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

Kenichi Yasunari, MD; Masakazu Kohno, MD; Hiroaki Kano, MD; Mieko Minami, MD; Junichi Yoshikawa, MD

**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 ≈70 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, \(N^{2-}[p\text{-bromocinnamylamino}]\text{ethyl}-5\text{-isoquinolinesulfonamide (H 89).}\) 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
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

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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-TGATTAGGAGCCAT-3′; sense D1A receptor, 5′-ATGGCTCCTAA-CACTTCTACC-3′; scramble D1A receptor, 5′-ATACTTCACGCC-GATGGTGAT-3′; antisense D1B receptor, 5′-CAGCATGTCGCG-CTGAGT-3′; sense D1B receptor, 5′-CTCAAGAGCCAGCTTGTA-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 Scheffe’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 D₁-Like Antagonist Sch 23390

The specific D₁-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).

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

Incubation of VSMCs with a PKA inhibitor, N-(2-[p-bromocinnamylamino]ethyl)-5-isquinolinesulfonamide (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 D1A and D1B subtypes of dopamine receptors in VSMCs using subtype-specific antibodies. D1A or D1B 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.

Figure 5. A, Effect of dopamine (DA) on migration of VSMCs stimulated with 10 μmol/L (M) phenylephrine (PE) or 0.1 μmol/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 μmol/L or angiotensin II 0.1 μmol/L in presence or absence of α1 receptor antagonist prazosin or AT1 receptor antagonist losartan. Oxidative stress was measured by flow cytometry and expressed as mean±SD (n=8) of fluorescence intensity. *P<0.05.

Figure 6. Effects of D1A (A) or D1B (B) receptor antisense (AS), sense (S), and scramble (RS) oligodeoxynucleotides on dopamine-induced prevention of increase in oxidative stress in cultured VSMCs. Both D1A and D1B antisense but neither sense nor scramble oligonucleotides at 5 μg/mL prevented dopamine (DA) 10 μmol/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.
Effects of Various Concentrations of Dopamine on Migration of and Oxidative Stress in Cultured VSMCs Stimulated With PDGF-BB

<table>
<thead>
<tr>
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<th>Migration, Cells/HPF</th>
<th>Oxidative Stress, Arbitrary Units</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.7 ± 2.4</td>
<td>22.4 ± 2.2</td>
</tr>
<tr>
<td>Control+DA 10 μmol/L</td>
<td>6.2 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>PDGF-BB 5 ng/mL</td>
<td>122.4 ± 10.4</td>
<td>NS</td>
</tr>
<tr>
<td>PDGF-BB 5 ng/mL+DA 0.001 μmol/L</td>
<td>118.2 ± 9.6</td>
<td>NS</td>
</tr>
<tr>
<td>PDGF-BB 5 ng/mL+DA 0.01 μmol/L</td>
<td>110.2 ± 6.4</td>
<td>*</td>
</tr>
<tr>
<td>PDGF-BB 5 ng/mL+DA 0.025 μmol/L</td>
<td>104.2 ± 4.2</td>
<td>*</td>
</tr>
<tr>
<td>PDGF-BB 5 ng/mL+DA 0.1 μmol/L</td>
<td>98.4 ± 8.8</td>
<td>*</td>
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<tr>
<td>PDGF-BB 5 ng/mL+DA 1 μmol/L</td>
<td>70.4 ± 7.2</td>
<td>*</td>
</tr>
<tr>
<td>PDGF-BB 5 ng/mL+DA 10 μmol/L</td>
<td>50.2 ± 6.2</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.

This study demonstrated that D1-like agonists inhibit oxidative stress in VSMCs stimulated with PDGF-BB through both Δ1A and Δ1B receptors (Figures 3 and 6). Dopamine is readily oxidized and therefore has direct radical-scavenging effects.12 However, the finding that Δ1A and Δ1B 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 Δ1-like receptor–mediated pathway. It has been reported that Δ1-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 Δ1-like receptors inhibits their activation.13 However, dopamine itself activated PKA,7 which decreased oxidative stress (Figure 4A), suggesting that the dopamine acting at Δ1-like receptors in this study was not totally oxidized.

Dose-dependent responses to Δ1-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.14,15 At present, we have no direct in vivo evidence that dopamine has an impact on atherogenesis. However, functional ablation of the Δ1A receptor gene produced diastolic hypertension in mice,16 and pathophysiological levels of dopamine may also decrease blood pressure.14,15 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.7 It has been reported that PLD and PKC play important roles in oxidative stress.2,3 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-receptor, antagonizes α1-mediated effects through D1-like receptors, suggesting that dopamine may act in the third peripheral catecholamine system.17 It is also interesting that dopamine antagonizes angiotensin II, which plays an important role in vascular remodeling,18 suggesting the possibility of local interaction between dopamine receptors and the renin-angiotensin system. Because activation of Δ1A receptors in rat juxtaglomerular cells has been reported to increase renin release,9 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 Δ1A and Δ1B receptors by activating PKA and suppressing PLD and PKC activities.

Acknowledgments
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References
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