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European Journal of Pharmacology 515 (2005) 28 – 33

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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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Received 22 March 2005; accepted 8 April 2005
Available online 13 May 2005

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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0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.04.013

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.