p21-Activated Kinase 1 Participates in Vascular Remodeling In Vitro and In Vivo

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Abstract—Vascular smooth muscle cell hypertrophy, proliferation, or migration occurs in hypertension, atherosclerosis, and restenosis after angioplasty, leading to pathophysiological vascular remodeling. Angiotensin II and platelet-derived growth factor are well-known participants of vascular remodeling and activate a myriad of downstream protein kinases, including p21-activated protein kinase (PAK1). PAK1, an effector kinase of small GTPases, phosphorylates several substrates to regulate cytoskeletal reorganization. However, the exact role of PAK1 activation in vascular remodeling remains to be elucidated. Here, we have hypothesized that PAK1 is a critical target of intervention for the prevention of vascular remodeling. Adenoviral expression of dominant-negative PAK1 inhibited angiotensin II–stimulated vascular smooth muscle cell migration. It also inhibited vascular smooth muscle cell proliferation induced by platelet-derived growth factor. PAK1 was activated in neointima of the carotid artery after balloon injury in the rat. Moreover, marked inhibition of the neointima hyperplasia was observed in a dominant-negative PAK1 adenovirus-treated carotid artery after the balloon injury. Taken together, these results suggest that PAK1 is involved in both angiotensin II and platelet-derived growth factor–mediated vascular smooth muscle cell remodeling, and inactivation of PAK1 in vivo could be effective in preventing pathophysiological vascular remodeling. (Hypertension. 2010;55:161-165.)

Key Words: angiotensin II □ platelet-derived factor □ signal transduction □ arterial injury □ restenosis

An increasing body of evidence strongly suggests that activation of a certain set of protein kinases is shared by multiple risk factors implicated in cardiovascular remodeling under hypertension or atherosclerosis.1 p21-activated protein kinase (PAK1), an effector kinase of small G proteins, could be one of these kinases, thus representing a novel therapeutic target. Except for plants, nearly all eukaryotes encode ≥1 PAK gene, indicating an important physiological function for this family of kinases.2 PAK has been implicated in cell growth, survival, and migration in noncardiovascular cells.2–5 In cultured vascular smooth muscle cells (VSMCs), PAK1 is the major isoform expressed. Multiple ligands implicated in pathological vascular remodeling, such as angiotensin II (AngII) and platelet-derived growth factor BB (PDGF-BB), have been shown to activate PAK1 in VSMCs.6–9 The PAK1 activation mechanism by these ligands in VSMCs has been extensively studied, which appears to include intracellular Ca2+ elevation, reactive oxygen species, protein kinase C-δ, small G protein Rac1, PYK2, c-Src, phosphoinositide-dependent kinase 1, and Nck.5,8,10,11

In contrast, limited information is available regarding the functional significance of PAK1 activation in VSMCs. Thus far, PAK1 has been shown to be required for VSMC hypertrophy and migration induced by AngII and PDGF-BB, respectively.8,10 However, the exact role of PAK1 activation in vascular remodeling remains to be elucidated. In the present study, we have hypothesized that PAK1 is a critical target of intervention for the prevention of vascular remodeling by inhibiting VSMC proliferation and migration. The hypothesis was tested in vitro with a primary culture of arterial VSMCs treated with AngII or PDGF-BB, as well as in vivo with a carotid artery after balloon angioplasty. Our data support that PAK1 is one of the critical protein kinases involved in pathological vascular remodeling.

Materials and Methods

Reagents
AngII was purchased from Sigma. PDGF-BB was purchased from R&D Systems. Phosphospecific antibodies to detect Ser192/204-phosphorylated PAK1 for immunoblotting and Thr423-phosphorylated PAK1 for immunohistochemistry were purchased from Cell Signaling Technology. Antibody to detect total PAK1 was purchased from Santa Cruz Biotechnology. Antibody against proliferation cell nuclear antigen (PCNA) was purchased from Chemicon.

Cell Culture
Isolation and characterization of rat aortic VSMCs in culture were described previously.12 Cells were subcultured in DMEM containing...
Adenoviral Infection

Generation and characterization of replication-deficient adenovirus encoding kinase-inactive/dominant-negative K299R/dnPAK1 were described previously. The adenovirus titer was determined by the Adeno-X Rapid Titer kit (BD Biosciences). VSMCs were infected with adenovirus for 2 days, as described previously. The infection efficiency was estimated to be 90% to 100%, as defined by infection with adenovirus (50 to 100 multiplicities of infection) encoding green fluorescent protein (GFP).

Immunoblotting

Immunoblotting was performed as described previously. Cell lysates were subjected to SDS-PAGE gel electrophoresis and electrothermally transferred to a nitrocellulose membrane. The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase-linked secondary antibody for 1 hour at room temperature, immunoreactive proteins were visualized by a chemiluminescence reaction kit. The results were quantified by densitometry in the linear range of film exposure using CanoScan N670U (Canon) and Un-Scan-It Gel 5.3 software (Silk Scientific). An example of data supporting the linearity has been demonstrated.

Wound Healing Assay

VSMC migration was measured using a monolayer-wounding protocol in which cells migrated from a confluent area into an area that was mechanically denuded of cells. VSMCs infected with adenovirus for 2 days were scraped by a metal dental pick (DenTek) and stimulated by 100 nmol/L of AngII for 24 hours with 5 mmol/L of hydroxyurea to completely block proliferation. VSMC migration was quantified as reported previously.

Cell Proliferation

VSMCs infected with adenovirus for 2 days were stimulated by 100 ng/mL of PDGF-BB for 72 hours, and then cell numbers were counted by a Coulter counter.

Balloon Angioplasty and Gene Transfer

Left common carotid artery balloon angioplasty was performed in male Sprague-Dawley rats (Charles River Breeding Laboratory) that were under pentobarbital sodium anesthesia, as reported previously. Subsequently, adenovirus encoding dominant-negative (dn)PAK or control GFP was delivered to the injured artery (1 × 10^9 pfu/mL). The vessels were harvested 14 days later and fixed, and histology was determined, as described previously. These investigations conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Temple University.

Immunohistochemistry and Morphometry

Immunohistochemistry was performed with a phosho-PAK1 antibody and PCNA antibody, as described previously. For vascular morphometry, digitized images were averaged from 3 representative stained tissue sections using Image Pro Plus (Media Cybernetics). The circumference of the lumen, the area encircled by the internal elastic lamina, and the external elastic lamina were quantified. The medial and intimal areas were then calculated.

Figure 1. Phosphorylation of PAK1 by AngII and PDGF-BB. VSMCs were stimulated with 100 nmol/L of AngII or 100 ng/mL of PDGF-BB for the indicated time periods. The cell lysates were immunoblotted with a phosphoselective antibody, which detects PAK1-Ser194/204 phosphorylation, and with an anti-PAK1 antibody. The bar graphs show quantification of the PAK1 phosphorylation by densitometry at 5 minutes and 30 minutes induced by AngII and PDGF-BB, respectively. Data are mean±SEM of 3 experiments. *P<0.05 vs the stimulated control.

Figure 2. dnPAK1 inhibited migration and proliferation of VSMCs. A, Confluent VSMCs infected with adenovirus (100 multiplicities of infection) encoding dnPAK1 or the control GFP vector were scraped by a metal dental pick and stimulated with AngII for 24 hours in the presence of 5 mmol/L of hydroxyurea to block cell proliferation completely. The nucleus was stained with Hoechst 33342 dye, and migrated VSMCs from the wound edge were counted in 4 independent view fields (× 100). Data are mean±SEM of 4 experiments. B, VSMCs were infected with adenovirus (100 multiplicities of infection) encoding control GFP vector or dnPAK1 for 48 hours. The cells were then stimulated with 100 ng/mL of PDGF-BB for 3 days and the cell number counted. Data are mean±SEM of 3 experiments. *P<0.05 vs the basal control. †P<0.05 vs the stimulated control.
In Vitro Experiments
To test whether AngII and PDGF-BB activate PAK1 in VSMCs, immunoblot analysis was performed with an antibody selectively recognizing Ser192/204-phosphorylated PAK1. As shown in Figure 1, both agonists markedly stimulated PAK1 phosphorylation in a time-dependent manner. The results are consistent with past publications.8,11 We have further questioned whether PAK1 is required for VSMC migration by AngII and proliferation by PDGF-BB in vitro. As shown in Figure 2A, infection of adenovirus encoding dnPAK1 completely inhibited VSMC migration induced by Ang II. Moreover, infection of dnPAK1 adenovirus completely inhibited VSMC proliferation induced by PDGF-BB (Figure 2B).

In Vivo Experiments
The above in vitro experiments prompted us to further test the effect of dnPAK1 on arterial neointimal hyperplasia after balloon angioplasty, because this type of vascular remodeling involves VSMC migration and proliferation. As shown in Figure 3, dnPAK1 gene transfer by adenovirus significantly inhibited neointima formation at the carotid artery 14 days after the injury, whereas it had no effect on the medial area of the injured artery. PCNA-positive cells were detected in the neointima of the carotid artery 14 days after the injury, especially at the lumen side, and were almost completely attenuated in the neointima treated with dnPAK adenovirus (Figure 4). These data suggest the crucial participation of PAK1 within pathological vascular hyperplasia. To confirm the causal role of PAK1 in vascular remodeling, activation of PAK1 in the artery 14 days after the injury was assessed by immunohistochemistry using a phosphospecific PAK1 antibody. Marked staining was specifically observed in the neointima region of the artery at the cytosol, as well as the nucleus, which was attenuated in the dnPAK1-treated artery (Figure 5).

Discussion
PAK1 has been shown to be required for the migration of cultured tracheal smooth muscle cells and VSMCs.3,8 We...
have reported previously that PAK1 activity is required for enhanced protein synthesis induced by AngII in cultured VSMCs. PAK1 was also implicated in the regulation of smooth muscle contraction and vascular permeability in VSMCs. PAK1 was also implicated in the regulation of enhanced protein synthesis induced by AngII in cultured VSMCs, as in the present study. Our in vivo data further highlighted PAK1 phosphorylation at the cytosol and nucleus in the neointima lesion, PAK1 has been shown to be involved in mitotic regulation at the nucleus. Therefore, an important future project will be the identification of the downstream target of PAK1, such as a nuclear substrate in VSMCs by which PAK1 supports neointima formation.

Perspectives
In addition to our present study suggesting the causal role of PAK1 in restenosis and potentially atherosclerosis, PAK1 is also implicated in contractile regulation of VSMCs. Therefore, further research on PAK1 in hypertensive animal models should be performed to expand our findings to lead to a better treatment of cardiovascular diseases associated with hypertension.

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Disclosures
None.

References


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