Dopamine D1-Like Receptor Stimulation Inhibits Hypertrophy Induced by Platelet-Derived Growth Factor in Cultured Rat Renal Vascular Smooth Muscle Cells

Kenichi Yasunari, Masakazu Kohno, Hiroaki Kano, Koji Yokokawa, Mieko Minami, Junuchi Yoshikawa

Abstract Vascular smooth muscle cell (VSMC) hypertrophy is believed to play some roles in atherosclerosis. To elucidate the role of vascular D1-like receptors in VSMC hypertrophy, the effects of dopamine and specific D1-like receptor agonists SKF 38393 and YM 435 on platelet-derived growth factor (PDGF) BB–mediated VSMC hypertrophy were studied. We observed that cells stimulated by PDGF-BB 5 ng/mL showed increased VSMC hypertrophy. These effects were prevented by concomitant with dopamine, SKF 38393, and YM 435 1-10 μmol/L, and this prevention was reversed by Sch 23390 1 to 10 μmol/L, a specific D1-like receptor antagonist. These actions are mimicked by forskolin 1 to 10 μmol/L, a direct activator of adenylate cyclase and 8-bromo-cAMP 0.1 to 1 mmol/L, and are blocked by a specific protein kinase A (PKA) inhibitor N-[2-(P-bromocinnamylamino)ethyl]-5-isouquinoline-sulfonamide (H 89) but not blocked by its negative control PDGF-BB (5 ng/mL)–mediated mitogen-activated protein kinase (MAPK) activity was significantly suppressed by concomitant with D1-like receptor agonists, which were reversed by PKA inhibitor H 89. These results suggest that vascular D1-like receptor agonists inhibit hypertrophy of VSMC, possibly through PKA activation and suppression of activated MAPK activity. 

Key Words platelet-derived growth factor • dopamine • vascular smooth muscle • hypertrophy

Excessive VSMC growth has been highlighted recently in the pathophysiology of hypertension and atherosclerosis. Indeed, one of the hallmarks of chronic hypertension is a generalized increase in the smooth muscle mass of the blood vessel wall. Whereas acute hypertensive models (such as aortic coarctation) or experimental injury models of atherosclerosis are characterized by VSMC proliferation (hyperplasia), chronic hypertensive models such as the Goldblatt two-kidney, one-clip hypertensive rats and spontaneously hypertensive rats exhibit aortic VSMC hypertrophy with an increase in polyplody without an increase in cell number.

PDGF is one of the major mitogens in serum and is responsible for proliferation of certain cell types, including VSMCs. It has been reported that PDGF-BB isoform is a potent inducer of hypertrophy as well as hyperplasia of VSMC. Two distinct classes of dopamine receptor exist in peripheral tissue and are designated D1-like and D2-like receptors. There are at least five dopamine receptor subtypes cloned from the brain. Type D1A and D1B are D1-like, whereas types D2, D3, and D4 are D2-like. Biochemical evaluation of D1-like receptors has been reported. Stimulation of D1-like receptors causes vasodilation. Vasodilator hormone such as atrial natriuretic peptide has been shown to act as antihypertrophic factors and antiproliferative factors. These findings led us to speculate that the antihypertrophic action of D1-like receptors on PDGF-BB induced VSMC hypertrophy.

Therefore, the present study was designed to investigate the possible role of the D1-like receptors on PDGF-BB–mediated VSMC hypertrophy and examine the potential therapeutic significance of D1-like receptor agonists on atherosclerosis.

Materials

PDGF (recombinant BB), 8-bromo cAMP, bovine serum albumin, and 3-isobutyl-1-methylxanthine were purchased from Sigma Chemical Co. DMEM, penicillin-streptomycin, trypsin EDTA (Versene), and FCS were purchased from GIBCO Laboratories. cAMP radioimmunoassay kits [3H]-thymidine and [γ-32P]ATP were purchased from Amersham Japan Co. Multwell pipettes and flasks were purchased from Becton Dickinson and Co. H-89, N-[2-(P-bromocinnamylamino)ethyl]-5-isouquinoline-sulfonamide and H-85, N-[2-(N-formyl-P-chlorocinnamyl amino)ethyl]-3-isouquinoline sulfonamide were purchased from Seskagaku Co. YM 435 was a gift from Yamanouchi Co. SKF 38393 is a gift from Smith Kline Beecham Co.

Cell Culture

VSMCs were grown from explants of 14-week-old normotensive Wistar rat renal arteries, with rats handled as described previously. Cells were identified as VSMCs according to their morphological and immunohistochemical characteristics as previously reported. Briefly, these cells showed a typical “hill-and-valley” growth pattern and had positive fluorescence with antibodies against α-smooth muscle actin but were negative
against factor VIII antigen. VSMCs were grown in DMEM supplemented with 10% FCS. Cells from passages 3 through 5 were used and were subcultured after trypsinization on a weekly basis because cells became confluent in 1 week. Each plate was replenished twice a week with fresh medium. To avoid the adrenergic effect of dopamine, propranolol 1 pmol/L was added to each dopamine stimulation. Propranolol 1 pmol/L alone did not affect cell size.

**Experimental Protocol**

VSMCs were placed into culture dishes at 2×10⁶ cells/mL and grown in DMEM containing 10% FCS for 1 week. After reaching confluence, the medium was aspirated, and the DMEM without FCS was applied for 48 hours to induce quiescence. Quiescent cells were stimulated by PDGF-BB 5 ng/mL with or without indicated doses of D₁-like receptor agonists (dopamine, SKF 38393, YM 435) or Sch 23390, 8-bromo-cAMP, or forskolin for 48 hours. Total incorporation of [³H]-leucine into protein and flow cytometric analysis of cell size were then performed to estimate vascular hypertrophy.

**Determination of Protein Synthesis**

Relative rates of synthesis were assessed by determination of [³H]-leucine incorporation into TCA-precipitable material. Quiescent VSMCs grown in 24-well culture dishes were pulsed for 4 hours with [³H]-leucine 10 μCi/mL, washed with calcium- and magnesium-free phosphate-buffered saline, and incubated with 5% TCA at 4°C for 10 minutes. Cells were dissolved in 1 N NaOH at 37°C for 30 minutes and neutralized. Radioactivity was determined by liquid scintillation counting.

**Flowcytometric Analysis of Cell Size**

Quiescent VSMCs grown in flasks were detached with 0.25% trypsin at 37°C for 5 minutes and then pelleted by centrifugation (1000 rpm for 5 minutes). The cells were resuspended in DMEM and applied to a flow cytometer (EPICS PROFILE) to measure cell size.

**MAPK Assay**

MAPK activity was measured by the Amersham MAPK assay system. Briefly, cell aliquots (1×10⁶ cells) were challenged with ligand at 37°C, and the reaction was terminated by directly adding lysis buffer containing 20 mmol/L Tris, pH 8, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 2 mmol/L EGTA, 2 mmol/L diethyldithiocarbamyl, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 0.1% Triton X-100 (final concentration) in a total volume of 200 μL. Extracts (15 μL) were then assayed by adding 10 μL substrate buffer (containing 6 mmol/L substrate peptide, 75 mmol/L HEPES, 300 μmol/L sodium orthovanadate, and 0.05% sodium azide, pH 7.4), and 5 μL ATP buffer (containing 0.3 mmol/L [γ-³²P]ATP [300 μCi/mL] and 90 mmol/L MgCl₂). The substrate peptide interacts with p34cdc2, PKC, 0.1% of MAPK activity. After 10 minutes of incubation at 37°C, 10 μL of 300 nmol/L orthophosphoric acid was added to terminate the reaction. Thirty microliters of each sample was spotted onto phosphocellulose disks, washed three times for 30 minutes in 0.5% phosphoric acid, and washed once for 5 minutes in distilled water. The radioactivity on each disk was then determined by scintillation counting.

**Statistical Analysis**

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

Antihypertrophic Action of D1-Like Receptor Agonists on Postconfluent VSMCs

Fig 1 shows the effect of D1-like receptor agonists on [3H]-leucine incorporations of postconfluent VSMCs in 0.1% FCS media or stimulated by PDGF-BB 5 ng/mL for 48 hours. The D1-like receptor agonists dopamine, SKF 38393, and YM 435 inhibited protein syntheses of VSMCs in a dose-dependent manner. These D1-like receptor agonists did not cause the loss of cells at the confluent state. After the addition of D1-like receptor agonists, <1% of cells were found to be present in the supernatant media. Cell viability was also checked by trypan blue staining, confirming that >98% of cells were alive.

Inhibition of Dopamine Action by the Specific D1-Like Antagonist Sch 23390

To further confirm that dopamine acts through D1-like receptors, a specific D1-like antagonist Sch 23390 was used. Sch 23390 significantly reversed the dopamine-induced decrease in [3H]-leucine incorporations (Fig 2).

Flowcytometric Analysis of Cell Size

Fig 3 shows the histograms of cell size of postconfluent VSMCs defined by flow cytometric analysis. The D1-like receptor agonist dopamine and YM 435 treatment tended to reduce the cell size and caused a significant left-hand shift in cell size in PDGF-BB-treated cell groups. This result further confirmed the inhibitory effect of the D1-like receptor agonists on cellular hypertrophy of VSMCs.

Effect of Forskolin and 8-bromo-CAMP on VSMC Hypertrophy Stimulated With PDGF-BB

To elucidate whether the inhibitory effect of dopamine on the hypertrophy of VSMCs after stimulation with PDGF is causally linked to the increase in cellular cAMP, we examined the effect of an activator of adenylate cyclase, forskolin, on VSMC hypertrophy treated with PDGF-BB. The addition of forskolin reduced PDGF-BB-induced VSMC hypertrophy in a dose-dependent manner (Fig 4). Furthermore, a CAMP analogue, 8-bromo-CAMP, also reduced VSMC hypertrophy treated with PDGF-BB. Inhibition of PDGF-BB-induced VSMC hypertrophy by dopamine could be reproduced by this analogue at concentrations of 100 μmol/L and 1 mmol/L (Fig 4).

Effect of PKA Inhibitor on D1-Like Agonists Mediated Suppression of [3H]-leucine Incorporation

Incubation of VSMC with PKA inhibitor H 89 significantly reversed D1-like agonists dopamine-, SKF 38393-, and YM 435-mediated suppression of [3H]-leucine incorporation by PDGF-BB (5 ng/mL) for 48 hours. This action was not observed H 85, a negative control of H 89 (Fig 4).

Inhibition of MAPK Activity by D1-Like Receptor Agonists

Incubation of VSMC with D1-like receptor agonists dopamine, SKF 38393, and YM 435 10 μmol/L significantly inhibited MAPK activation by 50%, 23%, and 48%, respectively (see the Table). Treatment of VSMC with D1-like agonists alone, at the concentration used in these experiments, did not alter the basal MAPK activity (data not shown). PKA inhibitor H 89 10 μmol/L significantly reversed the dopamine-, SKF 38393-, and YM 435-mediated decrease in MAPK activity stimulated by PDGF-BB 5 ng/mL for 10 minutes.

Discussion

This study has demonstrated for the first time that D1-like receptor agonists inhibit the hypertrophy of VSMCs stimulated with PDGF in a concentration-dependent manner. PDGF-BB-induced VSMC hypertrophy was significantly inhibited by 1 to 10 μmol/L dopamine. Dopamine and YM 435 completely and SKF 38393 partially prevented PDGF-BB (5 ng/mL)-induced hypertrophy of VSMCs estimated by [3H]-leucine incorporation and cell size measured by flow cytometry. Although it has been reported that immunoreactive dopamine is present in human and rat plasma, the plasma free dopamine concentrations (~0.1 to 1 nmol/L) are much lower than those of dopamine that inhibited VSMC hypertrophy significantly in our in vitro study. Although there is no evidence to indicate that free dopamine is available to the VSMCs, local levels of dopamine in vascular tissues can be much higher than plasma concentration because it has been shown that considerable amount of dopamine is present in the conjugated form (0.1 to 1 μmol/L) and it has been reported that free dopamine may be formed through a deconjugation reaction when necessary. Taking that matter into account, our results suggest that dopamine may inhibit the hypertrophy of VSMCs after stimulation with such factors as PDGF. Consequently, it is possible
Fig 3 A, Histogram of relative size of postconfluent cells as measured by flow cytometric analysis. VSMCs were plated on T-25 flasks and cultured in DMEM with control, PDGF-BB 5 ng/mL, PDGF-BB 5 ng/mL plus dopamine 10 μmol/L, and PDGF-BB 5 ng/mL plus YM 435 10 μmol/L until confluent. Cultures were maintained in serum free media for 2 days to induce quiescence. Data presented are typical of four such experiments. The y axis shows cell numbers, the x axis represents cell size (log scale). B, Inhibitory effect of D1-like receptor agonists on chronic PDGF-BB-induced hypertrophy of VSMCs. Relative cell size was measured by flow cytometry as follows: Cell Size = \sum \frac{\text{Size of Each Channel} \times \text{Cell Number of the Channel}}{\text{Total Cell Number}}. Mean ± SD of mean cell size is described in figure *P < 0.05.

Fig 4 A, Incorporation of [3H]-leucine into protein after PDGF-BB stimulation for 48 hours of quiescent VSMC with the indicated doses of 8-bromo-cAMP and forskolin. B, Relative cell size evaluation after PDGF-BB stimulation for 48 hours of quiescent VSMCs with the indicated doses of 8-bromo-cAMP and forskolin. *P < 0.05.
that at least exogenous dopamine may antagonize the development of these vascular lesions as a hypertrophic factor for VSMCs, although we have no direct evidence in vivo at this time.

In the present study, dopamine did not inhibit the basal hypertrophic activity of nonstimulated VSMC. In trypan blue exclusion tests, dead cells stained with trypan blue were not found 24 hours after treatment with 10 μmol/L dopamine. Based on these observations and the finding that cultured VSMCs actively produce cAMP induced by dopamine,30,31 it is unlikely that the inhibitory effect of dopamine on VSMC migration observed in this study was due to its cytotoxicity.

We have obtained some evidence for a causal link between increase in cAMP production and the inhibition of VSMC hypertrophy treated with PDGF-BB. As shown in Fig 4, forskolin, an activator of adenylate cyclase and 8-bromo-cAMP, a cAMP analog, prevented a PDGF-induced increase in [3H]-leucine incorporation and relative cell size. Moreover, as shown in Fig 5, PKA inhibitor H 89 reversed this D1-like receptor agonist-mediated inhibition of protein synthesis. These results suggest that dopamine inhibits VSMC hypertrophy stimulated with PDGF-BB, probably through a cAMP-dependent process and PKA activation.

It has been reported that not only proliferative agents but also hypertrophic agents such as thromboxane A232 or angiotensin II33 induce MAPK activation. And it has also been reported that PKA antagonizes PDGF-induced signaling by MAPK in human VSMCs.34 We have obtained the results that PKA activation by the stimulation of D1-like receptors reduced MAPK activity (the Table), suggesting that this inhibition may play some role in antihypertrophic action of D1-like receptor agonists.

In conclusion, activation of D1-like receptors suppresses PDGF-BB-mediated VSMC hypertrophy through PKA activation and inhibition of activated MAPK activity. Further studies are required to clarify the exact cellular mechanisms of the inhibition by dopamine of VSMC hypertrophy and to elucidate the potential clinical significance of these findings.

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Effects of Dopamine, SKF 38393, and YM 435 on PDGF-Induced Activation of MAPK Activity

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<th>MAPK Activity, cpm/10^4 cells</th>
<th>Control</th>
<th>PDGF 5 ng/mL</th>
<th>PDGF 5 ng/mL + dopamine 10 μmol/L</th>
<th>PDGF 5 ng/mL + YM 435 10 μmol/L</th>
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<tr>
<td></td>
<td>1096 ± 16.0</td>
<td>509 ± 28.8</td>
<td>259 ± 11.0</td>
<td>263 ± 13.6</td>
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*Compared with control, p < 0.05.
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