of avidin-biotin–alkaline phosphatase complex (Vector Laboratories) system with biotinylated goat anti-rabbit IgG (DAKO) as secondary Ab at a dilution of 1:300. Alkaline phosphatase was developed with the use of Vector Red (Vector Laboratories). Rabbit anti-phospho-p38 MAPK polyclonal Ab diluted 1:50 was used as the primary Ab. The sections were counterstained with methyl green. For negative controls, normal rabbit IgG (DAKO) was used instead of primary Ab to stain the injured arteries.

To assess the effects of FR167653 (10 mg · kg⁻¹ · d⁻¹) on the initial medial proliferation of VSMCs, immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) was performed as previously described.10 The carotid arteries were removed 48 hours after injury, fixed with 10% neutral buffered formalin, and embedded in paraffin. The embedded arteries were cut into six 4-μm-thick sections from proximal, middle, and distal segments perpendicular to the short axis and deparaffinized. Immunohistochemistry was performed according to an avidin biotin–horseradish peroxidase complex method (Vector Laboratories) with biotinylated rabbit anti-mouse IgG diluted 1:500 (DAKO). Peroxidase was visualized with 3,3′-diaminobenzidine. Mouse MAb against PCNA (PC10, YLEM, MA) was used as the primary Ab at 1:50 dilution. The sections were counterstained with hematoxylin. In control animals, the primary Ab was omitted. Cell number was counted at a ×400.

Light Microscopic Examination and Morphometry of Neointima

In these experiments, FR167653 was administered at dosages of 3 or 10 mg · kg⁻¹ · d⁻¹. On day 14 after arterial denudation, the rats were anesthetized before the injection of 200 μL of 2% Evans’ blue dye in PBS and heparin (100 U/rat) into the tail vein. The animals were perfusion-fixed with 10% neutral buffered formalin as previously described.10 The carotid arteries were removed and fixed further. The central portions of the blue-stained areas were divided into five 1-mm-thick sections embedded in paraffin. The sections were stained with elastic-aluminovan Gieson’s stain. The intimal and medial areas were measured with an image analyzer (LUZEX3U; Nikon). In each animal, the mean intimal and medial areas of each artery were calculated from these 5 sections.

RNA Preparation and cDNA Synthesis

Rats assigned to the treatment group received 10 mg · kg⁻¹ · d⁻¹ FR167653. The carotid arteries were harvested at 1, 2, 4, 8, 24, 72, or 120 hours after balloon injury (n = 5 at 8 hours and n = 3 at all other time points). Intact left common carotid arteries of Sprague-Dawley rats (n = 3 at 0 hour) were used as controls. Total RNA was prepared from the arteries according acid to the guanidinium thiocyanate-phenol-chloroform-isooamylalcohol isolation method, and 0.3 μg of total RNA template was subjected to first-strand cDNA synthesis with dNTP (Takara) and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), under the conditions recommended by the manufacturer. The synthesized cDNA was stored at −20°C.

Quantitative Competitive PCR

To quantitatively estimate IL-β mRNA expression, competitive PCR was performed as previously described.10 A constant amount of cDNA was amplified by PCR with serially diluted nonhomologous DNA fragments that contained primer template sequences as an internal control, according to the manufacturer’s instructions (PCR MIMIC Construction Kit; Clontech Laboratories Inc).

To determine the exact amount of the target mRNA species, the internal control was diluted 2-fold. A sense primer (A) and an antisense primer (B) were synthesized for each with use of the published cDNA sequences for rat IL-β1 and rat GAPDH as follows: IL-1β(A), 5′-AGTGGCAGCTTCTGAGACACT-3′; IL-1β(B), 5′-CAGGACAGGTATAATATTACTCAAACCTTT-3′; GAPDH (A), 5′-TGAAAGTCTGGTGAAGCAGATTG-3′; and GAPDH (B), 5′-CATGTAGCCATGAGTTCCACAC-3′. Each PCR contained 100 μmol/L dNTP, 0.5 μmol/L concentration of each specific primer, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% gelatin, and 0.25 U Taq polymerase (Perkin–Elmer Cetus) in a volume of 25 μL. [α-32P]dCTP was included in the reaction to quantify the PCR products. Both IL-1β and GAPDH cDNA were analyzed with 40 and 32 cycles of amplification in a thermal cycler (Perkin–Elmer Cetus). The cycle for IL-1β cDNA consisted of denaturation at 94°C for 40 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 90 seconds. The cycle for GAPDH cDNA consisted of denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds, and extension at 72°C for 90 seconds. A portion of each PCR product was electrophoresed on a 5% polyacrylamide gel, and the densitometric values of32 P-labeled target and internal control were analyzed with the FUJIX bioimaging analyzer (BAS 2000, Fuji Photo Film Co, Ltd). The molar ratio of target to internal control was calculated as target/internal control = (VT/VC)×(CC/CT), where VT and VC represent the densitometric value of the PCR product from target and internal control, respectively, and CC and CT represent the dCTP content in the PCR product from internal control and target. The amount of target gene was determined as that of the internal control at the point of an equal molar ratio between target and internal control. The amounts of IL-1β were divided by the amounts of GAPDH to correct for the efficiency of cDNA synthesis.

Cell Culture

VSMCs were isolated according to a previously described explant method,13 from thoracic aortas of 8- to 10-week-old male Sprague-Dawley rats. Cells were grown in DMEM (Nissui) supplemented with 10% FCS, 100 μg/mL streptomycin, and 100 U/mL penicillin (GIBCO BRL) in a humidified atmosphere (5% CO₂/95% air) at 37°C. Cells were used between passages 4 and 10 for all experiments.

Immunoprecipitation and p38 MAPK Activity Assay

VSMCs cultured at 80% to 90% confluence in 100-mm dishes were made quiescent through incubation with DMEM supplemented with 0.1% FCS for 24 hours. Cells were treated in the absence or presence of FR167653 for 2 hours and then stimulated with LPS (1 μg/mL) at 37°C. After stimulation, the cells were washed twice with cold PBS on ice, scraped, and extracted in 500 μL of cold lysis buffer that contained 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. The extracts were sonicated and centrifuged at 15 000 g for 10 minutes at 4°C, and the supernatants were immediately used for immunoprecipitation or stored at −80°C. p38 MAPK activity was measured with use of the p38 MAPK assay kit (New England Biolabs). Two hundred micrograms of cell lysates (400 μg protein) was incubated with 20 μL of immobilized phospho-p38 MAPK MAb overnight at 4°C. The beads were washed twice with 500 μL of lysis buffer and twice with 500 μL of kinase buffer containing 25 mmol/L Tris, pH 7.5, 5 mmol/L β-glycerophosphate,
2 mmol/L dithiothreitol, 0.1 mmol/L sodium orthovanadate, and 10 mmol/L MgCl₂ and then resuspended to 50 μL with kinase buffer. ATP (200 μmol/L) and ATF-2 (2 μg), as substrate, were added to the suspension. The kinase activity was assayed for 30 minutes at 30°C, terminated by the addition of 3× SDS sample buffer, and boiled for 5 minutes. The samples were resolved on 12% SDS-PAGE, electroblotted onto PVDF membranes, and probed with phospho-ATF-2 Ab (1:1000). The immunoreactivity was detected with enhanced chemiluminescence and determined with densitometry (NIH Image 1.61).

**Immunoblotting**

VSMCs lysates were prepared as described earlier. The proteins (30 μg) were separated on 10% polyacrylamide gels with SDS-PAGE followed by Western blotting with goat anti-p38 MAPK Ab (1:1500). Immunocomplexes were visualized through ECL detection with HRP-conjugated anti-goat Ab used as secondary Ab (1:1000). The kinase activity was assayed for 30 minutes at 30°C, terminated by the addition of 3 mmol/L ATP (200 μmol/L) and ATF-2 (2 μg/mL). After 24 hours of incubation, the supernatants were harvested. IL-1β in the supernatants was determined with specific ELISA kits (Biosource International). All measurements were performed in duplicate.

**Statistical Analysis**

Values are expressed as mean±SEM. Multiple comparisons were performed by ANOVA. Paired data were analyzed by the 2-tailed unpaired Student’s t test. Values for relative IL-1β mRNA levels at 8 hours after injury were compared using the Mann-Whitney U test, because they were nonparametrically distributed. A P value of <0.05 was considered statistically significant.

**Results**

**Immunolocalization of Phosphorylated p38 MAPK in Injured Carotid Arteries**

No positive immunostaining was detectable in intact carotid arteries (please see Figure 1, published online at http://atvb.ahajournals.org). In contrast, the majority of medial cells were intensely stained for phosphorylated p38 MAPK in sections of the arteries obtained at 10 minutes after balloon injury (Figures 1A and I). A representative stained section from a control experiment is shown in Figure 1B.

**Effects of Inhibition of p38 MAPK on Neointimal Hyperplasia**

Significant neointimal hyperplasia was observed in all left common carotid arteries 14 days after injury (Figures 2A and 2B). The oral administration of FR167653 inhibited intimal thickening (Figures 2C and 2D). In the group treated with 10 mg · kg⁻¹ · d⁻¹, neointimal hyperplasia was significantly inhibited compared with the untreated group. The mean intima-to-media ratio was reduced to 0.697±0.057 versus 0.988±0.066 in the untreated group, representing a 29.4% inhibition (P<0.05, Figure 3).

**Effect of Inhibition of p38 MAPK on Initial Medial Proliferation After Mechanical Injury**

At 48 hours after injury, immunohistochemical staining with anti-PCNA Ab showed a significant decrease in the population of medial proliferating cells in the FR167653-treated group compared with the untreated group (Table). The percentage of PCNA-positive cells was 19.0±0.9 in the FR167653-treated group versus 25.4±1.2 in the untreated group (P<0.05).

**IL-1β Gene Expression in Injured Arteries**

IL-1β mRNA in the balloon-injured arteries was measured with quantitative competitive RT-PCR. Coamplification of sample cDNA with serially diluted mimic cDNA showed a close correlation between the amount of mimic cDNA and the target cDNA-to-mimic cDNA ratio for each reaction (please see Figure II, published online at http://atvb.ahajournals.org). The induction of IL-1β gene expression was demonstrable as early as 1 hour and peaked at 8 hours after injury in the injured arterial wall of the untreated rats. IL-1β gene expression level decreased at 24 hours, and although it was relatively low but upregulated, IL-1β gene expression was still present at 120 hours after injury (Figure 4).

**Effect of Inhibition of p38 MAPK on IL-1β Gene Expression in Injured Carotid Arteries**

IL-1β gene expression in injured carotid arteries peaked at 8 hours after injury (Figure 4). At 8 hours after injury, the relative gene expression of IL-1β in the FR167653 (10 mg · kg⁻¹ · d⁻¹) treatment group was reduced to 18.1% of that measured in the vehicle-treated, control group (P<0.05, Figure II).

**p38 MAPK Activity by LPS-Stimulated VSMCs**

LPS-stimulated VSMCs were used in vitro to evaluate the inhibitory effect of p38 MAPK activity by FR167653.
the time course of p38 MAPK activity after LPS stimulation at a concentration of 1 \( \mu \text{g/mL} \) was determined. LPS induced a rapid activation of p38 MAPK in VSMCs, reaching its peak at 15 minutes and returning to baseline at 120 minutes (please see Figure III, published online at http://atvb.ahajournals.org). At 15 minutes, the amount of p38 MAPK activation by LPS in VSMCs was 3.1\( \pm \)0.3-fold that measured in unstimulated controls. In addition, LPS-induced p38 MAPK activity was inhibited by FR167653 in a concentration-dependent manner, reaching a 63.3\% reduction at 0.1 \( \mu \text{mol/L} \) \( (P<0.01 \text{ versus stimulated control; Figures III and 5}) \). The total protein level of p38 MAPK in these p38 MAPK activity experiments was not significantly modified. Comparable results were reproduced in 3 separate experiments.

**IL-1\( \beta \) Production by LPS-Stimulated VSMCs**

LPS caused an increase in IL-1\( \beta \) production by VSMCs at a concentration of 1 \( \mu \text{g/mL} \) \( (103.4\pm0.45 \text{ versus } 18.1\pm0.1 \text{ pg/mL at baseline, } P<0.01) \). IL-1\( \beta \) production was inhibited by FR167653 in a concentration-dependent manner, reaching 75.9\% reduction at 1 \( \mu \text{mol/L} \) \( (P<0.01 \text{ versus stimulated control; Figure 6}) \). Three separate experiments were performed.

**Effect of FR167653 on the Initial Proliferative Medial Activity 48 h After Balloon Injury**

The effects of FR167653 on the initial proliferative medial activity 48 h after balloon injury of the left common carotid artery were measured with morphometry, and the mean intima-to-media ratio was calculated as described in the text. *\( P<0.05 \) vs the group treated with the vehicle only (ANOVA). In addition, LPS-induced p38 MAPK activity was inhibited by FR167653 in a concentration-dependent manner, reaching a 63.3\% reduction at 0.1 \( \mu \text{mol/L} \) \( (P<0.01 \text{ versus stimulated control; Figures III and 5}) \). The total protein level of p38 MAPK in these p38 MAPK activity experiments was not significantly modified. Comparable results were reproduced in 3 separate experiments.
The activation of p38 MAPK is involved in intracellular signaling pathways that lead to a variety of cellular responses, including inflammatory cytokine production, cell growth, cell differentiation, and cell death. Previous in vitro studies have shown that the activation of p38 MAPK is induced in VSMCs and ECs by proatherogenic stimuli such as mechanical stress, TNF-α or IL-1β, or oxidative stress.7,14,15

In the present study, FR167653, a selective inhibitor of p38 MAPK, was used to examine the effects of p38 MAPK inhibition in vivo on the acute (ie, cytokine gene expression) and chronic (ie, neointimal hyperplasia) responses to mechanical injury in the arterial wall. FR167653 possesses the pyridine and fluorophenyl rings that are essential for binding to p38 MAPK. Recent crystallographic studies have revealed that inhibitors of p38 MAPK compete with ATP for binding to p38 MAPK via these structures and produces a selective, potent inhibition of p38 MAPK activity.16 p38 MAPK has been prominently implicated in the development of atherosclerosis.17 It regulates the production of several cytokines or chemokines, such as IL-1β, IL-6, TNF-α, and MCP-1, that play important roles in atherogenesis.8,18,19 Among them, we focused on IL-1β to examine the role of p38 MAPK in acute inflammatory responses.

The results of the in vivo experiments revealed that p38 MAPK inhibition significantly reduces neointimal hyperplasia in injured rat carotid arteries. This may be in part due to an inhibition of the initial medial VSMC proliferation, although inhibition of initial medial cell proliferation through another method was unsuccessful in limiting neointimal hyperplasia.20 Injured arterial walls contain a variety of mitogens for VSMCs,20,21 with the main one being PDGF-BB.21 In vitro, however, FR167653 did not inhibit the mitogenic response of VSMCs to PDGF-BB. Besides the inhibitory effect of p38 MAPK on the mitogenic response of VSMCs, a decrease in IL-1β gene expression may also be associated with reduced neointimal hyperplasia. In pigs, mechanical injury induces an abundant expression of IL-1β mRNA throughout the coronary arterial walls.22 A particularly prominent expression of IL-1β gene was found in inflammatory cells accumulated in the adventitia.22 Thus, in injured arterial walls exposed to endothelial denudation, both adventitial inflammatory cells and medial smooth muscle cells may be major sources of IL-1β.

The precise role of IL-1β in the development of neointimal hyperplasia is uncertain; other investigators, however, have reported that both IL-1α and IL-1β genes are expressed in human atherosclerotic lesions23 and in LPS-stimulated rabbit vascular tissue.24 Furthermore, chronic treatment with exogenous IL-1β induced coronary intimal hyperplasia in a porcine model.25 These observations suggest that IL-1β plays an important role in chronic inflammation of the arterial wall and promotes neointimal hyperplasia in rat balloon-injured carotid arteries.

In our model, the activation of p38 MAPK was required for the induction of IL-1β mRNA in injured arteries, because pharmacological inhibition of p38 MAPK markedly reduced IL-1β gene expression at its peak. The inhibitory effect of p38 MAPK inhibition on IL-1β production by VSMCs was...
also observed in the in vitro experiments, and the decrease of IL-1β production correlated with a decrease in p38 MAPK activity. This also suggests an important role of the intracellular signaling pathway that involves p38 MAPK in IL-1β gene expression in VSMCs. Other investigators recently reported that p38 MAPK regulates the production of IL-1β at the transcriptional and translational levels in monocytes.\(^1,2,6,26\) In the transcriptional regulation, p38 MAPK phosphorylates and activates transcriptional factors, such as ATF-2, CHOP, and MEF2C, thereby inducing cytokine gene expression.\(^1,3\) p38 MAPK may play a similar regulatory role in IL-1β gene expression in VSMCs.

Previous studies have suggested the existence of a positive feedback loop between p38 MAPK and IL-1β.\(^3,8\) Accordingly, interruption of this positive feedback loop is one of the possible mechanisms responsible for the inhibitory effects of p38 MAPK inhibition on IL-1β gene expression, as well as for neointimal hyperplasia. Thus, in the present study, multiple effects were identified of p38 MAPK inhibition on the acute responses of arterial walls to mechanical injury. Redundant and complicated use of intracellular signaling pathways by numerous cytokines and growth factors may prevent treatments that target these growth factors from being successful in the prevention of restenosis. Instead, targeting downstream intracellular signaling molecules such as p38 MAPK may be a more appropriate strategy in the development of new treatments aimed at the prevention of restenosis after angioplasty, because several signals from different receptors share similar intracellular signaling pathways, whereas a single intracellular signaling molecule is implicated in multiple cellular responses.\(^2,7,28\)

In conclusion, we found that the activation of p38 MAPK is induced by mechanical injury of arterial walls and promotes neointimal formation. Further studies are needed to clarify the precise mechanism by which p38 MAPK promotes neointimal hyperplasia and to test the effects of a selective inhibition of p38 MAPK in the prevention of restenosis after balloon angioplasty.

Acknowledgments

This work was supported by a research grant from the Ministry of Health and Welfare of Japan and a grant-in-aid for general scientific research from the Ministry of Education, Science, and Culture of Japan. We thank Fujisawa Pharmaceutical Co., Ltd, for generously providing FR167653 needed to perform the experiments. We are grateful to Shoko Sakai for preparation of the manuscript.

References

Role of p38 Mitogen-Activated Protein Kinase in Neointimal Hyperplasia After Vascular Injury
Naohiro Ohashi, Akira Matsumori, Yutaka Furukawa, Koh Ono, Masaharu Okada, Atsushi Iwasaki, Tadashi Miyamoto, Atsushi Nakano and Shigetake Sasayama

Arterioscler Thromb Vasc Biol. 2000;20:2521-2526
doi: 10.1161/01.ATV.20.12.2521
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/12/2521

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2000/12/14/20.12.2521.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Figure Legends of the Online Publication

Figure I:
Immunohistochemical analysis of phosphorylated p38 MAPK in intact and balloon-injured rat carotid arteries. **A.** Intact right common carotid artery. **B.** Left common carotid arteries at 10 min after injury. Most of the medial cells (arrows) are stained for phosphorylated p38 MAPK. Bar=20 μm.

Figure II:
Quantitative competitive RT-PCR analysis of IL-1β mRNA expression in the rat carotid arterial wall. **A.** Representative quantitative RT-PCR analysis of IL-1β gene expression using internal controls. See text for detailed description of the method. **B.** IL-1β gene expression in carotid arteries 8 h after injury treated with the vehicle versus FR167653, 10 mg/kg/day. Values are expressed as mean±SEM of 5 animals. *P<0.05 versus the group treated with the vehicle only (Mann-Whitney U test).

Figure III:
Effect of FR167653 on p38 MAPK activity by LPS-stimulated VSMCs. Growth-arrested VSMCs stimulated with LPS, 1 μg/mL, were harvested. p38 MAPK activity was then measured by an immune complex kinase assay, using ATF-2 as substrate. **A.** Time course of p38 MAPK activity and expression of p38 MAPK. Cells were stimulated for the indicated times. The experiment was repeated 3 times with similar results. **B.** Inhibitory effect of FR167653 on p38 MAPK activity and expression of p38 MAPK. Cells were stimulated for 15 min after pretreatment with FR167653 (0-0.1 M). Cont. indicates unstimulated control.