Trapidil Inhibits Platelet-Derived Growth Factor–Stimulated Mitogen-Activated Protein Kinase Cascade

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Abstract—Trapidil, an antiplatelet drug, has been shown to reduce restenosis after angioplasty. It exerts its action, at least in part, by inhibiting vascular smooth muscle cell proliferation, antagonizing platelet-derived growth factor (PDGF). We examined its site of action on PDGF cellular signaling. Exposure of cultured rat vascular smooth muscle cells to increasing concentrations of trapidil for 18 hours resulted in a dose-dependent reduction in PDGF-BB–stimulated [3H] thymidine incorporation. Trapidil (400 μg/mL) increased PDGFβ-receptor protein by 28±8%, whereas PDGF-induced tyrosine phosphorylation of PDGFβ-receptor remained unchanged. PDGF-induced tyrosine phosphorylation of phospholipase Cγ, the p85 regulatory subunit of phosphatidyl-inositol 3 kinase, Ras GTPase–activating protein, and an adaptor molecule Shc were also not altered. On the other hand, trapidil inhibited PDGF-stimulated mitogen-activated protein kinase (MAP kinase) activity by 35±6% at 10 minutes and by 32±10% at 6 hours. Activation of Raf-1, an upstream activator of MAP kinase, by PDGF was also attenuated by trapidil. Moreover, protein content of MAP kinase phosphatase-1, which inactivates MAP kinase, was elevated in trapidil-treated cells. These actions of trapidil may be mediated by cAMP. Thus, there was a 1.9-fold increase in cellular cAMP generation in trapidil-treated cells. The present results demonstrate that trapidil antagonizes PDGF-induced mitogenesis and MAP kinase activation in vascular smooth muscle cells, probably through cAMP. (Hypertension. 1998;31:665-671.)

Key Words: trapidil ■ growth factors, platelet-derived ■ restenosis ■ kinases ■ mitogenesis ■ cyclic AMP

Restenosis after angioplasty, occurring in 30% to 50% of patients, is the major limitation for the long-term effectiveness of this procedure. It is a complex phenomenon that is characterized by neointimal hyperplasia resulting from VSMC migration and proliferation.1 PDGF is one of the growth factors and substances released by the damaged intimal surface and platelets that are implicated as mediators of these processes. In support for the role of PDGF, previous studies have demonstrated that anti-PDGF antibody ameliorated neointimal smooth muscle cell accumulation after balloon injury in rats.2 Furthermore, the PDGF antagonist trapidil (triazolopyrimidine) has been shown to reduce restenosis in both animals and humans.3,4 Trapidil is an antiplatelet drug as well as a coronary vasodilator that exerts its action by inhibiting thromboxane A2 and stimulating the synthesis and release of prostacyclin.5,6 Moreover, trapidil has been shown to antagonize PDGF action. It inhibits PDGF-stimulated proliferation of several cell lines, including VSMC both in vivo and in vitro.7,8 Its mechanism of action has been considered to be the competitive blockade at the receptor level. Thus, Gesualdo et al.,8 using mesangial cells, demonstrated that trapidil interferes with the binding of PDGF to its receptor after short-term incubation of 2 hours. However, 48-hour exposure to trapidil caused a marked increase in PDGF binding. Because the effect of trapidil in vivo has been demonstrated after subacute to chronic administration, blockade at the receptor level seems unlikely to be the mechanism.3,4

Cellular signaling for PDGF requires ligand activation of receptor, intrinsic receptor tyrosine kinase activation, and autophosphorylation of receptor. Autophosphorylation on multiple tyrosine residues increases the ability to bind and phosphorylate other proteins such as PLCγ, Ras GAP, PI3K, and an adaptor molecule Shc.9 Recent works suggest that complexes of Shc, Grb2, and the guanine nucleotide releasing factor Sos lead to the activation of the MAP kinase cascade, which is considered to be a critical step for proliferation.10 PDGF-induced signal transduction cascades were studied to examine the mechanisms of action for trapidil. This information may provide clues to the signaling events that can be targeted for the intervention of restenosis.

Methods

Materials

Human recombinant PDGF-BB, MBP, protein kinase inhibitor, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and BSA were purchased from Sigma Chemical Co. Monoclonal anti-
phosphotyrosine antibody (PY20) was from Transduction Laboratories. Anti-human PDGFR, anti-human Shc, anti-Pi3K (p85 subunit), anti-human GAP, anti-Bovine PLC, and anti-rat MAP kinase R2 from were from Upstate Biotechnology Inc. Anti-Raf-1 (C-12) and anti-MKP-1(C-19) were from Santa Cruz Biotechnology, Inc. Wheat germ lectin-Sepharose 6MB and protein A Sepharose 4 fast flow were from Pharmacia. IgG sorb was from The Enzyme Center. N,N,N’,N’-triacetylchitotriose was from EY Laboratories Inc. RPMI-1640, fetal bovine serum, penicillin, streptomycin, and trypsin-EDTA were from Gibco Laboratories. Trapidil was a gift from Mochida Pharmaceutical Co.

Cell Culture

Rat aortic smooth muscle cells were isolated and cultured from 100- to 180-g male Sprague-Dawley rats by enzymatic dispersion as previously described.11 Cells were grown in RPMI containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. At passage 4 to 8, confluent cells were serum deprived, treated with trapidil or vehicle for 18 hours, and stimulated with PDGF-BB (10 ng/mL, final concentration) for 10 minutes or 6 hours at 37°C. Cell viability was not affected by trapidil as indicated by the Trypan blue dye exclusion test. Cells were washed with cold PBS and lysed in solubilization buffer containing 20 mmol/L HEPES (pH 7.2), 1% Triton-100, 10% glycerol, 20 mmol/L sodium fluoride, 1 mmol/L MgCl2, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Insoluble material was removed by centrifugation (10,000g for 10 minutes). The protein content in cell lysates was measured with a DC protein assay (Bio-Rad Laboratories).

[3H] Thymidine Incorporation

Cells were grown in a 24-well dish and then made quiescent by serum deprivation. After 24 hours, cells were treated with 200 to 800 μg/mL trapidil or vehicle. Then 1 μCi [3H] thymidine was added to the wells with PDGF 10 ng/mL. After incubation for an additional 24 hours, cells were washed with ice-cold PBS and 5% trichloroacetic acid, solubilized in 0.2 N NaOH, and counted by a liquid scintillation counter.

Immunoblot Analysis

Cleared lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp). Non-specific binding sites were blocked in TBS buffer (10 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl) containing 5% BSA overnight at 4°C. Antibodies were added to TBS with 5% BSA in saturating titers and incubated with mixing for 2 hours at 25°C. Blots were washed twice and then developed (anti-phosphotyrosine antibody). For analyses with anti-MAP kinase, anti-Raf-1, and anti-MKP-1 antibodies, blots were further incubated with goat anti-rabbit IgG/alkaline phosphatase conjugate for 1 hour. Membranes were washed once in substrate buffer (0.1 mol/L Tris-HCl, pH 9.5, 0.1 mol/L NaCl, and 5 mmol/L MgCl2) and then developed by the addition of fresh substrate (20 ng nitroblue tetrazolium in 60 mL of substrate buffer, mixed immediately before blot exposure with 10 mg of 5-bromo-4-chloro-3-indolyl phosphate in 200 μL N,N-dimethylformamide). Blots were scanned and quantitatively analyzed by NIH Image.

Lectin Affinity Step and Tyrosine Kinase Assay

Cell lysates were incubated with wheat germ lectin-Sepharose 6MB with continuous mixing for 2 hours at 4°C; lectin-affinity–recovered proteins were eluted in kinase buffer (20 mmol/L HEPES, 20 mmol/L MnCl2, 150 mmol/L NaCl, 0.1% Triton-100, 1 mmol/L sodium orthovanadate, and 1 mmol/L PMSF) containing 3 mmol/L N,N,N’,N’-triacetylchitotriose. PDGF tyrosine kinase activity was assayed in a final volume of 50 μL kinase buffer containing 20 mmol/L MgCl2, PDGF (50 ng/mL) was added for 15 minutes at 0°C before the addition of 20 μmol/L ATP. Trapidil was added at the same time as ATP. After 30 minutes of incubation at 22°C, reactions were stopped by the addition of 13 μL of 5X Laemmli’s SDS-PAGE sample buffer. Immunoblotting with anti-phosphotyrosine was performed as described above.

Immunoprecipitation

Cell lysates containing 400 μg of protein were incubated with 2 μg of anti-phosphotyrosine. Immunoprecipitates were collected by incubation with 25 μL of protein A Sepharose for 1 hour. Immunoprecipitated proteins were released from protein A Sepharose by incubation at 95°C in 10 μL of Laemmli’s buffer. Samples were analyzed by immunoblotting with indicated antibodies as described above.

Immune Complex MAP Kinase Assay

Cell lysates (500 μg) were incubated with 2 μL of anti-MAP kinase antibody. Immunoprecipitates were collected by incubation with protein A Sepharose. Assays were performed at 25°C for 15 minutes in a final volume of 50 μL containing 25 mmol/L Tris-HCl, pH 7.4, 10 mmol/L MgCl2, 1 mmol/L DTT, 1 mmol/L EGTA, 1 μmol/L protein kinase inhibitor, 50 μmol/L ATP, 2 μCi γ-[32P] ATP, 20 μg MBP, and 20 μL of Sepharose beads. The reactions were terminated by the addition of 13 μL Laemmli’s buffer. MBP was phosphorylated and phosphorylated protein was separated by SDS-PAGE. After autoradiography to visualize phosphorylation of MBP, radioactivity was determined with a liquid scintillation counter.

cAMP Generation Studies

After preincubation with 400 μg/mL trapidil or vehicle for 18 hours, cells were washed with ice-cold PBS and extracted with 0.5 mL of 10% trichloroacetic acid. The acid extracts were treated four times

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**Figure 1.** Trapidil inhibits PDGF-stimulated [3H] thymidine incorporation in VSMC. Data are presented as mean±SE (n=3). *P<.01 vs PDGF alone.
with 1.5 mL of water-saturated ether, lyophilized, and kept at 20°C.
cAMP was determined with the cAMP assay system from Amersham.

Statistical Analysis
The results are expressed as mean±SE. Statistical analysis was
performed with Student’s t test or ANOVA followed by multiple
comparisons as appropriate. Differences were considered statistically
significant at P<.05.

Results
Effect of Trapidil on [3H]
Thymidine Incorporation
PDGF-stimulated [3H] thymidine incorporation was signifi-
cantly attenuated by trapidil (Fig 1). Thus, incubation of
VSMC with increasing concentrations of trapidil (200, 400,
and 800 µg/mL) for 18 hours resulted in a dose-dependent
reduction in PDGF-stimulated [3H] thymidine incorporation
(62±3%, 68±1%, and 85±1% inhibition, respectively; n=3,
P<.01 versus PDGF alone).

Effect of Trapidil on PDGF Protein and
Tyrosine Kinase Activity
Exposure to 400 µg/mL trapidil for 18 hours caused an
increase in PDGF protein by 28±8% (n=4, P<.05) as
detected by immunoblotting with anti-PDGFR (Fig 2A).
However, tyrosine phosphorylation of 180 kD PDGFR, after
incubation with PDGF 10 ng/mL for 10 minutes, was not
different between trapidil-treated and untreated cells (Fig 2B).
Similarly, tyrosine phosphorylation of other cellular protein
was not altered by trapidil. Trapidil also had no direct effect on
in vitro tyrosine kinase activity of PDGFR, as assessed with
PDGFR purified by lectin-affinity chromatography (Fig 2C).
Thus, the addition of 200 to 800 µg/mL trapidil during in
vitro incubation with ATP did not alter tyrosine phosphory-
lation of PDGFR.

Effect of Trapidil on PDGF-Induced Tyrosine
Phosphorylation of PLCγ, GAP, PI3K, and Shc
The effect of trapidil on PDGF-induced tyrosine phosphory-
lation of PLCγ, GAP, PI3K, and Shc was examined by

Figure 2. Effect of trapidil on PDGFR. A, Trapidil increases PDGFR protein. Quiescent cells were treated with vehicle (lane 1) or 400
µg/mL trapidil for 18 hours (lane 2). Cell lysates were immunoblotted with anti-PDGFR antiserum; representative of four experiments.
Three independent isolations of VSMC were used. B, Trapidil does not affect PDGF-stimulated PDGFR tyrosine kinase activity in vivo.
Cells were exposed to vehicle (lanes 1 and 2) or 400 µg/mL trapidil for 18 hours (lane 3) and stimulated with 10 ng/mL PDGF for 10
minutes at 37°C (lanes 2 and 3). Cell lysates were immunoblotted with anti-phosphotyrosine (anti-PTyr); representative of three experi-
ments. Two independent isolations of VSMC were used. Left margin indicates Mr ×10^-3. C, Trapidil does not affect PDGFR tyrosine
kinase activity in vitro. Lectin affinity recovered proteins from unstimulated cell lysates were assayed for kinase activity. PDGF (50
ng/mL) was added for 15 minutes at 0°C; 20 µmol/L ATP then was added with or without trapidil (200, 400, or 400 µg/mL). After 30
minutes at 22°C, reactions were stopped and analyzed by immunoblotting with anti-PTyr.
which were attenuated by trapidil. Thus, the phosphorylated/unphosphorylated p42 MAP kinase ratio was 1.0 in trapidil-treated cells compared with 2.5 for PDGF alone, with the ratio for p44 MAP kinase being 1.5 and 3.8, respectively.

**Effect of Trapidil on PDGF-Induced Activation of Raf-1**

We then examined the effect of trapidil on an upstream activator of MAP kinase, Raf-1. PDGF-induced hyperphosphorylation of Raf-1, which parallels its activation, was observed at 10 minutes (Fig 4C). The electrophoretic mobility shift was partially inhibited by trapidil.

**Effect of Trapidil on MKP-1 Expression**

MAP kinase activity is determined by the balance of phosphorylation and dephosphorylation of threonine and tyrosine residues on the enzyme. The protein expression level of MKP-1, a dual-specificity phosphatase that inactivates MAP kinase, was increased in trapidil-treated cells by \( \approx 40\% \) (Fig 4D). The results suggest that trapidil affects dephosphorylation as well as phosphorylation of MAP kinase.

**Effect of Trapidil on cAMP Generation**

Trapidil has been reported to inhibit phosphodiesterase and to stimulate prostacyclin production, both of which lead to an increase in cAMP generation. Because cAMP has been shown to block PDGF cellular signaling by inhibiting Raf-1, we examined whether trapidil increased cAMP generation. As shown in Fig 5, baseline cellular cAMP generation was significantly higher in trapidil-treated cells (22.3 \( \pm \) 1.0 versus 12.0 \( \pm \) 1.3 mmol/mg protein, \( n=6 \), \( P<.05 \)). When stimulated with PDGF, cAMP generation decreased significantly at 10 minutes and returned toward the baseline values at 6 hours in trapidil-treated cells. Although the difference became smaller after PDGF stimulation, cAMP levels were still 1.3- and 1.5-fold higher in trapidil-treated cells than in control cells.

**Discussion**

The present study demonstrates that trapidil inhibits PDGF-induced mitogenesis and cellular signaling by MAP kinase in VSMC. The antagonism by trapidil was not at the level of PDGFR as previously thought. Gesualdo et al demonstrated that trapidil acutely inhibited PDGF binding to its specific surface receptors in mesangial cells. However, 48 hours of incubation with trapidil increased both PDGF binding and PDGFR mRNA transcript level. In agreement with their data, we observed an increase in PDGFR protein after 18 hours of incubation with trapidil in VSMC. To examine the functional consequences of the increased PDGFR in trapidil-treated cells, we performed both in vivo and in vitro PDGFR tyrosine kinase assays. In vivo PDGF stimulation caused similar increases in tyrosine phosphorylation of PDGFR as well as other cellular proteins in trapidil-treated cells compared with controls. Likewise, direct addition of trapidil had no effect on in vitro PDGFR protein kinase activity. These data indicate that although trapidil may inhibit PDGF binding to its receptor, it does not prevent tyrosine kinase activation of PDGFR, a first step for PDGF signaling. PDGFR has been shown to be downregulated by its own ligand. Thus, an increase in...
PDGFR after chronic exposure to trapidil may be a compensatory response to inhibited PDGF binding rather than the mechanism for the action of trapidil.

Because the blockade at the receptor level is unlikely to be the cause of the growth inhibition by trapidil, we next examined tyrosine phosphorylation of PDGFR tyrosine kinase substrates such as PLCγ, GAP, PI3K, and Shc. A principal signal transduction pathway by which PDGF stimulates cell growth involves the activation of Ras guanine nucleotide–binding proteins. Recent data suggest that an adaptor molecule Shc acts as a docking protein for Grb2 to bind to PDGF.\(^\text{17}\) Grb2 then forms a complex with the guanine nucleotide–releasing factor Sos.\(^\text{10}\) Complexes of Shc, Grb2, and Sos activate Ras, which in turn activates Raf-1 kinase, an upstream activator of MAP kinase.\(^\text{18}\) Ras activity is negatively regulated by GAP. PLCγ is an enzyme that produces diacylglycerol and inositol trisphosphate, leading to protein kinase C activation and an increase in intracellular calcium. PLCγ and PI3K (another PDGFR substrate) are also implicated in PDGF-induced mitogenesis.\(^\text{19}\) In our study, trapidil had no effect on tyrosine phosphorylation or protein content of any of these PDGF substrates, suggesting that the site of action for trapidil is downstream from these signaling proteins.

MAP kinase, also known as extracellular signal–regulated kinase, is a critical enzyme used by many growth factors and substances to regulate various cellular functions including proliferation.\(^\text{20}\) MAP kinase is activated by phosphorylation on both tyrosine and threonine and inactivated by dephosphorylation of either residue.\(^\text{12}\) The direct upstream activator of MAP kinase is MAP kinase kinase (MEK), which is in turn activated by upstream serine/threonine kinase Raf-1. PDGF-induced MAP kinase activation is reported to be biphasic in VSMC as well as in mesangial cells.\(^\text{21,22}\) Thus, a rapid increase in activity, maximal at 5 to 10 minutes, is followed by a lower sustained activity at 4 to 6 hours.\(^\text{22}\) The second sustained activation of MAP kinase appears to be essential for proliferation. First, angiotensin II, a hypertrophic rather than a hyperplastic substance, causes only a transient activation of MAP kinase.\(^\text{23}\) Second, blockade of the sustained MAP kinase

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**Figure 4.** Effect of trapidil on PDGF-stimulated MAP kinase cascade. A, Trapidil inhibits PDGF-induced MAP kinase activity. Cells were treated with vehicle (CON) or 400 μg/mL trapidil for 18 hours and stimulated with 10 ng/mL PDGF for 10 minutes or 6 hours at 37°C. Cell lysates were immunoprecipitated with anti-MAP kinase antibody, and \(^{32}\)P ATP incorporation into MBP was measured; representative autoradiography of three experiments. B and C, Trapidil attenuates PDGF-induced gel mobility shifts of MAP kinase and Raf-1. Immunoblotting with anti-MAP kinase antibody (B), anti-Raf-1 (C). Representative of three experiments. D, Trapidil increases protein expression of MKP-1. Anti-MKP-1 immunoblotting. Representative of three experiments.

**Figure 5.** Trapidil increases cellular cAMP generation. Cells were treated with vehicle (CON) or 400 μg/mL trapidil for 18 hours and stimulated with PDGF for 10 minutes or 6 hours (n=6). *P<.05 vs control (CON).
activation by the transient expression of an MAP kinase antisense RNA or a dominant-negative mutant inhibited cell proliferation. In the present study, trapidil inhibited MAP kinase activity similarly at 10 minutes and 6 hours after PDGF stimulation.

MAP kinase activity is regulated by upstream kinases such as MEK and Raf-1, by MAP kinase phosphatases, and by an abundance of the enzyme. Trapidil had no effect on the protein expression of MAP kinase. On the other hand, PDGF-induced mobility shift of MAP kinases as well as Raf-1 were attenuated in trapidil-treated cells. The results suggested that trapidil inhibits MAP kinase cascade at the level of Raf-1. Recent data showed that cAMP inhibits MAP kinase activation by several growth factors by preventing activation of Raf-1.16,17 In the present study, trapidil increased cellular cAMP generation at baseline as well as after PDGF stimulation. We have not added phosphodiesterase inhibitors to the incubation medium because inhibition of phosphodiesterase by trapidil has previously been reported and may account for cAMP generation.11 Although trapidil-induced increases in cAMP accumulation were relatively small (1.3–1.9-fold), a recent study by Matousovic et al18 using mesangial cells showed that phosphodiesterase inhibitors suppressed cellular mitogenesis by activating PKA without a detectable increase in cellular cAMP levels. The authors speculate that a specific cAMP compartment metabolized by certain phosphodiesterases is linked to PKA, which in turn phosphorylates Raf-1, leading to MAP kinase cascade blockade. Their results are compatible with our finding that a small increase in cAMP results in the marked inhibition of mitogenesis. In addition to the inhibition of phosphodiesterase, stimulation and release of prostacyclin may also be responsible for the increase in cAMP by trapidil.19 Taken together, the actions of trapidil shown in the present study are most likely mediated by the cAMP-PKA pathway.

MAP kinase activity is negatively regulated by phosphatases. MKP-1 is a member of the dual-specificity phosphatase family, which exhibits dual catalytic activity toward phosphotyrosine and phosphothreonine. MAP kinase has been demonstrated to be dephosphorylated and inactivated by MKP-1 in vivo in various cell lines, including VSMCs.20,21 Of note, cAMP-elevating agents have been shown to induce MKP-1 in fibroblasts.22 Thus, trapidil inhibits PDGF-stimulated MAP kinase activity by both inhibiting the activation and enhancing the inactivation of the enzyme, probably through cAMP.

The redundancy in growth-factor signaling pathways has led to the suggestion that targeting a single growth factor may not be effective in vascular therapy.23 In this regard, trapidil has been shown to block proliferation stimulated by fetal bovine serum, epidermal growth factor, and basic fibroblast growth factor in addition to PDGF.4 The MAP kinase cascade is activated by these growth factors, as well as by angiotensin II, endothelin, or thrombin, other substances implicated in the pathogenesis of restenosis.24 Our results, together with the clinical effectiveness of trapidil, may underscore the importance of targeting components of the signaling cascade that are shared by many growth factors as a therapeutic approach.

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References
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