Dopamine D₁ Receptor Augmentation of D₃ Receptor Action in Rat Aortic or Mesenteric Vascular Smooth Muscles

Chunyu Zeng, Dan Wang, Zhiwei Yang, Zheng Wang, Lareano D. Asico, Christopher S. Wilcox, Gilbert M. Eisner, William J. Welch, Robin A. Felder, Pedro A. Jose

Abstract—Dopamine is an important modulator of blood pressure, in part, by regulating vascular resistance. To test the hypothesis that D₁ and D₃ receptors interact in vascular smooth muscle cells, we studied A10 cells, a rat aortic smooth muscle cell line, and rat mesenteric arteries that express both dopamine receptor subtypes. Fenoldopam, a D₁-like receptor agonist, increased both D₁ and D₃ receptor protein in a time-dependent and a concentration-dependent manner in A10 cells. The effect of fenoldopam was specific because a D₁-like receptor antagonist, SCH23390 (10⁻⁷ M/24 h), completely blocked the stimulatory effect of fenoldopam (10⁻⁷ M/24 h) (D₁ receptor: control=21±1 density units [DU]); SCH23390=23±2 DU; fenoldopam=33±2 DU; fenoldopam+SCH23390=23±2 DU; n=10). D₁ and D₃ receptors physically interacted with each other because fenoldopam (10⁻⁷ M/24 h) increased D₁/D₃ receptor coimmunoprecipitation (35±5 versus 65±5 DU; n=8). A D₃ receptor agonist, PD128907, relaxed mesenteric arterial rings independent of the endothelium, effects that were blocked by a D₁ receptor antagonist, U99194A. Costimulation of D₁ and D₃ receptors led to additive vasorelaxation. We conclude that the D₁ receptor regulates the D₃ receptor by physical interaction and receptor expression. D₁ receptor stimulation augments D₃ receptor vasorelaxant effects. An interaction of D₁ and D₃ receptors may be involved in the regulation of blood pressure. (Hypertension. 2004; 43:673-679.)

Key Words: receptor ■ dopamine ■ arteries ■ blood pressure

Dopamine, a well known neurotransmitter in the central nervous system, has recently been characterized as an important modulator of blood pressure, sodium balance, and renal and adrenal function and is relevant to the pathogenesis and/or maintenance of hypertension.¹⁻⁴ Dopamine receptors are classified into 2 families: the D₁-like receptor subfamily includes the D₁ and the D₃ receptor, while the D₂, D₄, and D₅ receptors belong to the D₂-like subfamily. Whereas the D₁-like receptors couple to the stimulatory G protein, Gₛ, and thus activate adenylyl cyclase, all of the D₂-like receptors couple to the inhibitory G protein, Gᵢ/Gₛ, and inhibit adenylyl cyclase and calcium channels and modulate potassium channels.¹⁻⁴

Both D₁ and D₃ receptors are expressed in vascular smooth muscle cells, and the activation of the D₁ receptor relaxes blood vessels and decreases blood pressure.⁵⁻⁶ However, the effect of D₁ receptors on resistance vessels is not clear. A low intravenous dose of R(+)-7-hydroxypipropripylaminotetralin (7-OH-DPAT), a D₁-like agonist with a 50-fold selectivity for the D₁ over the D₃ receptor, constricts postglomerular arterioles without affecting systemic blood pressure.⁴ A higher dose, however, decreases blood pressure.⁴ To test the hypothesis that D₁ and D₃ receptors interact in vascular smooth muscle cells, we studied the effect of fenoldopam, a D₁-like receptor agonist, on D₁ and D₃ receptor expressions in rat thoracic aorta-derived smooth muscle cell line (A10).⁷ We also studied the effect of D₁ and D₃ receptor agonists on the wall tension of rat mesenteric arterial rings.

Methods

Cell Culture and Sample Preparation

Embryonic thoracic aortic smooth muscle cells⁷,⁸ (passage 10 to 20) from normotensive Berlin-Druckrey IX (A10; CRL 1476, ATCC) were cultured at 37°C in 99% air/5% CO₂ atmosphere in Dulbecco modified eagle medium.

A10 cells (80% confluence) and mesenteric arteries from sodium pentobarbital-anesthetized Wistar-Kyoto (WKY) rats (Taconic, Germantown, NY) were homogenized in ice-cold lysis buffer (5 mL/g tissue) (20 mmol/L Tris-HCl, pH 7.4; 2 mmol/L EDTA, pH 8.0; 2 mmol/L EGTA; 100 mmol/L NaCl; 10 μg/mL leupeptin; 10 μg/mL aprotinin; 2 mmol/L phenylmethylsulfonyl fluoride; 1% NP-40), sonicated, kept on ice for 1 hour, and centrifuged at 16 000g for 30 minutes. All samples were stored at −70°C until use. All experiments were approved by the Georgetown University Animal Use and Care Committee.
Immunoblotting
The antibodies are polyclonal, purified, and antipeptides. The rat D1 receptor immunizing peptide (299-GSEEFTQPFPC-307) (Research Genetics)9-14 and the rat D3 receptor immunizing peptide (228-QPP SPG QTH GGL KRY YSI C-306) are located on the third extracellular loop of their corresponding receptors.13,14 The specificity of these antibodies has been reported.9-14 A10 cells were treated with vehicle (dH2O), a D1-like receptor agonist (fenoldopam) (Sigma, St. Louis, Mo).9,10,12,13,15 at the indicated concentrations and times. The transblots were probed with the D3 (1:250) (Alpha Diagnostic International, San Antonio, Tex) or the D1 receptor antibody (Research Genetics) (1:800). The amount of protein transferred onto the membranes was determined by Ponceau-S staining and immunoblotting for α-actin.

Immunoprecipitation
A10 cells were incubated with vehicle (dH2O) or fenoldopam (10^-7 M) for 24 hours, as described.9,14 Equal amounts of cell lysates (200 μg protein/mL supernatant) were incubated with affinity-purified anti-D1 receptor antibodies (2 μg/mL) for 1 hour and protein-G agarose at 4°C for 12 hours. The immunoprecipitates were suspended in sample buffer (2X sample buffer: 100% glycerol 2.0 mL; 0.5 mmol/L Tris-HCl 2.5 mL, pH 6.8; 10% SDS 4 mL [final concentration=0.14 mmol/L]; 2 mg/mL bromophenol blue 0.5 mL; 2-mercaptoethanol 0.5 mL, added dH2O until total volume 10 mL), boiled for 10 minutes, and subjected to immunoblotting with the D3 receptor antibody. To determine the specificity of the bands found on the immunoblots, preimmune serum of D1 receptor antibody (negative control) and D1 receptor antibodies (positive control) were used as the immunoprecipitates instead of the D3 receptor antibodies.

Immunohistochemistry
The rat mesenteric artery, cleared of blood with ice-cold oxygenated saline and kept in Histochrome (Amresco, Solon, Ohio) for 1 to 2 days at 4°C, were sectioned (4 μm), embedded in paraffin, and mounted on slides.17 The tissue was deparaaffinized and rehydrated by successive incubations in xylene, 100% ethanol, 95% ethanol, 75% ethanol, and phosphate-buffered saline. The tissue was then briefly treated with 0.1% Triton X-100, 10 mmol/L sodium citrate (pH 6.0), and blocked sequentially with 5% goat serum in phosphate-buffered saline overnight at 4°C. The tissue sections were incubated with anti-D3 receptor antibody (1:200) at room temperature for 1.5 hours. After staining with antibody (1:250), the tissue sections were incubated with the D1 receptor antibodies (Figure 1, lane 2), because they were no longer visible when the antibody was preadsorbed with the immunizing peptide (1:20 w/w incubation for 12 hours). The molecular sizes are given.

Results
To test the action of D1 and/or D3 receptors, cumulative concentrations (10^-10 to 10^-7 M) of a D1-like receptor agonist, fenoldopam, and/or D3 receptor agonists, PD128907 (Sigma)20,21 and 7-OH-DPAT (Sigma)20,22 were added to the arterial rings preconstricted by norepinephrine or KPSS. The receptor specificities of drug effect were determined by incubating the vessels for 30 minutes with a D1-like agonist, SCH23390 (Sigma8,15) or the D3 receptor antagonists, U99194 (10^-6 M), or the D1 receptor antagonists, (+)-UH232 (Tocris Cookson Inc, Ellisville, Mo) (10^-6 M),20,24 To investigate the mechanisms of ligand-mediated vasorelaxation, studies were performed in vessels preincubated for 30 minutes with nifedipine (Sigma)20 or apamin (Sigma) (10^-6 M; a small conductance calcium-activated K+ channel blocker)26 and charybdotoxin (Sigma) (10^-6 M; a large conductance calcium-activated K+ channel blocker).26

To determine the endothelium dependence of any D1 or D3 receptor agonist-induced relaxation, vessels with or without endothelium were studied. The endothelium was removed by pulling a hair along the vessel; successful denudement of the endothelium was confirmed by an absence of relaxation by acetylcholine.27 To test any interaction between D1 or D3 receptors, PD128907 (10^-10 to 10^-7 M) studies were performed in vessels preincubated for 30 minutes with fenoldopam (10^-7 M). To confirm the D3 receptor-mediated vasorelaxation, we used 2 other D3 receptor ligands, 7-OH-DPAT20,22 (agonist), and (+)-UH23220,24 (antagonist).

Statistical Analysis
The data are expressed as mean±SEM. Comparison within groups was made by repeated measures ANOVA (or paired t test when only 2 groups were compared), and comparison among groups (or t test when only 2 groups were compared) was made by factorial ANOVA with Duncan test. Corresponding periods between 2 different groups were analyzed by independent t test. Fenoldopam and PD128907 sensitivity are expressed as pED50, which is the (–log) concentration of the drug required to produce 50% of the maximum response. Relaxation responses to fenoldopam and PD128907 are expressed as a percentage decrease from the maximum contractile response. A value of P<0.05 was considered significant.

Results
Specificity of Receptor Antibodies
Specific D1 receptor (~45 kDa) bands were found in A10 cells (Figure 1, lane 2), because they were no longer visible when the
antibodies were pre-adsorbed by the immunizing peptide. The immunoblots for the D₃ and D₁ receptors were similar to those in our previous reports.⁹,¹¹,¹³,¹⁴,¹⁷

D₁ Receptors Increase D₃ Receptor Protein Expression in A10 Cells

The D₁-like receptor agonist, fenoldopam, increased D₃ receptor protein in a concentration-dependent and time-dependent manner. The stimulatory effect at 24 hours was evident at 10⁻⁹ M (Figure 2A). The stimulatory effect of fenoldopam (10⁻⁷ M) was noted at 16 hours and maintained for at least 24 hours (Figure 2B).

The specificity of fenoldopam as a D₁-like receptor agonist was determined by studying the effect of a D₁-like receptor antagonist, SCH23390. Consistent with the data shown in Figure 2A and 2B, fenoldopam (10⁻⁷ M/24 h) increased D₁ receptor protein (control=21±1 DU, fenoldopam=33±2 DU; n=10; P<0.05). SCH23390 (10⁻⁷ M) by itself had no effect on D₁ receptor protein (23±2 DU) but reversed the stimulatory effect of fenoldopam on D₁ receptor protein expression (fenoldopam+SCH23390=23±2 DU; n=10) (Figure 2C). To determine whether there is mutual receptor interaction, A10 cells were treated with PD128907 (10⁻¹⁰ M to 10⁻⁵ M) for 24 hours and immunoreactive D₁ receptor protein was measured. Unlike the stimulatory effect of D₁ receptors on D₃ receptor expression, we did not find any effect of D₃ receptors on D₁ receptor expression (data not shown).

D₁-Like Agonist Increases D₁ Receptor Protein Expression in A10 Cells

To investigate the effect of a D₁-like receptor agonist on the protein expression of its own receptor, A10 cells were incubated with fenoldopam. Immunoblots showed that fenoldopam also increased D₁ receptor protein in a time-dependent (2 to 30 hours) and concentration-dependent (10⁻¹¹ to 10⁻⁶ M) manner. The stimulatory effect (24 hours) was evident at 10⁻⁸ M. The stimulatory effect of fenoldopam (10⁻⁷ M) was noted at 8 hours and maintained for at least 30 hours (Figure 3A and B).

D₁ Receptor Physically Interacts With the D₃ Receptor in A10 Cells

To determine whether there is a physical interaction between the D₃ and the D₁ receptor, D₁ receptors were first immunoprecipitated with anti-D₁ receptor antibodies, and then the immunoprecipitates were probed with anti-D₃ receptor antibodies. The 45 kDa band, representing the coimmunoprecipitated D₃ and D₁ receptors, was increased by a 24-hour treatment with fenoldopam (10⁻⁷ M) (control=35±5 DU, fenoldopam=65±5 DU; P<0.01, n=8) (Figure 4).

D₁ Receptor Exists in Rat Mesenteric Artery

We also determined whether the D₁ receptor is expressed in rat mesenteric arteries. Consistent with the results in A10 cells, the D₁ receptor immunoblots of mesenteric artery homogenates also showed a 45-kDa band (Figure 1, lane 1). Immunohistochemistry showed D₁ receptors in the tunica media and intima (Figure 5). Both the immunoblotting and immunohistochemistry results were specific, because the immunoblot (Figure 1, lane 1) and immunostaining (Figure 5) were no longer visible when the antibodies were pre-adsorbed with the D₁ receptor immunizing peptide.

Costimulation of D₁ and D₃ Receptors Has an Additive Vasorelaxant Effect in the Rat Mesenteric Artery

We next determined the effect of D₁ and D₃ receptor agonists on the wall tension of rat mesenteric artery rings. The D₁...
fenoldopam: norepinephrine=4±0.2, KPSS=5±0.2, P<0.05; pED_{50}: PD128907: norepinephrine=4±0.2, KPSS=5±0.1, n=12, P<0.01). The D_{1} and D_{3} receptor agonist vasorelaxant effects were endothelium-independent, because the vasorelaxant effects of fenoldopam and PD128907 were not affected by the presence or absence of the endothelium (10^{-6} M PD128907: with endothelium=25%±4%, without endothelium=24%±6%; 10^{-8} M fenoldopam: with endothelium=29%±5%, without endothelium=26%±6%, P>0.05, n=12) (Figure 6B and C).

**Effect of Antagonists**
The vasorelaxant effects of D_{1} and D_{3} receptors were specific, because the D_{1}-like receptor antagonist, SCH23390 (2%±1%), D_{3} receptor antagonists, U99194A (2%±0.02%) and (+)-UH232 (9%±3%), did not have any effect by themselves but blocked the vasorelaxant effects of their respective agonists: fenoldopam (10^{-6} M) (fenoldopam=29%±5%, fenoldopam+SCH23390=3%±3%, P<0.01, n=12); PD128907 (10^{-6} M) (PD128907=24%±6%, PD128907+U99194A=46±2%, P<0.01, n=12), 7-OH DPAT (7-OH-DPAT=90%±2%, 7-OH-DPAT+U99194A=11±4%, P<0.01, n=4) (Figure 6B, C, and D).

Effect of combined D_{1} and D_{3} receptor stimulation is described herein. PD128907-induced vasorelaxation was increased in vessel preincubated with fenoldopam with vehicle (% relaxation at 10^{-6} M: fenoldopam=40%±5%, vehicle=24%±6%, n=12, P<0.01; pED_{50}: fenoldopam=6±0.1, vehicle=5±0.1, n=12, P<0.05; Figure 6E). These data indicate that D_{1} and D_{3} receptor have additive vasorelaxant effects.

Mechanism of D_{1} and D_{3} receptor-induced vasorelaxation is described herein. In norepinephrine-preconstricted vessels, fenoldopam-induced and PD128907-induced vasorelaxation was increased in vessel preincubated for 30 minutes with nifedipine^{25} (10^{-6} M), indicating that the decreased vasorelaxant effects of fenoldopam and PD128907 in norepinephrine-preconstricted vessels may be related to a norepinephrine-induced increase in intracellular calcium (Figure 6F and G). The vasodilatory effect of PD128907 was abrogated by apamin and charybdotoxin, indicating involvement of small-conductance and/or large-conductance calcium-activated potassium channels (Figure 6G).
Discussion

The effect of D₁ receptors on arterial vascular tone is not well understood. Quinpirole, a D₃ receptor agonist with a 35-fold selectivity over the D₂ receptor, has no vascular relaxant effect on mesenteric arteries. Another D₃ receptor agonist, pramipexole, with a 60 selectivity over the D₂ receptor, decreases vascular resistance, although part of the effect could be accounted for by an interaction with the D₁ receptor. In anesthetized rats, the systemic infusion of 7-OH-DPAT, a D₃ receptor agonist with a 50-fold selectivity over the D₂ receptor, decreases renal blood flow by postglomerular constriction, resulting in an increase in glomerular filtration rate. In conscious dogs, the intrarenal arterial infusion of quinpirole also decreases renal blood flow, but glomerular filtration rate is decreased as well. The reason for these apparent discrepancies is not clear. However, the effect of D₃-like receptors on vascular tone may vary depending on the resisting tone and the arterial segment being studied. For example, D₁ receptor agonist increases the vascular tone of the rat tail artery. In other vessels, including the renal artery, D₃ receptors are vasodilatory. Prejunctival D₂-like receptors are vasodilatory whereas postjunctival D₂-like receptors can induce vasoconstriction when resting vascular tone is low or vasodilation when resting vascular tone is high.

Our studies show that in the maximally vasorelaxed mesenteric artery, 2 different D₃ receptor agonists, PD128907, with a 120-fold selectivity over the D₂ receptor, and 7-OH-DPAT, have no effect on vascular contractility. However, when the mesenteric rings are preconstricted, the PD128907, 7-OH-DPAT, and fenoldopam, by themselves,
cause vasorelaxation. As compared with vessels preconstricted with norepinephrine, the D1 and D3 vasorelaxation is greater in vessels preconstricted with potassium chloride. The ability of norepinephrine to impair the vasodilatory effects of dopamine has been reported.33 The decreased vasodilator effect of D3 agonists in vessels preconstricted with norepinephrine may be caused by an increase of intracellular calcium by activation of α1-adrenergic receptors.34-35 Our studies show that the vasorelaxant effects of fenoldopam (D1 receptor agonist) and PD128907 (D3 receptor agonist) are enhanced by calcium channel blockade with nifedipine. The vasodilatory effect of D1 receptors may also involve potassium channels (small-conductance and/or large-conductance calcium activated potassium).36,37 Additionally, norepinephrine can also compete with the agonists for dopamine receptor occupancy.38,39

The vasorelaxant effect of dopamine in the rabbit pulmonary artery has been reported to be endothelium-dependent and endothelium-independent.40 However, in our studies, despite the presence of D3 receptors in the intima, the vasodilatory effects of the D1 and D3 receptor agonists are endothelium independent. The function of the D3 receptor in endothelial cells is not clear, but D3 receptors have cell-protective properties.41 In human brain endothelial cells,42 the D3 agonist, PD128907, inhibits superoxide production, determined by cytochrome C reduction test,43 an effect that is blocked by a D3 antagonist, U99194A, which by itself does not have any effect (Z. Yang, C. Zeng, K.S. Kim, P.A. Jose, unpublished data, 2003).

The simultaneous stimulation of D1 and D3 receptors causes a vasorelaxation that is additive rather than synergistic. This may indicate that D1 and D3 receptors induce vasorelaxation by different mechanisms. Our studies also indicate that the vasorelaxation of the mesenteric artery is probably caused by activation of postjunctional rather than prejunctional dopamine receptors, because neither D1 receptor nor D3 receptor is expressed in the tunica adventitia, where the nerve supply terminates. The reason for the inability of others to demonstrate D3 receptors in vascular smooth muscles is not clear.44

We find that D1 and D3 receptors coimmunoprecipitate, and that coimmunoprecipitation is increased by D1 receptor stimulation. There are 2 possible explanations for this observation: increased D1 and D3 receptor expression, per se, or an increased physical interaction between D1 and D3 receptors. This interaction occurs only after several hours of treatment and may explain the absence of synergism in the acute studies in mesenteric vessels. Further studies are needed to determine whether the increased interaction between these 2 receptors is a direct or an indirect mechanism. However, homooligomerization or hetero-oligomerization of several G protein-coupled receptors have been reported.45,46

In summary, we have demonstrated that the D3 receptor regulates the D1 receptor by physical interaction and receptor expression. D1 receptor stimulation augments D3 receptor vasodilatory effects. A D1/D3 receptor interaction may be involved in the regulation of blood pressure.

**Perspectives**

The vasodilator effect of dopamine via D1-like receptors is mediated mainly by cAMP/protein kinase A.47,48 It seems paradoxical that the D1 receptor dilates the mesenteric artery because D1-like receptors decrease while D3-like receptors increase cAMP production.1-3 D1 receptors linkage to adenyl cyclase inhibition is weak, unless adenyl cyclase V is present.49 Adenyl cyclase isoform V has been shown in rat pulmonary arterial smooth muscles but not in aortic smooth muscles.50 Therefore, it is likely that the D1 receptor-mediated vasorelaxation in vascular smooth muscle cells is caused by its ability to stimulate K+ channels.36 Under these conditions, the vasorelaxant effects of D1-like receptors caused by cAMP/PKA may be additive to the vasorelaxant effect of D3 receptors caused by stimulation of K+ channels.36,37

**Acknowledgments**

These studies were supported in part by grants from the National Institutes of Health, HL 074940, HL 23081, DK 39308, HL 08686, DK 52612, and HL 62211.

**References**


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Hypertension, published online February 9, 2004;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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