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Increased phosphodiesterase 3A/4B expression after angioplasty and the effect on VASP phosphorylation ☆

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ABSTRACT

The beneficial effects of coronary angioplasty are limited by the proliferation and migration of vascular smooth muscle cells leading to restenosis. We hypothesized that increased activity of phosphodiesterase (PDE) after angioplasty in response to growth factors such as platelet-derived growth factor (PDGF)-BB and fibroblast growth factor (FGF), leads to reduced cAMP levels, which, in turn, may contribute to vascular smooth muscle cell proliferation. In rats subjected to angioplasty, aortic expression and activity of PDE3/PDE4 were increased within 24 h and associated with reduced phosphorylation of vasodilator-stimulated phosphoprotein (VASP), a substrate for cAMP-dependent protein kinase A (PKA). Inhibition of PDE3 increased VASP phosphorylation in aortic rings from rats subjected to angioplasty, whereas inhibition of PDE4 or stimulation of adenylate cyclase with isoproterenol was without effect; however, combined inhibition of PDE3 and PDE4 produced a synergistic effect on VASP phosphorylation. In cultured vascular smooth muscle cells, exposure to PDGF-BB resulted in increased expression of PDE3, which was prevented by an inhibitor of PI3 kinase but not by inhibitors of the MAP kinase signaling pathway. In contrast, FGF increased the expression of PDE4 in vascular smooth muscle cells but did not influence expression of PDE3. This study shows that angioplasty results in increased expression/activity of PDE, possibly arising from stimulation by PDGF-BB and FGF, and decreased cAMP levels, which may promote restenosis. These results provide a rational explanation for the beneficial effects of PDE inhibitors.

1. Introduction

Proliferation and migration of vascular smooth muscle cells in response to mitogens contribute to restenosis following coronary angioplasty and limit the benefit of this procedure. Platelet-derived growth factor (PDGF)-BB and fibroblast growth factor (FGF) are two major factors responsible for this process (Levitzki, 2005). Accordingly, there have been efforts directed at the development of agents to inhibit restenosis. One group of agents is the inhibitors of phosphodiesterase (PDE) 3 and cilostazol is undergoing clinical trials and has shown promise in countering restenosis (Douglas et al., 2005). These agents act by inhibiting degradation of cAMP by PDE3 and, thereby, increase increasing levels of cAMP, which have been shown to inhibit vascular smooth muscle cell proliferation (Indolfi et al., 1997). There are eleven mammalian PDE families but differential splicing leads to at least 50 isozyme variants (Maurice et al., 2003). Of these, the PDE3 and PDE4 families are of particular relevance because they contribute to the regulation of vascular smooth muscle cell migration and proliferation. PDE3 exhibits activity against both cAMP and cGMP but *in vivo* kinetics favor cAMP (Maurice, 2005), whereas PDE4 is selective for cAMP (Conti et al., 2003). Inhibitors of PDE3 and PDE4 reduce DNA synthesis in bovine (Osinski and Schror, 2000) and porcine aortic smooth muscle cells (Souness et al., 1992) and decrease PDGF-BB-stimulated proliferation of mesangial smooth muscle cells (Chini et al., 1997). PDE3 and PDE4 inhibitors, also, show a synergistic effect on inhibition of PDGF-BB-induced vascular smooth muscle cell migration (Palmer et al., 1998). Changes in PDE3 expression have been reported for several cardiovascular diseases such as diabetes (Matsumoto et al., 2003), pulmonary hypertension (Wagner et al., 1997; Murray et al., 2002) and heart failure (Smith et al., 1997; Ding et al., 2005) and PDE4 deficiency promotes heart failure and arrthymias in rats (Lehnart et al., 2005), whereas stroke is associated with the *PDE4D* gene (Brophy et al., 2006; Markus and Alberts, 2006; Gretarsdottir et al., 2003). Our recent study showed that PDE3 activity was increased 24 h after balloon angioplasty and affected vasoreactivity of rat aortic rings (Zhao et al., 2007). However, there are no reports of changes in PDE expression after balloon angioplasty and which growth factors may be potentially responsible for these changes.

cAMP regulates cellular processes mainly by activating protein kinase A (PKA), for which there are multiple targets that affect smooth

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muscle cell migration/proliferation. One of the newly discovered targets is vasodilator-stimulated phosphoprotein (VASP), which has complex effects on cell motility, migration and adhesion (Krause et al., 2003; Kwiatkowski et al., 2003). VASP can be phosphorylated by both PKA and protein kinase G (cGMP-dependent protein kinase; PKG). PKA prefers Ser^{157} of VASP, followed by Ser^{239}, whereas PKG prefers Ser^{239} *in vitro*, but phosphorylates both Ser^{157} and Ser^{239} with similar kinetics *in vivo*. Thr^{278} can be phosphorylated by both PKA and PKG but only after both Ser^{157} and Ser^{239} are phosphorylated (Butt et al., 1994). Phosphorylation of Ser^{157} but not Ser^{239} or Thr^{278} results in a VASP mobility shift from 46 to 50 kDa on SDS-PAGE (Smolenski et al., 1998) and Ser^{157} phosphorylation is used to monitor PKA activity in cells.

The current studies were undertaken to examine the expression and activity of PDE3 and PDE4 isoforms following balloon angioplasty and to address a potential role for PDGF-BB and FGF. We postulated that one mechanism for the proliferative effect of PDGF-BB and FGF could be induction of PDE to reduce cAMP levels. In cultured vascular smooth muscle cells we found that PDGF-BB led to increased expression of PDE3 while FGF led to increased expression of PDE4. Similarly, we found increased expression/activity of PDE3 and PDE4 after balloon angioplasty that was correlated with decreased phosphorylation of VASP, which was used as an index of PKA activity. Phosphorylation of VASP was enhanced by PDE inhibitors, further indicating the importance of inhibiting PDE activity after balloon angioplasty.

2. Materials and methods

2.1. Angioplasty

Male Sprague Dawley rats (326–350 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed for at least 3 days prior to experiments. Animal use was approved by the New York Medical College Institutional Animal Care and Use Committee. Rats were anaesthetized by intra-peritoneal injection of 70 mg/kg Nembutal sodium and a 2F Fogarty embolectomy balloon catheter (Edwards Life Sciences, Irvine, CA) was introduced through the left femoral artery to the aortic arch and pulled back 10 mm. The balloon was then inflated to 750–850 mm Hg and the catheter was withdrawn to the branch of the femoral artery; this process was repeated three times. Hematoxylin-eosin staining showed that 24 h after balloon angioplasty, the endothelium was removed and the innermost layers of smooth muscle fibers showed slight disorganization. For sham rats, the uninflated catheter was inserted only once into the left femoral artery without entering the aorta.

2.2. Preparation of isolated aortic rings and VASP phosphorylation assay

The thoracic aorta was excised and cut into 2.5 mm rings in ice-cold Krebs solution as described (Schafer et al., 2003). Rings for PDE expression were frozen in liquid nitrogen. For the VASP phosphorylation assay, freshly isolated rings were shaken for 10 min in 1 ml prewarmed Krebs solution at 37 °C, and then incubated for a further 10 min with the vehicle or the PDE3 inhibitor, OPC3911 (2 μM, *N*-cyclohexyl-*n*-(2-hydroxyethyl)-4-((1,2,3,4-tetrahydro-2-oxo-6-quinolinyl)oxy)butyramide), or the PDE4 inhibitor, Ro20-1724 (20 μM, 4-(3-Butoxy-4-methoxyphenyl)methyl-2-imidazolidone), or both. Isoproterenol (1 μM) was then added for 5 min and the reaction was stopped by freezing the rings in liquid nitrogen. The rings were homogenized in VASP homogenate buffer (20 mM Tris pH 7.5, 250 mM sucrose, 5 mM EGTA, 20 mM EDTA, 25 mM $Na_4P_2O_7$, 25 mM NaF, 1% Triton with 3 mM benzamidine, 10 μg/ml pepstatin, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.8 mM PMSF) and centrifuged at 16,000 ×g for 20 min. Forty μl of the supernatants was mixed with 10 μl 5× sample buffer (312.5 mM Tris–HCl pH 6.8, 10% SDS, 25% glycerol, 0.05% bromophenol blue) and incubated at 95 °C for 5 min and then subjected to Western blot analysis.

2.3. Primary cell cultures and PDGF-BB/FGF treatment

Rat aortic vascular smooth muscle cells were cultured as described (Waldbilling and Pang, 1992). All drug treatments were carried out using cells from passage 4 to 8. For PDGF-BB treatment, cells reaching 90% confluence were rendered quiescent for 48 h in 0.1% BSA and then treated with PDGF-BB (100 ng/ml) added to the medium in the presence or absence of inhibitors of PI3-kinase (LY294002, 200 μM, 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one-hydrochloride), p38 MAP kinase (SB203580, 10 μM, 4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1*H*-imidazole), and MEK (U0126, 40 μM, 4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene) for 12 h. Cells treated with FGF (50 ng/ml) were incubated for 5 h under similar conditions. Cells were homogenized in buffer containing 290 mM sucrose, 10 mM MOPS, 1 mM EGTA, 3 mM NaN_3, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM DTT, and protease inhibitors as above. The homogenate was centrifuged at 16,000 ×g for 5 min and the protein concentration in the supernatant was determined (Bio-Rad, Hercules, CA). Twenty-five μg of protein was mixed with 2× sample buffer and incubated at 95 °C for 5 min and then subjected to immunoblotting.

2.4. Medial smooth muscle cell preparation and PDE activity assay

Medial smooth muscle cells were prepared as described (Miano et al., 1990). Briefly, aortas were rapidly excised from the animal, rinsed in Medium 199 with Hank's balanced salt solution and incubated for 25 min in the same medium plus 1% collagenase, 0.25% elastase, and 1% soy bean trypsin inhibitor. After incubation, the adventitia and endothelium were stripped away from the media. Medial smooth muscle cells were snap-frozen in liquid nitrogen and stored at −70 °C. Frozen tissues were then powdered, homogenized on ice with homogenate buffer and subjected to differential centrifugation; the 40,000 ×g supernatants, the cytosolic fractions, were used for the PDE assay.

For the cAMP PDE activity assay, ^{3}H-cAMP (100,000 dpm) was added to 200 μl buffer containing 50 mM Hepes, 0.1 mM EGTA, 8.3 mM $MgCl_2$ and 0.1 μM cAMP. Various concentrations of the test sample in 100 μl were added to start the reaction and the mixture was incubated at 30 °C for 15 min. The reaction was stopped by adding 100 μl stop mix (7.5 mM cAMP/3.33 mM 5′AMP in 0.17 N HCl). Samples were neutralized with 100 μl buffer containing 250 mM Tris, 250 mM NaOH, pH 8.0. Then, 100 μl venom mix (0.1875% Crotalus atrox in 100 mM Tris, pH 8.0) was added and the samples were incubated at 30 °C for 20 min. The sample was then applied to a DEAE-Sephadex A-25 column and the eluate collected in scintillation vials containing 10 ml Beckman Readi-Gel. The column was washed with 3.6 ml H_2O and the eluate was also collected into the same vial and the radioactivity was measured in a liquid scintillation counter. PDE3 or PDE4 activities were determined as the activities susceptible to the PDE3 inhibitor OPC3911 (1.5 μM) or the PDE4 inhibitor Ro20-1724 (15 μM), respectively, added to the assay mix.

2.5. Western blot analysis

Samples were subjected to SDS-PAGE in Mini-PROTEAN 3 Electrophoresis Cells (Bio-Rad) and transferred to PVDF membranes (Millipore, Billerica, MA) in Mini Trans-Blot Cells (Bio-Rad). The membranes were washed, incubated with antibodies to PDE3A, PDE4B, Ser^{239} phosphorylated VASP and total VASP and developed with ECF Vistra Reagent (GE Health, Piscataway, NY) according to the procedure supplied by the company. We compared several antibodies directed against PDE3A from different sources (see below) to confirm the identity of PDE3A as we evaluated the 98 kDa isoform rather than the 120 kDa isoform that is most often reported for vascular smooth muscle cells.