Increase of PTP levels in vascular injury and in cultured aortic smooth muscle cells treated with specific growth factors

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Chang, Yingzi, Daming Zhuang, Chunxiang Zhang, and Aviv Hassid. Increase of PTP levels in vascular injury and in cultured aortic smooth muscle cells treated with specific growth factors. Am J Physiol Heart Circ Physiol 287: H2201–H2208, 2004. First published July 22, 2004; doi:10.1152/ajpheart.00520.2004.—Migration and proliferation of vascular smooth muscle cells are key events in injury-induced neointima formation. Several growth factors and ANG II are thought to be involved in neointima formation. A recent report indicated that vascular injury is associated with increased mRNA levels of protein tyrosine phosphatase (PTP)-1B (PTP-1B). In the present study, we tested the following hypotheses: 1) rat carotid artery injury induces the expression of PTP-1B, Src homology-2 domain phosphatase (SHP-2), and PTP-proline, glutamate, serine, and threonine sequence (PEST) protein; and 2) polypeptide growth factors as well as ANG II increase the levels of tyrosine phosphatases in cultured rat aortic smooth muscle cells. We found that vascular injury induced by balloon catheter increases the protein levels of aforementioned phosphatases and that these effects occur in a PTP specific, as well as temporally and regionally specific, manner. Moreover, treatment of cultured primary rat aortic smooth muscle cells with PDGF or bFGF, but not with IGF1, EGF, or ANG II, increases PTP-1B, SHP-2, and PTP-PEST protein levels. These results suggest that increased PDGF and bFGF levels, occurring after vascular injury, may induce expression of several PTPs.

angiotensin II; protein tyrosine phosphatase

VASCULAR REOCCLUSION is a significant problem occurring after percutaneous transluminal angioplasty. Proliferation and migration of vascular smooth muscle cells induce intimal thickening, an event thought to be triggered by several growth factors released after vascular injury, including basic FGF (bFGF), PDGF, EGF, or IGF-1. Upregulation of bFGF, PDGF, and EGF receptors is also thought to contribute to neointimal expansion (15, 23, 33). Moreover, attenuation of cell proliferation, migration, and intimal thickening by growth factor antagonists, acting at various levels of signal transduction cascades (31, 34, 37), supports the involvement of growth factors in injury-induced intimal thickening.

Tyrosine phosphorylation of growth factor receptor kinases, followed by activation of multiple signaling pathways, is thought to lead to cell proliferation and migration. Receptor tyrosine kinase signaling can be regulated by several protein tyrosine phosphatases (PTPs) in positive or negative fashion. In a previous study (32), we showed that overexpression of PTP-1B attenuates insulin-induced cultured vascular smooth muscle cell motility in a manner that was correlated with dephosphorylation of the insulin receptor. Separately, we showed that overexpression of PTP-proline, glutamate, serine, and threonine sequence (PEST) attenuates serum-induced migration of cultured vascular smooth muscle cells by inducing dephosphorylation of adapter protein p130cas and decreasing the association of p130cas and adapter protein crk (21), and that ectopic expression of a third phosphatase, Src homology-2 domain phosphatase (SHP-2), induces smooth muscle cell migration by eliciting a decrease of small GTPase Rho activity (5). Other recent studies (26, 30) have shown that SHP-2, PTP-1B, or PTP-PEST associate with growth factor receptors (PDGFR, EGFR, IGF-1R, or insulin receptor), suggesting that these tyrosine phosphatases may be involved in regulation of receptor tyrosine kinase signaling.

The purpose of the present study was to determine the effect of vascular injury on the relative protein levels of nonreceptor PTPs (PTP-1B, PTP-PEST, or SHP-2) in injured rat carotid artery and the potential involvement of growth factors in upregulation of aforementioned phosphatases. We also investigated the effect of ANG II on PTP protein levels in cultured cells because receptor levels for ANG II are increased after vascular injury (35) and ANG II receptor blockers markedly attenuate neointima formation (19, 20).

MATERIALS AND METHODS

Sprague-Dawley rat pups were bred in the University of Tennessee vivarium. Type I collagenase, soybean trypsin inhibitor, fetal bovine serum, proteinase inhibitor cocktail containing aprotinin, leupeptin, bestatin, pepstatin A, 4-(2-aminoethyl)benzenesulfonyl fluoride, and E-64, monoclonal antibodies against α- and β-actin were purchased from Sigma (St. Louis, MO). DMEM/F-12 (1:1) culture medium was obtained from Gibco-BRL (Grand Island, NY). Fetal bovine serum was purchased from Cellgro (Herndon, VA). Porcine pancreatic elastase was from Calbiochem (San Diego, CA). Monoclonal antibodies against PTP-1B was obtained from Oncogene (San Diego, CA), SHP-2 antibody was from Transduction Laboratories (San Diego, CA), and rabbit antisera against PTP-PEST was raised in our laboratory, using an oligopeptide immunogen corresponding to PTP-PEST amino acid residues 444–457 in the mouse sequence or 445–458 in the human sequence, because the rat allele has not yet been cloned.

Rat carotid artery balloon injury model. Rat carotid artery injury was generated via a standard procedure with the use of a balloon catheter (39). Briefly, male Sprague-Dawley rats (weighing 250–300 g, from Charles River Laboratories, Wilmington, MA) were anesthetized with an intraperitoneal injection of xylazine (5 mg/kg) and ketamine (60 mg/kg). Balloon catheter injury was performed with a 2-F Fogarty catheter (Baxter) in the right carotid arteries. The uninjured left carotid artery served as a control. Animals were euthanized 3, 7, or 14 days after injury, carotid arteries were isolated and

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longitudinally opened, the endothelium (control) was denuded using a

| cotton-tipped applicator, and the neointima (injury), media, and |
| adventitia (control and injury) were dissected under a stereo- |
| microscopy. Each sample was pooled from six rats. Tissues were |
| homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.25% Na-deoxycholate, 0.1% SDS, 2 mM sodium orthovanadate, 1 mM sodium fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 50 μg/ml PMSF). This protocol and others involving the use of rats were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23).

**Cell culture.** Smooth muscle cells were isolated from thoracic aortas of Sprague-Dawley rat pups (6–9 days old), as described previously (21). All tissue culture experiments were performed via the use of primary cells that were never subcultured. Experimental treatments were administered for 24 h. At the end of each experiment, cells were washed with PBS twice to stop the treatment and lysed in buffer composed of 188 mM Tris-HCl, pH 6.8, 15% glycerol, 3% SDS, 1 mM sodium orthovanadate, 1 mM EDTA, 10 mM sodium pyruvate, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 μg/ml pepstatin.

**Immunoblotting.** Immunoblotting was performed via a published procedure (21). The blots were sequentially probed with antibodies directed against PTP1B, SHP2, PTP-PEST, and α- and β-actin (cultured cells), or α- and β-actin (carotid arteries). Western blot band densities were quantitated using NIH Image software. All raw densitometric values were normalized to the values found in lysates of control artery medial layer, in the same blot ( uninjured contralateral artery).

**RESULTS**

**Vascular injury induces a sustained increase in protein levels of PTP-1B.** A previous study (36) reported that carotid artery injury induces upregulation of PTP-1B mRNA levels. Moreover, we (32) have reported that PTP-1B targets insulin or IGF-1 receptors in vascular smooth muscle cells and that this phosphatase decreases insulin- or IGF-1-induced cell motility in cultured cells. In the present study, we found that upregulation of PTP-1B protein levels occurred in media and adventitia of injured rat carotid, as early as 3 days after injury, remaining above control levels in these regions for at least 7 days after injury. These increases occurred not only in media or adventitia (3 days after injury, Fig. 1A), but also in intima (7 and 14 days after injury, Fig. 1, B and C). PTP-1B protein levels were increased by 8- and 16-fold in adventitia and media, respectively, 3 days after injury (Fig. 1A). As expected, no neointima was apparent, 3 days after injury. Seven days after injury, PTP-1B levels were increased by 6-fold in adventitia and by 12-fold in media and intima, respectively (Fig. 1B).

As expected, the thickness of neointima increased significantly between 7 and 14 days (not shown). PTP-1B protein levels in adventitia returned to control values, 14 days after injury, whereas the levels of PTP-1B in media and intima were still ninefold (7 days, Fig. 1B) and sevenfold (14 days, Fig. 1C) above control levels.

**Vascular injury induces a transient increase of SHP-2 protein levels.** A previous study (36) reported no significant change in mRNA levels of SHP-2, after injury of rat carotid artery. In the present study, we determined the effect of vascular injury of rat carotid artery on protein levels of SHP-2. As shown in Fig. 2A, SHP-2 protein levels increased by ~50% in medial cells, 3 days after injury. Interestingly, adventitial cells expressed little or no SHP-2, either in control or injured vessels. This is consistent with a report (1) indicating the predominant localization of SHP-2 in smooth muscle cells and the deficit of smooth muscle cells in adventitia. Seven days after injury, medial SHP-2 protein levels were back to control, whereas SHP-2 levels in neointima were increased by about twofold (Fig. 2B). We (5) have previously reported that ectopic expression of SHP-2 can induce motility of cultured rat aortic smooth muscle cells, suggesting that upregulation of SHP-2 protein may be involved in smooth muscle cell migration from media to neointima. SHP-2 protein levels in both media and intima were decreased significantly, relative to values in control media, 14 days after injury (Fig. 2C), indicating that upregulation of SHP-2 is transient and suggesting that SHP-2 may be involved only in the initiation of neointima formation.

**Vascular injury increases protein levels of PTP-PEST in transient fashion in media and adventitia but in sustained fashion in neointima.** Wright et al. (36) reported that mRNA levels of PTP-PEST did not change significantly after carotid artery injury. In this study, we found that PTP-PEST protein levels were increased in both adventitia and media by about

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**Fig. 1.** Injury-induced upregulation of protein tyrosine phosphatase-1B (PTP-1B) protein levels in rat carotid arteries. A: 3 days after injury; B: 7 days after injury; and C: 14 days after injury. Top, representative Western blots; bottom, summaries of all Western blot experiments. Results are means ± SE from three independent experiments normalized to control (C) values in uninjured (contralateral) media. #P < 0.05 and ##P < 0.01 compared with control adventitia. *P < 0.05 and **P < 0.01 compared with control media [ANOVA followed by Fisher’s protected least-significant difference (PLSD) test].
threefold, 3 days after injury (Fig. 3A), but the levels were back to control values, 7 days (Fig. 3B) and 14 days (Fig. 3C), respectively, after injury. On the other hand, neointimal PTP-PEST protein levels were increased, 7 and 14 days after injury, by three- and fourfold, respectively. Together with a recent report (21) from our laboratory, indicating that ectopic expression of PTP-PEST attenuates the motility of cultured rat aortic smooth muscle cells, these results suggest that PTP-PEST could play a counterregulatory role in vascular injury.

Differential effects of vascular injury on α- or β-actin protein levels. Previous studies (6) have reported that the actin isoform pattern is switched from predominant α-actin to β-actin expression in proliferating smooth muscle cells. Moreover, vascular injury-induced neointima formation is associated with dedifferentiation of proliferating vascular smooth muscle cells, corresponding to a decrease of smooth muscle α-actin levels but an increase of nonsmooth muscle β-actin levels (4). On the basis of these findings, we measured relative protein levels of...
α- or β-actin in a positive control-type experiment, to provide validation of our Western blot analysis methodology for the measurement of PTP protein levels. We found that α-actin protein levels were decreased only in adventitia, 3 days after injury (Fig. 4A) but in all layers of carotid arteries, both 7 days (Fig. 4B) and 14 days after injury (Fig. 4C). On the other hand, β-actin protein levels were increased in both adventitia and media, 3 days after injury (Fig. 5A), remaining high in all layers of carotid arteries 7 days after injury (Fig. 5B) but only in neointima 14 days after injury (Fig. 5C). These findings indicate that, as expected, smooth muscle dedifferentiation occurred in the process of vascular remodeling and indirectly validate our Western blot analysis of PTP.

PDGF or bFGF, but not EGF, IGF-1, or ANG II, induces protein tyrosine phosphatase expression in cultured rat aortic smooth muscle cells. Several polypeptide growth factors/cytokines, including ANG II, are upregulated after vascular injury and are thought to be involved in neointima formation. Because vascular injury increases the expression of several protein tyrosine phosphatases, as documented by the aforementioned experiments, we hypothesized that treatment of cultured cells with either polypeptide growth factors or ANG II would increase the expression of several PTPs. We found that PDGF or bFGF increased PTP-1B, PTP-PEST, and SHP-2 protein levels in a concentration-dependent manner (Figs. 6 and 7). On the other hand, IGF-1, EGF, or ANG II failed to increase PTP protein levels significantly (data not shown). Because of these negative results, we also measured the phosphorylation levels of Erk after treatment of cells with EGF or ANG II, to confirm the intrinsic responsiveness of our cultured cells to these agonists. We found that both EGF and ANG II increased threonine/tyrosine phosphorylation levels of Erk significantly (data not shown), which is consistent with previous reports (7, 27) indicating the capacity of EGF or ANG II to activate this signaling pathway in vascular smooth muscle cells. Taken together, our findings indicate that PDGF and/or bFGF may be the most important agents that induce an increase of PTP-1B, PTP-PEST, and SHP-2 protein levels after balloon injury. Our in vitro results mimic those of early stage (3 and 7 days) balloon injury. On the basis of reports (8, 24, 31, 37) indicating that PDGF and bFGF are major contributors to vascular injury-induced neointima formation, our results using cultured cells support a potential involvement of these growth factors in upregulation of PTP levels in vascular injury.

DISCUSSION

Smooth muscle cell proliferation and migration are key events in vascular injury-induced neointima formation. Several studies have implicated PDGF and bFGF as the principal
agents that induce cell proliferation and migration, leading to neointima formation (16, 17, 31). It is well recognized that signaling events downstream of polypeptide growth factor receptors involve an increase of intrinsic receptor tyrosine kinase activity. Moreover, recent studies (9, 12, 25, 28) indicate that steady-state tyrosine phosphorylation levels generally represent the balance of tyrosine kinase activity opposed by tyrosine phosphatase activity. Although mechanisms related to tyrosine kinases have undergone extensive scrutiny, relatively little is known regarding mechanisms related to tyrosine phosphatase function.

Several studies have found that growth factor tyrosine kinases are recognized as substrates by various PTP, both in vitro and in vivo. Thus, receptors for IGF-1, insulin, or PDGF are targeted by PTP-1B or PTP-PEST (9, 12). SHP-2 has been reported to induce dephosphorylation of the IGF-1 receptor (25), and the phosphatase appears to be necessary for PDGF-induced cell migration (29). We (32) have reported that PTP-1B targets insulin or IGF-1 receptors and that ectopic expression of PTP-1B attenuates insulin or IGF-1-induced cell motility in cultured rat aortic smooth muscle cells. We (5) have also found that ectopic expression of SHP-2 induces cell motility, in cultured smooth muscle cells.

An important recent study (36) indicated that vascular injury induces upregulation of mRNA levels of several PTP, including a maximal 15-fold increase of PTP-1B mRNA levels in rat carotid arteries. However, the aforementioned study did not investigate phosphatase protein levels. We were therefore prompted to test the following two hypotheses: 1) rat carotid artery injury induces upregulation of protein tyrosine phosphatase protein levels, and 2) polypeptide growth factors induce upregulation of protein tyrosine phosphatase protein levels in cultured rat aortic smooth muscle cells.

We report the novel finding that vascular injury induces upregulation of at least three protein tyrosine phosphatase proteins and that this phenomenon occurs in a temporally and regionally selective manner for various PTP. We also report the novel finding that specific growth factors have the capacity to induce upregulation of PTP protein levels in cultured aortic smooth muscle cells.

Of the three PTP investigated in this study, PTP-1B elevation is quantitatively the most impressive, represented by a 10- to 20-fold increase. This finding is consistent with the study of Wright et al. (36), reporting a 15-fold maximal increase of mRNA levels, as determined by RT-PCR. It is also interesting to note that the levels of PTP-1B protein remain high in all layers of blood vessels at all experimental time points, except in adventitia, for at least 14 days after injury.

PTP-1B, a nonreceptor PTP, is widely expressed and localized to focal adhesions and the endoplasmic reticulum (3, 10). Ectopic expression of PTP-1B in cultured cells has revealed downregulation of motogenic signaling induced by activation...
Fig. 6. PDGF-BB induces PTP-1B, PTP-PEST, and SHP-2 expression in rat aortic smooth muscle cells. Cells were treated with different concentrations of PDGF-BB for 24 h, followed by lysis, as described in MATERIALS AND METHODS. PTP protein levels were measured via Western blot analysis using antibodies directed against PTP-1B, SHP-2, PTP-PEST, or \( \alpha \)-actin. Measurement of \( \alpha \)-actin protein levels was used to verify equivalent loading of samples onto gels. A–D: representative Western blots. E–G: summaries of all Western blot experiments. Results are the means ± SE of three independent experiments. All data are normalized to control values (no treatment). \(* P < 0.05; ** P < 0.01\) compared with control (ANOVA and Fisher’s PLSD test).

Fig. 7. Basic FGF (bFGF) induces PTP-1B, PTP-PEST, and SHP-2 protein expression in rat aortic smooth muscle cells. Cells were treated with different concentrations of bFGF for 24 h, followed by lysis, as described in MATERIALS AND METHODS. PTP protein levels were detected via Western blot analysis using antibodies directed against PTP-1B, SHP-2, PTP-PEST, or \( \alpha \)-actin. Measurement of \( \alpha \)-actin protein levels was used to verify equivalent loading of different samples onto gels. A–E: representative Western blots. F–H: summaries of all Western blot experiments. Results are the means ± SE of 4 (F) or 3 (G and H) independent experiments. All data are normalized to control values (no treatment). \(* P < 0.05; ** P < 0.01\) compared with control (ANOVA and Fisher’s PLSD test).
of insulin or IGF-1 receptors (32). A recent study (13) has found that PTP-1B interacts with activated (internalized) growth factor (EGF and PDGF) receptor tyrosine kinases, thus potentially contributing to signal termination and indicating that the phosphatase may serve as negative regulator in growth factor signaling.

Of the many growth factors released after vascular injury induced by balloon catheter, PDGF and bFGF seem to be the most important contributors to neointimal thickening (8, 22, 24, 31). In the current study, we found that PDGF and bFGF increase PTP-1B protein levels in cultured smooth muscle cells, suggesting that release of these growth factors could potentially explain balloon injury-induced upregulation of PTP-1B. These findings also suggest that upregulation of PTP-1B may play a counterregulatory role by opposing augmented protein tyrosine kinase activity via tyrosine dephosphorylation of signaling proteins, including that of receptor tyrosine kinases. Experiments to test this hypothesis are currently in progress.

We also report that the protein levels of another nonreceptor PTP, PTP-PEST, are upregulated after balloon injury. Similar to PTP-1B, PTP-PEST is thought to serve as negative regulator of signaling (11). Overexpression of PTP-PEST is associated with decreased motility in fibroblasts and vascular smooth muscle cells (11, 21). In the current study, we found that PTP-PEST protein levels are increased in adventitia and media, 5 days after balloon injury. Seven days after injury, PTP-PEST levels in adventitia and media return to normal but the levels remain high in neointima for at least 14 days. These results, taken together with the reported lack of increased PTP-PEST mRNA levels (36), support the view that the mechanism involved in PTP-PEST protein upregulation may be nontranscriptional. Moreover, our previous study (21), showing that overexpression of PTP-PEST decreases smooth muscle cell motility, by specifically dephosphorylating focal adhesion adapter protein p130cas, suggests that PTP-PEST, together with PTP-1B, may play a counterregulatory role in vascular injury-induced neointima formation. Upregulation of PTP-PEST after vascular injury is also likely to occur via PDGF and/or bFGF-stimulated signal transduction pathways, because our in vitro experiments found that PDGF and bFGF both induce upregulation of PTP-PEST, albeit at higher concentrations than those that induce upregulation of PTP-1B. It is also noteworthy that treatment of cultured rat aortic smooth muscle cells with nitric oxide induces upregulation of PTP-PEST activity but not protein levels (21). Furthermore, upregulation of inducible nitric oxide synthase in vascular injury is also well established (14, 18, 38). However, the role played by nitric oxide in upregulation of PTP-PEST activity in vivo remains to be determined.

Among vascular cells, SHP-2 is predominantly expressed in smooth muscle cells (1); moreover, we have previously reported that upregulation of SHP-2 increases vascular smooth muscle cell motility (5). Others have found that SHP-2 is associated with the PDGF receptor (26) and that SHP-2 is necessary for FGF receptor-3-induced NIH-3T3 cell transformation (2). The current study shows that both FGF and PDGF markedly induce SHP-2 expression in cultured smooth muscle cells, which is similar to in vivo results indicating that SHP-2 protein levels are increased in media 3 days, and in neointima 7 days, respectively, after injury. These observations are consistent with the view that SHP-2 may be involved in cell motility induced by FGF or PDGF, leading to neointima formation. If upregulation of SHP-2 were causally related to neointima formation, then termination of neointimal expansion could be explained, at least in part, by downregulation of SHP-2, 14 days after injury.

Although ANG II, IGF-1, and EGF and their receptors also seem to be involved in neointima formation in animal models, these agents are less likely to be involved in injury-induced upregulation of PTP, because our in vitro experiments show that treatment of smooth muscle cells with ANG II, IGF, or IGF-1 failed to increase the levels of several PTP. However, this conjecture remains to be validated in vivo.

Vascular smooth muscle cell migration and proliferation are important events regulated by growth factors released after vascular injury. Phosphorylation of receptor tyrosine kinases is a key event in growth factor signaling. Our current results indicate that expression of PTP-1B, PTP-PEST, or SHP-2 is upregulated by FGF or PDGF. Therefore, it is likely that release of FGF and/or PDGF can explain upregulation of aforementioned PTP after vascular injury. The current results also suggest that PTP-1B and/or PTP-PEST may play a counterregulatory role in growth factor-induced cell migration, whereas SHP-2 may be involved in growth factor-induced neointima formation, via stimulation of cell motility. Experiments are in progress to determine the specific roles of various PTP in vascular injury.

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