Dopamine as a Novel Antioxidative Agent for Rat Vascular Smooth Muscle Cells Through Dopamine D1-Like Receptors

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Background—To elucidate the roles of vascular D1-like receptors in atherosclerosis, the effects of the specific D1-like agonists on platelet-derived growth factor (PDGF)-BB–mediated oxidative stress in vascular smooth muscle cells (VSMCs) were studied.

Methods and Results—Immunohistochemical studies demonstrated the coexistence of D1A and D1B dopamine receptors in VSMCs. Western blotting revealed a band of \( \approx 70 \, \text{kDa} \) for D1A and D1B dopamine receptors. VSMCs stimulated by PDGF-BB exhibited increased oxidative stress directly measured by flow cytometry. These effects were prevented by dopamine, SKF 38393, or YM 435, and this prevention was reversed by Sch 23390. These effects were blocked by a specific protein kinase A (PKA) inhibitor, \( N\)-(2-[\text{p}-\text{bromocinnamylamino}]ethyl)-5-isoquinolinesulfonamide (H 89). The PDGF-BB–mediated increase in oxidative stress of VSMCs was significantly suppressed by the indirect phospholipase D (PLD) inhibitor suramin or the specific protein kinase C (PKC) inhibitor calphostin C. Both antisense but neither sense nor scrambled oligonucleotides to D1A and D1B receptors inhibited dopamine-induced suppression of increase in oxidative stress of VSMCs induced by PDGF-BB.

Conclusions—These findings suggest that vascular D1-like receptors (D1A and D1B receptors) inhibit any increase in oxidative stress of VSMCs, possibly through activation of PKA and suppression of PLD and PKC.

**Key Words:** catecholamines | muscle, smooth | atherosclerosis | receptors | hypertension | kidney

Increased oxidative stress in vascular smooth muscle cells (VSMCs) enhances VSMC migration\(^1\) and proliferation.\(^2\) In fact, intracellular oxidative stress plays an important role in growth factor–mediated signal transduction and VSMC migration.\(^3\) In addition, increased migration and proliferation of VSMCs may lead to enhanced atherosclerosis.\(^4\) A critical linkage therefore exists among oxidative stress, migration, and atherosclerosis.

At least 5 dopamine receptor subtypes have been cloned from the brain. Types D1A and D1B are D1-like, whereas types D2, D3, and D4 are D2-like.\(^4\) Furthermore, D1A receptors have been detected in coronary artery VSMCs,\(^5\) and D1-like receptors have been evaluated biochemically.\(^6\) We recently demonstrated that D1-like receptors inhibit migration and proliferation of VSMCs, possibly through activation of protein kinase A (PKA) and suppression of protein kinase C (PKC), suggesting that D1-like receptors have an antiatherosclerotic effect.\(^7\)

The present study was therefore designed to investigate the role of D1-like receptors in platelet-derived growth factor (PDGF)–mediated increase in oxidative stress in VSMCs and to examine the potential therapeutic effect of D1-like receptor agonists on atherosclerosis.

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**Methods**

**Cell Culture**

VSMCs were grown from explants of 14-week-old normotensive Wistar rat renal arteries. Cells were identified as VSMCs on the basis of their morphological and immunohistochemical characteristics as previously reported.\(^7\)

**Immunohistochemistry**

Antibodies against D1A and D1B dopamine receptors were purchased from Calbiochem, Inc. Polyclonal antibodies were raised against 2 synthetic peptide sequences: (1) Ac-M-D-G-T-G-L-V-V-E-R-D-F-S-C-COOH, amino acids (Ac-9 to 21-Cys 22 ) within the D1A receptor, and (2) L-P-P-G-S-N-G-T-A-Y-C, amino acids (2 to 11-Cys 12 ) within the D1B receptor.

For light microscopic immunohistochemistry, VSMCs were cultured in slides until confluence. The VSMCs were washed in PBS and then incubated for 30 minutes with 3% normal goat serum and 1% nonfat dry milk in PBS and were incubated overnight at 4°C with either (1) D1A receptor primary antiserum, (2) D1B receptor antiserum, or (3) preadsorption serum. After washes in PBS, immunostaining was detected with an avidin-biotin immunoperoxidase reaction (Vectastain ABC Kit, Vector Laboratories) and visualized with diaminobenzidine (Fast DAB tablets, Sigma Chemical Co) or the peroxidase chromogen method (AEC Kit, Beomeda Co). For double staining with D1A/D1B receptors, the following incubations were performed in the indicated order: D1A receptor antibody, biotinylated goat anti-rabbit immunoglobulin (DAKO), immuno-
staining detection and visualization with DAB and nickel (black); and D1B receptor antibody, biotinylated goat anti-rabbit immunoglobulin, immunostaining detection and visualization by AEC (red).

**Immunoblotting**

Immunoblotting was performed as previously described. 7

**Migration Assay**

Migration of VSMCs was assayed by a modification of a Boyden’s chamber method as previously reported. 7

**Assay of Intracellular Oxidative Stress**

Intracellular oxidative stress levels were measured with carboxydi-chlorofluorescein diacetate bis-acetoxymethyl (AM) ester (Molecular Probes) as previously reported. 8

**Antisense Oligonucleotides**

Phosphothioate-modified oligodeoxynucleotides for the rat D1A and D1B receptors were designed as reported 9,10 and synthesized and purified by high-performance liquid chromatography by Japan Bio Service Co. D1A sense and D1B sense oligodeoxynucleotides are from +1 to +21 of the rat D1A receptor cDNA and from −12 to +6 of rat D1B receptor cDNA, respectively, as follows: antisense D1A receptor, 5’-GGTAGAAG-TGTTAGGAGCCAT-3’; sense D1A receptor, 5’-ATGGCTCCTAA-CACTTCTACC-3’; scramble D1A receptor, 5’-ATACCTTCAACGCC-GATGGTGAT-3’; antisense D1B receptor, 5’-CAGCATGTGCG-CTGAGT-3’; sense D1B receptor, 5’-ACTCAGCGCGACATGCTG-3’; and scramble D1B receptor, 5’-CTAAAGAGCAGCTTGTTA-3’. These oligodeoxynucleotides were added to serum-free DMEM 24 hours before the start of PDGF-BB stimulation with transfection by cationic compound; lipofectin reagent (Gibco BRL) and oligonucleotides were effectively taken up by VSMCs.

**Statistical Methods**

Statistical analysis was performed by ANOVA and Scheffé’s modified t test. 11 Values of P<0.05 were considered significant.

**Results**

**Immunohistochemistry**

Figure 1A demonstrates the existence of D1A and D1B receptors on VSMCs. Double staining demonstrated the coexistence of D1A and D1B receptors in the same cells. In VSMCs exposed to preadsorption antisera, no specific staining was observed. Western blotting revealed a band of ~70 kDa (lanes 1 and 3). VSMCs with preadsorbed antisera (lane 2, D1A preadsorbed; lane 4, D1B preadsorbed) exhibited nonspecific staining.

**Effects of D1-Like Receptor Agonists on VSMC Migration Stimulated by PDGF**

The effects of the D1-like receptor agonists dopamine, SKF 38393, and YM 435 on migration of VSMCs stimulated with 5 ng/mL PDGF-BB for 4 hours are shown in Figure 2. D1-like receptor agonists inhibited PDGF-BB–induced VSMC migration.

**Effects of D1-Like Receptor Agonists on Oxidative Stress Stimulated by PDGF**

The effects of dopamine, SKF 38393, and YM 435 on oxidative stress in VSMCs treated with PDGF-BB for 4 hours are shown in Figure 3A. D1-like receptor agonists significantly inhibited PDGF-BB–induced increase in oxidative stress. Representative flow cytometry results are shown in Figure 3B.
Inhibition of Effects of Dopamine by D<sub>1</sub>-Like Antagonist Sch 23390

The specific D<sub>1</sub>-like antagonist Sch 23390 alone had no effect on oxidative stress (data not shown) but significantly reversed the dopamine-induced decrease in oxidative stress (Figure 3A).

Possible Involvement of PKA in Oxidative Stress in VSMCs Stimulated by PDGF

Forskolin 1 µmol/L or 8-bromo-cAMP 100 µmol/L reduced PDGF-BB–induced increase in oxidative stress (Figure 4A).

Figure 3. Effects of dopamine (DA), SKF 38393, and YM 435 on oxidative stress of VSMCs incubated with PDGF-BB 5 ng/mL in presence or absence of D<sub>1</sub>-specific antagonist Sch 23390 or PKA-specific antagonist H 89 (A) and representative flow cytometry results (B). Relative mean fluorescence intensity (mean oxidative stress) was measured by flow cytometry as follows: Mean fluorescence intensity = \( \frac{\sum(\text{fluorescence of each channel} \times \text{cell number of channel})}{\text{total cell number}} \). Values are mean ± SD (n=8) of a single, representative experiment from among 3 independent experiments. *P<0.05.

Possible Involvement of PLD and PKC in Oxidative Stress in VSMCs Stimulated by PDGF

Incubation of VSMCs with a PKA inhibitor, \( N-(2-[p\text{-bromocinnamylamino}]\text{ethyl})-5\text{-isouquinolinesulfonamide} \) (H 89) at 10 µmol/L significantly reversed dopamine-mediated suppression of oxidative stress in VSMCs (Figure 3A). Representative flow cytometry results are shown in Figure 4B.

Possible Involvement of PLD and PKC in Oxidative Stress in VSMCs Stimulated by PDGF

A phospholipase D (PLD) inhibitor, suramin 10 µmol/L, and a PKC inhibitor, calphostin C 0.1 µmol/L, each significantly prevented the increase in oxidative stress induced by
PDGF-BB (Figure 4A). Representative flow cytometry results are shown in Figure 4B.

Effects of D₁-Like Agonists on Phenylephrine- or Angiotensin II–Induced Increase in Oxidative Stress in VSMCs
Dopamine 10 μmol/L prevented migration (Figure 5A) and the increase in oxidative stress (Figure 5B) induced through α₁- or angiotensin II AT₁ receptors. Receptor specificities were confirmed by use of prazosin, a specific α₁-antagonist, and losartan, a specific angiotensin II AT₁ receptor antagonist.

Inhibition of D₁-Like Receptor Activation by Antisense Oligonucleotides
Antisense oligonucleotides to both D₁A and D₁B receptors at 5 μmol/L inhibited the dopamine-induced suppression of increase in oxidative stress induced by PDGF. However, neither sense nor scrambled oligonucleotides to D₁A and D₁B receptors at 5 μmol/L had significant effects (Figure 6).
Discussion

We examined the cellular distribution of the closely related D\textsubscript{1A} and D\textsubscript{1B} subtypes of dopamine receptors in VSMCs using subtype-specific antibodies. D\textsubscript{1A} or D\textsubscript{1B} staining was observed in cultured VSMCs from renal artery. Double immunostaining experiments revealed that the 2 receptors were frequently coexpressed in single VSMCs from renal artery.

![Diagram](image1.png)

**Figure 5.** A, Effect of dopamine (DA) on migration of VSMCs stimulated with 10 \textmu mol/L (M) phenylephrine (PE) or 0.1 \textmu mol/L angiotensin II (A II) for 4 hours. Values are mean±SD of 8 replicate measurements in a single representative experiment. B, Effect of dopamine on oxidative stress of cultured VSMCs incubated with phenylephrine 10 \textmu mol/L or angiotensin II 0.1 \textmu mol/L in presence or absence of \alpha\textsubscript{1} receptor antagonist prazosin or AT\textsubscript{1} receptor antagonist losartan. Oxidative stress was measured by flow cytometry and expressed as mean±SD (n=8) of fluorescence intensity. *P<0.05.

![Diagram](image2.png)

**Figure 6.** Effects of D\textsubscript{1A} (A) or D\textsubscript{1B} (B) receptor antisense (AS), sense (S), and scramble (RS) oligodeoxynucleotides on dopamine-induced prevention of increase in oxidative stress in cultured VSMCs. Both D\textsubscript{1A} and D\textsubscript{1B} antisense but neither sense nor scramble oligonucleotides at 5 \mu g/mL prevented dopamine (DA) 10 \textmu mol/L (M)-mediated inhibition of increase in oxidative stress induced by PDGF-BB (5 ng/mL). Oxidative stress was measured by flow cytometry and expressed as mean±SD (n=8) of fluorescence intensity. *P<0.05.
This study demonstrated that D₁-like agonists inhibit oxidative stress in VSMCs stimulated with PDGF-BB through both D₁A and D₁B receptors (Figures 3 and 6). Dopamine is readily oxidized and therefore has direct radical-scavenging effects. However, the finding that D₁A and D₁B antisense oligonucleotides and the PKA inhibitor H 89 reduced the antioxidative effects of dopamine suggests that the antioxidative effect of dopamine is, at least in part, mediated by a D₁-like receptor–mediated pathway. It has been reported that D₁-like receptors coupled to adenylyl cyclase on rat VSMCs possess a thiol group at or near the agonist binding site and that the oxidation of a thiol group of D₁-like receptors inhibits their activation. However, dopamine itself activated PKA, which decreased oxidative stress (Figure 4A), suggesting that the dopamine acting at D₁-like receptors in this study was not totally oxidized.

Dose-dependent responses to D₁-like receptor agonists showed that suppression of VSMC migration and oxidative stress was present at 0.025 μmol/L (Table), which is 25 times higher than the normal plasma concentrations. This concentration is observed in humans in pathophysiological conditions. At present, we have no direct in vivo evidence that dopamine has an impact on atherogenesis. However, functional ablation of the D₁A receptor gene produced diastolic hypertension in mice, and pathophysiological levels of dopamine may also decrease blood pressure. Therefore, the blood pressure–lowering effect of dopamine in vivo may affect atherosclerosis in vivo.

We have already demonstrated that PDGF-BB enhances VSMC migration and proliferation through PLD, PKC, and mitogen-activated protein kinase activation. It has been reported that PLD and PKC play important roles in oxidative stress. We examined the effects of inhibitors of PLD or PKC and found that PLD and PKC play important roles in mediating the increased oxidative stress induced by PDGF-BB (Figure 4).

We demonstrated that angiotensin II and phenylephrine increased oxidative stress through AT₁ and α₁-receptors, respectively. D₁-like receptor agonists also prevented the increase in oxidative stress induced by angiotensin II and phenylephrine. Interestingly, dopamine, which is the precursor of norepinephrine, an α₁-agonist, antagonizes α₁-mediated effects through D₁-like receptors, suggesting that dopamine may act in the third peripheral catecholamine system. It is also interesting that dopamine antagonizes angiotensin II, which plays an important role in vascular remodeling, suggesting the possibility of local interaction between dopamine receptors and the renin-angiotensin system. Because activation of D₁A receptors in rat juxtaglomerular cells has been reported to increase renin release, this dopamine antagonism of angiotensin II effects may be a feedback mechanism responding to increased renin release.

In conclusion, our findings indicate that D₁-like receptor agonists suppress PDGF-BB–mediated increase in oxidative stress through D₁A and D₁B receptors by activating PKA and suppressing PLD and PKC activities.

Acknowledgments
This study was supported by Yoshitomi Research Foundation and Kimura Memorial Foundation. We thank Atsumi Ohnishi and Yuka Inoshita for excellent technical assistance.

References

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<th>Dose</th>
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<tr>
<td>Control</td>
<td>7.7±2.4</td>
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<td>PDGF-BB 5 ng/mL</td>
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DA indicates dopamine; NS, not significantly different. Migration activities are expressed as the number of cells per high-power field (HPF). Oxidative stress was measured by flow cytometry. Values are given as mean±SD of 8 replicate measurements in a single representative experiment. *P<0.05.
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Circulation. 2000;101:2302-2308
doi: 10.1161/01.CIR.101.19.2302

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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