Vascular Neuronal NO Synthase Is Selectively Upregulated by Platelet-Derived Growth Factor
Involvement of the MEK/ERK Pathway

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Objective—We demonstrated recently that neuronal NO synthase (NOS) is expressed in arteriosclerotic lesions and exerts important vasculoprotective effects in vivo. In this study, we examined the molecular mechanism(s) for vascular neuronal NOS (nNOS) expression.

Methods and Results—In cultured rat aortic smooth muscle cells, treatment with platelet-derived growth factor (PDGF) selectively upregulated nNOS expression but not inducible NOS (iNOS) or endothelial NOS (eNOS) expression. Treatment with PDGF also significantly caused activation of mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), p38MAPK, and c-Jun N-terminal kinase (JNK). ERK kinase (MAPK kinase [MEK]) inhibitors inhibited PDGF-induced nNOS expression, whereas a p38MAPK inhibitor or JNK inhibitor was without effects. Importantly, gene transfer of MEK per se elicited nNOS induction, and gene transfer of dominant-negative MEK abolished PDGF-induced nNOS expression. In isolated aortas of wild-type, eNOS−/−, and iNOS−/− mice, but not in those of nNOS−/− mice, treatment with PDGF significantly enhanced nNOS expression and nitrite plus nitrate production, both of which were again attenuated by a MEK inhibitor.

Conclusions—These results provide the first evidence that vascular nNOS expression is upregulated selectively in response to PDGF through the MEK/ERK pathway. Upregulated nNOS may play an important compensatory role under arteriosclerotic/inflammatory conditions associated with eNOS dysfunction to maintain vascular homeostasis. (Arterioscler Thromb Vasc Biol. 2005;25:2502-2508.)

Key Words: ERK ■ mitogen-activated protein kinase ■ neuronal nitric oxide synthase ■ nitric oxide ■ platelet-derived growth factor

N O has multiple important actions that contribute to the maintenance of vascular homeostasis.1–4 NO is synthesized by 3 different isoforms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). eNOS and nNOS are constitutively expressed mainly in endothelial cells and nitrergic nerves, respectively, synthesizing a small amount of NO in a calcium-dependent manner either under basal conditions or on stimulation.1–4 In contrast, iNOS is expressed by inflammatory stimuli such as microbial endotoxins and certain proinflammatory cytokines, producing a large amount of NO in a calcium-independent manner.1–4

Although the roles of eNOS5–8 and iNOS8–11 in the development of arteriosclerosis have been investigated extensively, little is known about the role of nNOS. We demonstrated recently that nNOS also exerts important vasculoprotective effects in vivo.12,13 In a carotid artery ligation model, nNOS−/− mice exhibited accelerated neointimal formation and constrictive vascular remodeling caused by blood flow disruption.12,13 In a rat balloon injury model, selective inhibition of nNOS activity potently enhanced vasoconstrictor responses to calcium-mobilizing stimuli, and exacerbated neointimal formation.12,13 In these models, nNOS was upregulated in vascular lesions and was expressed predominantly in the neointimal and medial smooth muscle cells (SMCs).12,13 These findings provide a new concept that nNOS can be regarded as an “inducible” enzyme in the vascular system, in contrast to the nervous system. However, the regulatory mechanism(s) for vascular nNOS expression remains to be fully elucidated.

Platelet-derived growth factor (PDGF) is synthesized not only in platelets but also in almost all vascular wall cells that

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constitute atherosclerotic lesions, including endothelial cells, vascular SMCs, fibroblasts, and macrophages. PDGF promotes vascular SMC proliferation and migration and plays a central role in the pathogenesis of atherosclerosis. Proliferation in most cell types is driven by activation of the mitogen-activated protein kinases (MAPKs), which mediates cellular responses to diverse extracellular stimuli. Thus, in this study, we tested our hypothesis that vascular nNOS expression is upregulated in response to PDGF via the MAPK pathway.

Methods
This study was reviewed and approved by the ethics committee of animal care and experimentation of the University of Occupational and Environmental Health, Japan.

Materials
PDGF-BB was purchased from Genzyme. PD98059 was from Calbiochem. U0126 was from Promega. SB203580 and SP600125 were from Biomol Research Laboratories. Hemagglutinin-tagged MAPK kinase-1 (MEK1) plasmids were a generous gift from Dr. Natalie G. Ahn (Department of Chemistry and Biochemistry, University of Colorado, Boulder). Angiotensin II, A23187, mouse monoclonal antibody against vinculin, and tetramethylrhodamine isothiocyanate (TRITC)–conjugated goat anti-mouse IgG were from Sigma, and interleukin-1 isoforms (IL-1β) from Peprotec. Rabbit polyclonal antibodies against nNOS, iNOS, and eNOS were from BD Biosciences. Mouse monoclonal antibodies against phospho–extracellular signal-regulated kinase 1/2 (ERK1/2), phospho–c-Jun N-terminal kinase (JNK), and rabbit polyclonal antibodies against ERK1/2 and phospho-p38MAPK were from Cell Signaling Technology. A mouse monoclonal antibody against hemagglutinin was from Roche Diagnostics. Fluorescein isothiocyanate (FITC)–conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch.

Cultured Rat Aortic SMCs
Vascular SMCs were isolated from the thoracic aortas of 8-week-old male Sprague-Dawley rats by enzymatic dissociation. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (GIBCO/BRL) at 37°C in a CO2 incubator. When phosphorylation of MAPKs was examined, the concentration of FBS in DMEM was reduced to 0.5%. Cultured rat aortic SMCs were harvested with 100 μL of Hanks’ balanced salt solution (HBSS) containing 0.1% Triton X-100, 0.1 mg/mL aprotinin at 4°C. The mouse aortas were homogenized at 4°C in 500 μL of buffer containing 1 μL of 0.1 M leupeptin, 0.7 μM aprotinin, 120 μM PMSF, 0.7 μM pepstatin, 1 mM 1-iodoacetamide, and 1 mM diisopropylfluorophosphate. Western blot analysis was performed as reported previously.

Plasmid Transfection
A total of 4 μg of each hemagglutinin-tagged MEK1 plasmid was transfected into cultured rat aortic SMCs using the cationic liposome-mediated transfection method (Lipofectamine 2000; Invitrogen Corp.). The effects of gene transfer of wild-type MEK were studied 24 hours after plasmid transfection. In an experiment with dominant-negative type MEK, PDGFs were added in culture medium after 6 hours of plasmid transfection and incubated for 24 hours.

Immunofluorescence
Cultured rat aortic SMCs were plated on gelatin-coated chamber slides and were incubated with 50 ng/mL PDGF at 37°C for 1 day in a CO2 incubator. The cells were fixed with 4% paraformaldehyde and were reacted with an nNOS antibody for 1 hour at room temperature. Then they were incubated with FITC-conjugated IgG for 1 hour at room temperature. For double staining, certain cells were further exposed to a vinculin antibody and TRITC-conjugated IgG. Fluorescence was visualized by confocal laser microscopy.

Nitrite Plus Nitrate Measurement
Mouse aortic rings with endothelium were freshly isolated and cultured in DMEM supplemented with 0.1% FBS in the presence and absence of 50 ng/mL PDGF or 100 μM sepiapterin at 37°C for 2 days in a CO2 incubator. The rings were then moved to Krebs-Ringer bicarbonate solution and were incubated with and without 1 μM sepiapterin. NOx concentrations were evaluated by NOx concentrations accumulated in culture medium for 2 days, and A23187-stimulated vascular NOx production was assessed by the difference in the NOx concentrations in Krebs solution with and without A23187 stimulation. NOx concentrations were analyzed by the Griess method, with the detection limit of 50 mmol/L.

Superoxide Measurement
Mouse aortic rings with endothelium were cultured in the presence of 50 ng/mL PDGF at 37°C for 2 days. Aortic superoxide generation was assessed by the lucigenin-enhanced chemiluminescence method.

Statistical Analysis
Results are expressed as mean±SEM. Statistical analyses were performed by unpaired t test or 1-way ANOVA followed by Fisher’s post hoc test. A value of P<0.05 was considered to be statistically significant.

Results
Effects of PDGF on NOS Expression in Cultured Rat Aortic SMCs
We first examined whether PDGF modulates expression of individual NOS isoform in cultured rat aortic SMCs. Treatment with PDGF significantly increased nNOS expression in mRNA and protein levels in time- and concentration-dependent manners (Figure 1A through 1C). The significant
increases were observed at 0.5 to 5 days of treatment (Figure 1B) and 10 to 200 ng/mL of concentrations (Figure 1C) in both levels. Based on these results, we used the concentration of 50 ng/mL of PDGF in the following experiments.

In contrast, treatment with PDGF (50 ng/mL) did not significantly affect iNOS or eNOS expression (Figure 1A). A faint increase in iNOS expression was seen at 0.5 days after treatment with PDGF; however, no further increase was detected thereafter (Figure 1A).

**Effects of PDGF on Phosphorylation of MAPK Family in Cultured Rat Aortic SMCs**

Activation of MAPK family was assessed by an increase in phosphorylation of the kinases by Western blot analysis. Treatment with PDGF (50 ng/mL; 10 minutes) significantly caused phosphorylation of MAPK family, including ERK, p38MAPK, and JNK (Figure 2). Simultaneous treatment with either PD98059 (25 μmol/L) or U0126 (1 μmol/L), ERK kinase (MEK) inhibitors, inhibited PDGF-induced ERK phosphorylation and nNOS protein expression (n=5). On the other hand, cotreatment with SB203580 (10 μmol/L), a p38MAPK inhibitor, or SP600125 (20 μmol/L), a JNK inhibitor, suppressed p38MAPK or JNK phosphorylation, respectively, but did not alter PDGF-induced nNOS protein expression (n=5). *P<0.05 vs without PDGF treatment; †P<0.05 vs with PDGF treatment.

**Effects of Gene Transfer of MEK Plasmids on ERK Phosphorylation and nNOS Expression in Cultured Rat Aortic SMCs**

Gene transfer of wild-type MEK per se elicited nNOS protein induction as well as ERK phosphorylation even in the absence of PDGF (Figure 3A and 3B). In addition, gene transfer of dominant-negative MEK abolished PDGF-induced ERK phosphorylation and nNOS protein expression (Figure 3C and 3D). Successful gene transfer of the hemagglutinin-tagged plasmids was confirmed by Western blotting for hemagglutinin (Figure 3A and 3C).
Effects of Angiotensin II and IL-1β on ERK Phosphorylation and nNOS Expression in Cultured Rat Aortic SMCs

Treatment with either angiotensin II (0.1 μmol/L; 1 day) or IL-1β (10 ng/mL; 1 day) significantly increased ERK phosphorylation (410±20% or 314±6%, respectively) and nNOS protein expression (311±5% or 393±26%, respectively) in cultured rat aortic SMCs (all P<0.05), all of which were blocked by gene transfer of dominant-negative MEK (99±11% or 101±9% and 95±11% or 87±24%, respectively; all P<0.05; n=5 to 6), as in the case with PDGF.

Intracellular Localization of nNOS in Cultured Rat Aortic SMCs

Immunofluorescence for nNOS (green) showed that the enzyme was upregulated predominantly in the cytoplasm, the cellular membrane, and the nuclear membrane in cultured rat aortic SMCs after treatment with PDGF (50 ng/mL; 1 day; Figure 4A). This upregulation was abrogated by simultaneous treatment with MEK inhibitor U0126 (n=5; A). Costaining for nNOS (green) and vinculin (red), a marker of the membrane, showed positive double immunofluorescence (yellow) in the cellular and nuclear membrane after treatment with PDGF (n=5; B). Bars=10 μm.

Effects of PDGF on NOS Expression, NOx Production, and Superoxide Generation in Isolated Aortas of Wild-Type and NOS−/− Mice

We next investigated whether PDGF also modulates nNOS expression in intact mouse arteries. In isolated aortas of wild-type mice, treatment with PDGF (50 ng/mL; 2 day) significantly increased nNOS protein expression but not eNOS or iNOS protein expression (Figure 5A). PDGF treatment simultaneously enhanced basal NOx production (as assessed by NOx accumulation in culture medium for 2 days; Figure 5B) and calcium ionophore A23187-stimulated NOx production (as evaluated by the difference of NOx concentrations in Krebs solution between with and without 1 μmol/L A23187 stimulation for 2 hours; Figure 5C) in isolated aortas of wild-type mice. The stimulatory effects of PDGF on basal and A23187-stimulated vascular NOx production were also observed in isolated aortas of iNOS−/− and eNOS−/− mice; however, none of the effects were noted in those of nNOS−/− mice (Figure 5B and 5C).

Superoxide generation did not significantly differ between wild-type and nNOS−/− mouse aortas after treatment with PDGF (Figure I, available online at http://atvb.ahajournals.org). On the other hand, supplementation of sepiapterin (100 μmol/L; 2 days), a precursor of tetrahydrobiopterin, significantly increased basal and A23187-stimulated NOx production in wild-type mouse aortas after treatment with PDGF (Figure I).
Discussion

The principal new findings of the current study are as follows: (1) in cultured rat aortic SMCs, treatment with PDGF causes nNOS expression and ERK activation, both of which are suppressed by either MEK inhibitors or dominant-negative MEK; (2) in isolated mouse aortas, treatment with PDGF also elicits nNOS expression as well as NOx production, both of which are again attenuated by a MEK inhibitor; and (3) in both tissues, neither iNOS nor eNOS expression was altered by PDGF. These results provide the first evidence that vascular nNOS expression is selectively upregulated in response to PDGF through activation of the MEK/ERK pathway.

Induction of Vascular nNOS Expression

nNOS can no longer be considered a constitutive enzyme because nNOS is induced in human atherosclerotic vascular lesions.29 Indeed, we recently demonstrated inducible expression of nNOS in several diseased blood vessels, including a mouse carotid artery ligation model,12 a rat balloon injury model,12 mouse coronary arteries after long-term treatment with Nω-nitro-L-arginine methyl ester,26 and mouse coronary arteries after long-term treatment with asymmetrical dimethylarginine.13,30 Thus, it is likely that nNOS is subject to expression regulation in the vascular system. However, little is known about the regulatory mechanism(s) for vascular nNOS expression.

Effect of PDGF on Vascular nNOS Expression

In the present study, treatment with PDGF significantly increased nNOS expression in cultured rat aortic SMCs and isolated mouse aortas. The stimulatory effects of PDGF were noted at mRNA levels as well as protein levels in cultured rat aortic SMCs, suggesting the expression regulation at transcriptional level. The effect of PDGF was selective to nNOS isoform, as evidenced by the fact that PDGF had little effect on iNOS or eNOS expression in cultured rat aortic SMCs or in isolated mouse aortas. Thus, PDGF appears to be a specific enhancer of vascular nNOS expression.

The effects of PDGF on vascular nNOS expression is not attributable to promotion of vascular SMC proliferation because vascular nNOS mRNA and protein levels were evaluated by the ratio of nNOS mRNA to GAPDH mRNA and by nNOS protein levels per fixed total protein amount (100 μg), respectively.

It is important to consider how relevant the concentrations of PDGF that we used in this study are to the in vivo arteriosclerotic situation. Although direct data for the in vivo concentrations of PDGF in arteriosclerotic lesions are still lacking, PDGF is synthesized in those lesions associated with nNOS expression.14–16 Thus, it is conceivable that the concentrations of PDGF we used have a clinical relevance because we were able to demonstrate that vascular nNOS expression is indeed increased along with vascular lesion formation in vivo.12,13,26,30

Role of MEK/ERK in PDGF-Induced Vascular nNOS Expression

MAPK family consists of 3 major components: ERK, p38MAPK, and JNK. MEK (MAPK/ERK kinase) is located upstream of ERK. In the present study, treatment with PDGF significantly phosphorylated and activated all of the 3 components in cultured rat aortic SMCs. MEK inhibitors (PD98059 and U0126), a p38MAPK inhibitor (SB203580), or a JNK inhibitor (SP600125) repressed only its pathway, confirming the specificity of these inhibitors. Of importance, inhibition of PDGF-induced increase in nNOS expression was noted only when activation of the MEK/ERK cascade was blocked by either PD98059 or U0126. Thus, it is conceivable that PDGF activates all of ERK, p38MAPK, and JNK; however, PDGF-induced nNOS expression is selectively mediated by a mechanism involving activation of the MEK/ERK cascade in vascular SMCs.
To further elucidate the molecular mechanism for PDGF-induced vascular nNOS expression, we next performed gene transfer experiments. Overexpression of dominant-negative MEK prevented PDGF-induced ERK activation and nNOS expression in cultured rat aortic SMCs. It also blocked angiotensin II- and IL-1β-induced ERK activation and nNOS expression in the cells. More important, overexpression of wild-type MEK per se caused ERK activation and nNOS expression. These results provide the first direct evidence for the essential role of MEK/ERK in nNOS expression in vascular SMCs.

**Intracellular Localization of Uregulated nNOS in Vascular SMCs**

It has been reported that under physiological conditions, intracellular localization of nNOS varies in different types of organs and tissues (eg, nNOS localizes the cytoplasm in the cerebellum, the cellular membrane in skeletal muscle, and the sarcoplasmic reticulum in cardiomyocytes). We therefore examined whether nNOS is upregulated in vascular SMCs by immunofluorescent technique. After treatment with PDGF, nNOS was detected mainly in the cytoplasm, the cellular membrane, and the nuclear membrane in cultured rat aortic SMCs. Costaining for nNOS and vinculin as a membranous marker corroborated the localization of nNOS in the cellular and nuclear membrane. This membranous localization is in agreement with a previous study that expression of nNOS is increased in the cellular membrane of cardiomyocytes after experimental myocardial infarction in senescent rats.

**Vascular NO Production**

To elucidate whether upregulated nNOS induced by PDGF treatment is functionally accompanied by enhanced NO production, we finally investigated NOX production in intact mouse aortas after treatment with PDGF. In wild-type mice, basal and A23187-stimulated NOX production was significantly higher in PDGF-treated than in untreated aortas. Similar results were also noted in eNOS−/− and iNOS−/− mice but not in nNOS−/− mice. Thus, it is evident that upregulation of vascular nNOS expression is indeed associated with enhanced NO production in mouse blood vessels. A MEK inhibitor, U0126, abrogated PDGF-induced basal and calcium-stimulated vascular NOX production as well as vascular nNOS expression, again confirming the important role of the MEK/ERK in PDGF-induced vascular nNOS expression.

In many disease states, there is not enough tetrahydrobiopterin around to allow proper NOS function such that the enzyme exerts important vasculoprotective actions in vivo.

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Figure I
Figure II

Panel A: Western blot showing nNOS expression in control, PDGF, and PDGF + U0126 treatments.

Panel B: Bar graph showing NOx accumulation in culture medium. PDGF treatment significantly increased NOx accumulation compared to control and PDGF + U0126.

Panel C: Bar graph showing A23187-stimulated NOx production. PDGF treatment significantly increased production compared to control and PDGF + U0126.