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Trapidil inhibits platelet-derived growth factor-induced migration via protein kinase A and RhoA/Rho-associated kinase in rat vascular smooth muscle cells

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Abstract

Trapidil suppresses platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell (VSMC) proliferation by inhibiting Raf-1/extracellular signal-regulated kinase (ERK) via cAMP/protein kinase A (PKA). We examined whether trapidil inhibits PDGF-induced VSMC migration and investigated its mechanisms of action. VSMC migration was inhibited to a similar extent by trapidil and forskolin. A PKA inhibitor *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H89) blocked the inhibition by forskolin to a greater degree than that by trapidil. Trapidil but not forskolin suppressed PDGF-stimulated RhoA activation. In the presence of both H89 and (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate, an inhibitor of Rho-associated kinase (ROCK), trapidil and forskolin inhibited migration to a similar extent. Thus, in addition to cAMP/PKA activation, trapidil inhibits RhoA/ROCK activation, which may be important in trapidil's inhibitory effect on migration.

Keywords: Rho GTP-binding protein; Angioplasty; Trapidil; Protein–serine–threonine kinase; Forskolin

1. Introduction

Restenosis is the major limitation for the long-term efficacy of angioplasty. Injury to the arterial wall induces endothelial denudation, vascular smooth muscle cell (VSMC) migration and proliferation, resulting in the formation of neointimal hyperplasia (Schwartz et al., 1992). Among several growth factors capable of stimulating VSMC migration and proliferation, platelet-derived growth factor (PDGF) plays a critical role in the development of restenosis (Ferns et al., 1991). Trapidil (triazolopyrimidine), an anti-platelet drug with broad biological activities, has been demonstrated to reduce restenosis after angioplasty in animals as well as in humans (Maresta et al., 1994; Ohnishi et al., 1982). It inhibits PDGF-stimulated proliferation of VSMCs both in vivo and in vitro (Ohnishi et al., 1982; Hoshiya and Awazu, 1998). Its mechanism of action has previously been considered to be the competitive blockade at the receptor level (Gesualdo et al., 1994). A study from our laboratory, however, showed that trapidil did not affect tyrosine kinase activity of PDGF β-receptor in VSMCs (Hoshiya and Awazu, 1998). Trapidil's mechanism of action was shown to be the inhibition of extracellular signal-regulated kinase (ERK), a key enzyme in a wide range of cellular processes including proliferation. Stimulation of Raf-1, an upstream activator of ERK, by PDGF was also attenuated by trapidil. These actions of trapidil were accompanied by an increase in cellular generation of cAMP. In view of the evidences that cAMP/protein kinase A (PKA) inhibits Raf-1 (Graves et al., 1993), trapidil may antagonize mitogenic action of PDGF through cAMP/PKA.

While VSMC proliferation is an important event in restenosis, VSMC migration precedes proliferation. RhoA, a member of Rho family small GTPases, has been shown to be important in mediating migration in various cell types (Takai et al., 1995). Upon stimulation, RhoA translocates

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from the cytosolic to the membrane fraction and stimulates downstream targets including Rho-associated kinase (ROCK). Recent evidence suggests that RhoA/ROCK plays an important role in neointimal formation after vascular injury (Sawada et al., 2000; Shibata et al., 2001).

In the present study, we examined whether trapidil inhibits PDGF-stimulated VSMC migration. Since trapidil's antiproliferative effect is mediated by cAMP/PKA, we compared the effects of trapidil with an adenylyl cyclase activator forskolin. We also examined the effects of trapidil and forskolin on PDGF-induced RhoA activation and proliferation, events important in neointimal formation. The role of ROCK in PDGF-stimulated migration and proliferation was also examined using a specific ROCK inhibitor (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632). The results will allow us to identify a therapeutic target for the prevention of restenosis.

2. Materials and methods

2.1. Materials

Human recombinant PDGF-BB and bovine serum albumin were purchased from Sigma (St. Louis, MO). 4-(2-Aminoethyl)-benzenesulphonyl fluoride (AEBSF) was from Molecular Probes, Inc. (Eugene, OR). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin, streptomycin, trypsin–ethylene diamine tetra-acetic acid (EDTA) and Hank's balanced salt solution (HBSS) were from Gibco Laboratories (Grand Island, NY). Trapidil was a gift from Mochida Pharmaceutical Co. (Tokyo, Japan). Rabbit polyclonal anti-RhoA (119) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit IgG and goat anti-mouse IgG were from Amersham (Buckinghamshire, UK). Y-27632 was from Calbiochem-Novabiochem Corp. (San Diego, CA).

2.2. Cell culture

Rat aortic smooth muscle cells were isolated and cultured from 100 g to 180 g male Sprague–Dawley rats by enzymatic dispersion as previously described (Cornwell and Lincoln, 1989). Cells were grown in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin.

2.3. Migration

Cell migration was assessed using modified Boyden chambers containing transwell filters (6.5 mm diameter, 8 μm pores, Corning Costar, Acton, MA) coated on the underside with collagen type 1 (Upstate Biotechnology, Lake Placid, NY). Cells (10^6 cell/ml) were added to the upper chamber of the transwell filter with DMEM placed in the lower chamber. PDGF 25 ng/ml, trapidil 400 μg/ml, forskolin 10 μM, a PKA inhibitor *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H89) 10 μM or a ROCK inhibitor Y-27632 10 μM was added to the lower chamber. This dose of forskolin has previously shown to inhibit PDGF-induced VSMC migration completely (Yasunari et al., 1997; Sun et al., 2002). The dose of trapidil was chosen because in our previous study it inhibited PDGF-induced proliferation by 68% (Hoshiya and Awazu, 1998). After 3 h, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells on the lower side were fixed with 3.7% paraformaldehyde and stained with Wright–Giemsa solution. The number of migrated cells was counted in 4 fields under 400× magnification.

2.4. RhoA activation

RhoA activation was determined by using Rho activation assay biochem kit (Cytoskeleton Inc., Denver). The pull-down assay has previously been described (Ren et al., 1999). Briefly, cells were treated with PDGF 25 ng/ml in the presence or absence of trapidil 400 μg/ml or forskolin 10 μM for 1 h. Cell lysates were rotated with a glutathione *S*-transferase (GST)-fusion protein of the Rho binding domain (RBD) of the Rho effector protein rhotekin for 1 h at 4 °C. Beads were washed twice and resuspended in Laemmli buffer. The amount of activated RhoA was detected by immunoblot analysis as described below.

2.5. Subcellular fractionation

Cells were treated with PDGF in the presence or absence of trapidil or forskolin for 1 h or 16 h. Cell-free lysates were prepared by adding 100 μl hypotonic lysis buffer containing 20 mM Tris (pH 8.0), 3 mM $MgCl_2$, 0.4 mM AEBSF, 5 μg/ml aprotinin, 2 μg/ml trypsin inhibitor and 20 μM leupeptin. After three cycles of freeze and thaw, samples were centrifuged at 100,000×*g* at 4 °C for 60 min. The supernatant was saved as a "soluble" fraction. Pellets were washed twice by the same lysis buffer and resuspended in 100 μl of the lysis buffer supplemented with 1% Triton X-100 and 0.1% sodium dodecylsulfate (SDS). Cell debris was separated by centrifugation (14,000 rpm at 4 °C, 20 min) and supernatant was saved as a "particulate" fraction.

2.6. [^{3}H]Thymidine incorporation

Cells were grown in a 24-well dish and made quiescent by serum deprivation. After 24 h, cells were treated with vehicle or 25 ng/ml PDGF in the presence or absence of 400 μg/ml trapidil, 10 μM forskolin or 10 μM Y-27632. [^{3}H]Thymidine 1 μCi was then added to the wells. After incubation for 16 h, cells were washed with ice-cold phosphate buffered saline (PBS) and 5% trichloroacetic acid, solubilized in 0.2 N NaOH and counted by a liquid scintillation counter.

2.7. Immunoblot analysis

The protein content in cell lysates was measured using DC protein assay (Bio-Rad Laboratories, Tokyo, Japan). Lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corp, Bedford, MA). Non-specific binding sites were blocked in TBS-T buffer (10 mM Tris–Cl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20) containing 5% skim milk overnight at 4 °C or for 1 h at 25 °C. Antibodies were added to TBS in saturating titers and incubated with mixing for 2 h at 25 °C. Blots were washed two times, then incubated with goat anti-mouse IgG for pull-down assay and goat anti-rabbit IgG for translocation assay for 1 h with mixing and washed. Bound antibodies were detected using the ECL Western blotting system (Amersham, Buckinghamshire, UK). At least three independent experiments were performed with similar results.

2.8. Statistical analysis

The results are expressed as mean±S.E. Statistical analysis was performed with Student's *t*-test or analysis of variance followed by multiple comparisons as appropriate. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Trapidil or forskolin inhibits PDGF-induced migration

Trapidil 400 μg/ml and forskolin 10 μM inhibited PDGF-stimulated VSMC migration to the same extent (20±2% and 20±2% of PDGF alone, respectively, Fig. 1). A specific ROCK inhibitor Y-27632 also inhibited PDGF-induced migration (28±3% of PDGF alone), which is in agreement with previous studies (Sawada et al., 2000). In the presence of a PKA inhibitor H89, the inhibition by forskolin was alleviated to 74±7% of PDGF alone, but that of trapidil was reduced to only 52±4% of PDGF alone ($P<0.05$ vs. forskolin). In the presence of both H89 and Y-27632, the inhibition of PDGF-stimulated VSMC migration by trapidil and forskolin were statistically equivalent (40±3% vs. 44±4% of PDGF alone, NS). Of note, H89 alone inhibited PDGF-stimulated VSMC migration (20±2% of PDGF alone).

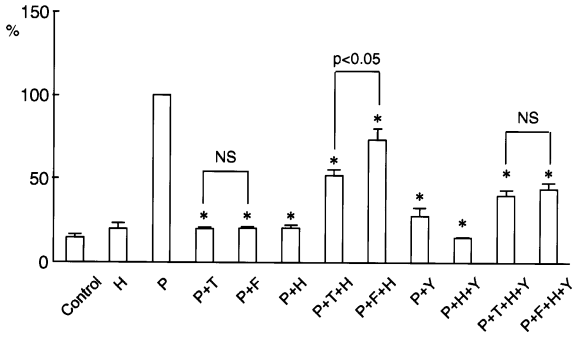

Fig. 1. Trapidil or forskolin inhibits PDGF-stimulated migration of rat vascular smooth muscle cells (VSMCs). VSMCs were treated with vehicle (control) or 25 ng/ml PDGF (P) in the presence or absence of 400 μg/ml trapidil (T), 10 μM forskolin (F), 10 μM H89 (H), a PKA inhibitor or 10 μM Y-27632 (Y), a ROCK inhibitor. Cell migration assay was performed as described in Section 2 ($n=4$, mean±S.E.). *$P<0.05$ vs. PDGF.

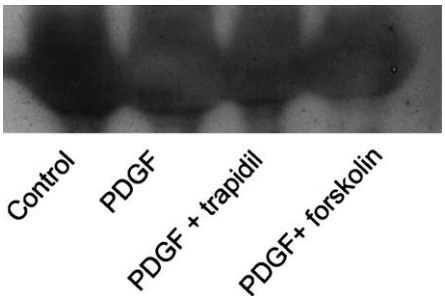

Fig. 2. Trapidil but not forskolin inhibits PDGF-stimulated RhoA activation. Quiescent VSMCs were incubated with vehicle (control) or 25 ng/ml PDGF, in the presence or absence of 400 μg/ml trapidil or 10 μM forskolin for 1 h. Cells were then lysed and incubated with a GST-fusion protein of the RBD of rhotekin for 1 h at 4 °C. Beads were washed twice and resuspended in Laemmli buffer. The amount of activated RhoA was detected by immunoblot analysis.

3.2. Trapidil but not forskolin inhibits RhoA activation

Since the small G protein RhoA is implicated in cell migration, we next examined the effects of trapidil and forskolin on PDGF-stimulated RhoA activation. As shown in Fig. 2, RhoA was activated by PDGF. While trapidil inhibited PDGF-induced RhoA activation, forskolin had no effect. Activation of RhoA was also examined indirectly. RhoA is known to be translocated from the soluble to the particulate fraction upon activation. Treatment with PDGF caused a significant increase (≈200%) in the RhoA content of the particulate fraction (Fig. 3). The translocation occurred at 1 h (data not shown) and was observed at least until 16 h after stimulation (Fig. 3). Trapidil completely

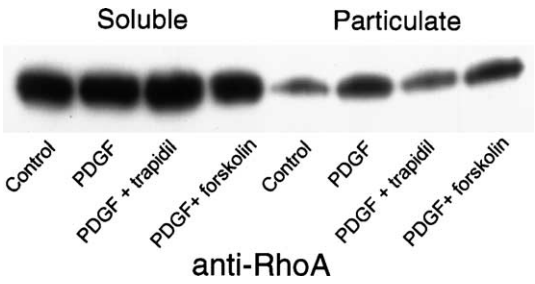

Fig. 3. Trapidil but not forskolin inhibits PDGF-stimulated RhoA translocation. Quiescent VSMCs were incubated with vehicle (control) or 25 ng/ml PDGF, in the presence or absence of 400 μg/ml trapidil or 10 μM forskolin for 16 h. Cells were then fractionated as described in Section 2 and immunoblotted with anti-RhoA antibody.

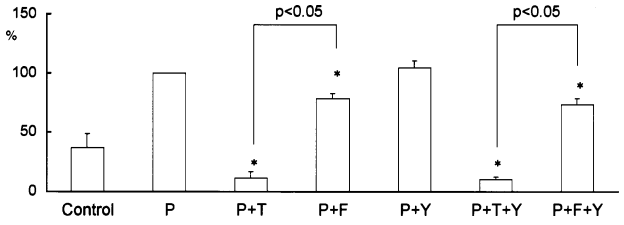

Fig. 4. Trapidil or forskolin inhibits PDGF-stimulated [^{3}H]thymidine incorporation. Quiescent VSMCs were treated with vehicle (control) or 25 ng/ml PDGF (P) in the presence or absence of 400 μg/ml trapidil (T), 10 μM forskolin (F) or 10 μM Y-27632 (Y). Cells were then incubated in the presence of [^{3}H]thymidine for 16 h ($n=4$, mean±S.E.). *$P<0.05$ vs. PDGF.

reversed the soluble to particulate translocation of RhoA, whereas forskolin had no effect.

3.3. Trapidil or forskolin inhibits PDGF-induced [^{3}H]thymidine incorporation

We next compared the effects of trapidil and forskolin on PDGF-induced [^{3}H]thymidine incorporation. Both trapidil and forskolin attenuated PDGF-stimulated [^{3}H]thymidine incorporation, but the degree of inhibition by trapidil was significantly greater than that by forskolin (12±1% vs. 77±4% of PDGF alone, $P<0.05$, Fig. 4). Cell viability was not affected as assessed by the Trypan blue dye exclusion test, indicating that the effect of trapidil is not due to nonspecific toxicity. Y-27632 did not inhibit PDGF-induced [^{3}H]thymidine incorporation nor affected the inhibition of DNA synthesis by trapidil or forskolin.

4. Discussion

The present study demonstrates that trapidil may inhibit PDGF-induced VSMC migration by activating PKA and inhibiting RhoA/ROCK. Thus, trapidil 400 μg/ml and an adenylyl cyclase activator forskolin 10 μM inhibited VSMC migration to the same extent. This dose of forskolin is known to exert a maximal effect on PDGF-induced VSMC migration (Yasunari et al., 1997; Sun et al., 2002). In the presence of a PKA inhibitor H89, trapidil inhibited VSMC migration to a greater degree than forskolin. In the presence of both H89 and a ROCK inhibitor Y-27632, there was no difference in the antimigratory effect between trapidil and forskolin. These results suggest that trapidil has two modes of action on cell migration, i.e. activation of PKA and inhibition of ROCK. In support of this notion, trapidil inhibited PDGF-stimulated activation of RhoA, an upstream activator of ROCK. Forskolin, on the other hand, exerted no effect on RhoA activation. Recently, blockade of RhoA/ROCK has been shown to suppress neointimal formation in animals. Thus, Sawada et al. (2000) and Shibata et al. (2001) reported that systemically given Y-27632 prevented neointimal formation of balloon-injured carotid arteries in rats. Y-27632 has also been shown to block thrombin- and PDGF-induced migration of cultured VSMCs (Seasholtz et al., 1999; Sawada et al., 2000).

The inhibition of VSMC migration by cAMP raising-agents has previously been demonstrated, but the cellular mechanism has not been elucidated (Palmer et al., 1998). The present study suggests that the inhibition of PDGF-stimulated VSMC migration by PKA does not involve RhoA/ROCK. Of note, H89 alone inhibited PDGF-stimulated migration. PDGF has been shown to stimulate PKA in human VSMC through ERK-mediated activation of cytosolic phospholipase A2 (Graves et al., 1996). In fibroblasts, inhibition of PKA blocked growth factor-stimulated migration (Edin et al., 2001). Thus, the antimigratory effect of H89 in cells treated with PDGF alone is most likely due to the inhibition of PDGF-stimulated PKA. In cells treated with trapidil or forskolin, H89 is considered to inhibit PKA stimulated by trapidil or forskolin, thus reversing the antimigratory effects of these agents.

RhoA has also been implicated in cell proliferation. Seasholtz et al. (1999) reported that RhoA partly mediates thrombin-stimulated DNA synthesis. Y-27632 inhibited thrombin- and PDGF-stimulated VSMC DNA synthesis suggesting a role of ROCK (Seasholtz et al., 1999; Sawada et al., 2000). In our study, however, Y-27632 did not inhibit PDGF-stimulated DNA synthesis. Shibata et al. (2001) also showed that Y-27632 did not affect the number of 5′-bromodeoxyuridine-positive (BrdU) cells in the injured intima. The reason for these discrepant results is unknown. In the study by Sawada et al. (2000) which showed the antimitogenic effect of Y-27632, the inhibition of migration by Y-27632 was significantly smaller than that in our study (72% compared with 28±3% of PDGF alone in our study). Thus, the involvement of ROCK in migration and proliferation may vary among different cell preparations.

The degree of inhibition of PDGF-induced [^{3}H]thymidine incorporation by trapidil was significantly greater than that by forskolin. While ROCK does not appear to explain the more potent antimitogenic action of trapidil, RhoA may have a role. Recent evidence showed that RhoA collaborate with Ras to promote cell cycle progression and proliferation (Weber et al., 1997; Olson et al., 1998; Seasholtz et al., 1999). RhoA has been shown to be necessary for Ras-induced DNA synthesis in fibroblasts (Weber et al., 1997; Olson et al., 1998). In VSMCs as well, RhoA and Ras have been shown to have synergistic effect on DNA synthesis (Seasholtz et al., 1999). RhoA has downstream target molecules other than ROCK. Thus, another intermediary pathway may be involved in the regulation of DNA synthesis. Alternatively, trapidil may have other modes of action than stimulation of PKA and inhibition of RhoA. One possibility is trapidil's inhibitory effect on phosphodiesterases (Bethke et al., 1991). Thus, the lower potency of forskolin than trapidil to inhibit mitogenesis may be due to the rapid break down of generated cAMP.

The mechanism of the inhibition of RhoA activation by trapidil remains to be determined. We previously speculated that trapidil's effect was mediated by cAMP. However, the increase in cellular cAMP generation by trapidil was relatively minor (Hoshiya and Awazu, 1998). Recently, Bönisch et al. (1998) reported that low concentrations of trapidil directly activated PKA without affecting cAMP generation or phosphodiesterase in VSMCs. They subsequently demonstrated that trapidil increased the activity of PKAII isoforms by a cAMP-sensitizing effect (Sichelschmidt et al., 2003). They also showed that cAMP did not fully activate PKAII. Thus, the different actions of trapidil and forskolin on RhoA may be explained by their divergent mechanism to activate PKA. It has been known that different subtypes of PKA with different substrate specificity are activated by specific agents (Scott, 1991), and a recent work by Gamm et al. (1996) has demonstrated that a certain isozyme of PKA is preferentially activated by cAMP. Alternatively, trapidil's action on RhoA may be explained by effects other than PKA activation such as inhibition of phosphodiesterases (Bethke et al., 1991) or thromboxane A2 generation (Ohnishi et al., 1981).

In conclusion, trapidil inhibits PDGF-induced cell migration and RhoA activation in VSMCs. These actions of trapidil are associated with more potent inhibition of DNA synthesis than forskolin. While activation of cAMP/PKA by 8-Br-cAMP or phosphodiesterase inhibitors has been shown to be efficacious in inhibiting VSMC proliferation induced by balloon injury in rats (Indolfi et al., 1997), their efficacy in humans has not yet been demonstrated. Trapidil is one of the few drugs proven to be effective for post angioplasty restenosis in humans (Maresta et al., 1994; Hirayama et al., 2003). Targeting RhoA/ROCK in addition to Raf-1/ERK pathway may be important in the treatment of postangioplasty restenosis.

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