Stent Implantation Activates Akt in the Vessel Wall
Role of Mechanical Stretch in Vascular Smooth Muscle Cells

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Objective—The long-term efficacy of stent implantation is affected by in-stent restenosis (ISR). Multiple factors can contribute to ISR, and the underlying mechanism remains elusive. We investigated the possible role of mechanical stretch and the associated molecular signaling in ISR.

Methods and Results—Stent implantation in rat abdominal aortas induced neointima formation. Immunohistochemical studies revealed the activation of Akt in the media and neointima of the stented vessels. Western blotting showed increased phosphorylation of Akt at both Thr308 and Ser473 and phosphorylation of GSK-3β in the stented vessels. A stretch device applying static equibiaxial stretch on cultured vascular smooth muscle cells was used to delineate the molecular mechanism underlying the stretch activation of Akt. Static mechanical stretch induced the sustained activation of Akt and its upstream phosphoinositide 3-kinase (PI3K) and the phosphorylation of GSK-3β, its downstream effector in vascular smooth muscle cells. LY294002, a PI3K inhibitor, and N-acetylcysteine, a scavenger of reactive oxygen species, inhibited the stretch activation of Akt. Furthermore, N-acetylcysteine and wortmannin, another PI3K inhibitor, reduced the neointima formation after stent implantation.

Conclusions—Mechanical stretch of the vascular wall during stent deployment may contribute to ISR by activating the Akt pathway. (Arterioscler Thromb Vasc Biol. 2003;23:2015-2020.)

Key Words: vascular smooth muscle cells ■ stent ■ Akt ■ mechanical stretch ■ in-stent restenosis

Stenosis of blood vessels leads to ischemia and infarction of the supplied tissues and organs. Besides surgical bypass, stent implantation has been a popular angioplasty procedure for many vascular occlusive diseases. Stents can be implanted in many different vessels, including coronary, carotid, iliac, mesenteric, and intracranial arteries, as well as the aorta. Despite the high success rate of stenting in the treatment of vascular occlusive diseases, the major drawback of this procedure is in-stent restenosis (ISR), which involves late arterial renarrowing at the site of stenting. With the increased usage of stents, ISR has become a common clinical problem and hence is presently under extensive investigation. ISR is largely a result of the formation of neointima, the cellular component of which is mainly the proliferated smooth muscle cells (SMCs). Some areas of neointima, especially those surrounding the stent struts, are composed of SMCs exclusively. Although factors such as thrombosis, inflammation, and stent strut stimulation are known to increase the risk of ISR, the underlying mechanism of ISR remains elusive.

Several recent studies have shown that medial damage and stent oversizing are positively correlated with the severity of ISR. The amount of stenting-induced intimal hyperplasia is proportional to the degree of circumferential vascular stretch. Stenting exerts its lumen-opening effect by mechanical distention and providing a static luminal scaffolding to eliminate recoil and keep the lumen patent. After stenting, the local vascular wall, including the regions in contact with and adjacent to the stent struts, is subjected to mechanical stretch as the aortic diameter is increased. The mechanical stretch imposed on the vessel wall by stent implantation can be both transverse and longitudinal. In addition to the direct contact effect of stent struts on the vascular wall, the mechanical stretch secondary to stenting may also play an important role in initiating intimal hyperplasia. This led us to hypothesize that static mechanical stretch caused by stenting promotes the formation of ISR through the initiation of the prohyperplasia signaling pathway in vascular SMCs (VSMCs).

Stretch devices that apply cyclic or static stretch to cultured cells have been used to investigate the effect of mechanical stretch on many cell types, including VSMCs and cardiac myocytes. With the advantage of well-controlled mechanical and chemical factors, such a system can precisely dissect the mechanotransduction events in response to mechanical...
stretch. Protein kinase Akt, also known as protein kinase B, plays an important role in mediating cell survival, proliferation, and migration. Akt can be activated in VSMCs by multiple stimuli, such as angiotensin II and platelet-derived growth factor (PDGF). Recent studies showed that Akt activation plays an important role in mechanotransduction in cells in the vascular system. Akt-mediated NOS activation is responsible for shear stress–induced NO production in endothelial cells. However, the role of Akt in the response of SMCs to mechanical stretch remains to be investigated. Reactive oxygen species (ROS) such as H$_2$O$_2$ and superoxide have been found to be modulators of signaling initiated by the activation of various receptors, including PDGF receptor. In VSMCs, ROS are required for Akt activation and cell proliferation. Our recent results revealed that many ROS-induced genes are upregulated in elastase-induced rat abdominal aortic aneurysm, which results in a large increase in aortic diameter. In contrast, antioxidant enzymes and proteins are downregulated. In view of the role of the Akt pathway in promoting VSMC proliferation and migration, we investigated the effect of stenting on the Akt pathway in vivo in the vascular wall. To explore additionally the molecular basis of the stretch activation of Akt, we used a stretch device to apply mechanical stretch to cultured VSMCs and investigated the possible mechanotransduction pathway.

Methods

Rat Model for Abdominal Aortic Stenting

Male Sprague-Dawley rats (~400 g) were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). The lower 2.5-cm segment of the abdominal aorta was exposed, and 2 vascular clips were placed proximal and distal to the segment to be stented. A small incision was made at the caudal end, and a 2.8-mm human coronary stent (kindly provided by Guidant, Temecula, Calif) was implanted in the isolated aortic segment at 10 atm. The control rats underwent a sham operation, during which no stents were implanted. NAC (Sigma) or wortmannin (Calbiochem) was administered to rats to investigate the inhibitory effects of these agents on the stent-induced neointima formation. The daily administration of these agents started 2 days before the stent implantation and lasted until aortas were harvested. NAC, dissolved in PBS, was administered intraperitoneally at a daily dose of 200 mg/kg, and wortmannin, dissolved in 2% DMSO/PBS, was administered by intravenous injection through tail veins at a daily dose of 10 µg per rat. The rats were killed at the indicated time by an overdose of pentobarbital, and the stented and sham-operated aortas were then harvested. All procedures were approved by the UCR Institutional Animal Care and Use Committee.

Morphometry and Immunohistochemistry

Specimens of abdominal aortas retrieved were fixed and cut longitudinally. After the removal of the implanted stent with microforces, the aortic segments were embedded in paraffin and serial cross-sections (7 µm) were obtained. Polyclonal anti-phospho-Akt(Thr308) (1:100 dilution, Cell Signaling) or FITC-conjugated anti-SMC α-actin mAb (1:100 dilution, Sigma) was used as primary antibodies. As a negative control, normal rabbit IgG was used instead of the primary antibody. The primary antibody was recognized by a rhodamine-conjugated donkey anti-rabbit secondary antibody (1:100 dilution, Jackson ImmunoResearch Laboratories). Immunohistochemical results were visualized under a fluorescence microscope (Nikon TE 300). The serial tissue section was then stained with H&E for morphological observation. Neointima formation was measured as the ratio of the thickness of the intima to that of media (I/M). Serial sections were obtained for every 200-µm segment spanning the entire length of the stent. For each segment, 5 sections were analyzed, and the I/M ratio was determined with a computerized digital image analysis system. The results were averaged from 8 different points of the cross section.

Preparation of Arterial Protein Extracts

Sections of aorta spanning the stented area were collected from the experimental animals, and aortas with the same size and from a similar location were harvested from the control group. The adhered fat and connective tissues were dissected from the isolated vessels, and the endothelial layer was denuded. Pooled aortic specimens were homogenized with use of a Polytron homogenizer. The homogenates were centrifuged at 10 000 g at 4 °C for 30 minutes. The protein concentrations of the supernatants were determined by the Bradford method.

Cell Culture and Stretch Experiments

Rat aortic SMCs were isolated by procedures previously reported and used between passages 2 through 5. VSMC cell line (A7r5) was obtained from ATCC. Cells were cultured in DMEM (Gibco-RBL) supplemented with 10% FCS. A specially designed stretch apparatus was used to apply uniform equibiaxial stretch to cells cultured on a deformable membrane that was coated with fibronectin (2.5 µg/cm², Sigma). After becoming confluent, SMCs were serum-starved for 12 hours and then subjected to static stretch with 5% or 20% area change. The stretch apparatus was kept in a humidified incubator with 95% air and 5% CO$_2$ at 37°C. The control cells were cultured in the stretch chambers under the same conditions but received no stretch. In some of the experiments, LY294002 (20 µmol/L), wortmannin (10 nmol/L), or NAC (20 µmol/L) was included in the medium during stretching. Cellular proteins were then isolated from the stretched and control cells.

Western Blotting and Kinase Activity Assay

Rat aortic protein extracts (50 µg) or whole VSMC lysates (40 µg) were analyzed by Western blotting with polyclonal anti-Akt, anti-phospho-Akt(Thr308), anti-phospho-Akt(Ser473), and anti-phospho-GSK-3β(Ser9). Horseradish peroxidase–labeled IgG secondary antibodies (Santa Cruz) with an enhanced chemiluminescence kit (Amersham) were used to visualize the protein bands. The intensities of the various protein bands were quantified by a densitometer. For the kinase assay of Akt and phosphoinositide 3-kinase (PI3K), please see the online supplement, available at http://atvb.ahajournals.org.

Statistical Analyses

Statistical analyses of results are expressed as mean±SD. The data were analyzed by 2-tailed unpaired Student’s t test. For experiments in which the control values were set to 100%, the SDs were reported by using the nontransformed raw data.

Results

Stenting Activates Akt in the Vascular Wall In Vivo

We implanted stents into rat abdominal aortas. ISR was observed in all stented arteries 3 weeks after implantation (Figure 1). VSMCs constituted the major cell type in the ISR, as indicated by the presence of antigens recognized by anti-SMC α-actin. Immunostaining with anti-phospho-Akt(Thr308) revealed Akt activation in the vessel wall of the stent-implanted aortas. Minimal immunostaining was found in control experiments in which only secondary antibody was used. The merged image in Figure 1 indicates that Akt activation was mainly in the media and neointima. The effect of stent implantation on Akt was also examined by Western blotting on protein extracts isolated from the stented and sham-operated arterial specimens 12 hours after the surgical
procedure. Compared with sham controls, stented vessels showed activated Akt, as demonstrated by the increased phosphorylation of Akt at both Thr308 and Ser473 (Figure 3). The phosphorylation of Ser9 of GSK-3β, an Akt effector, was also increased in the stented vessels.

Because ROS can activate Akt in VSMCs, we examined whether NAC can inhibit ISR. As shown in Figure 2, the I/M ratio in the NAC-administered animals was reduced to 1.3 ± 0.2, compared with 2.0 ± 0.3 in the controls. Because Akt is a downstream effector of PI3K, we also administered wortmannin, an inhibitor specific for PI3K, to the stented rats intravenously. In line with the NAC experiments, wortmannin, at a dose previously shown to inhibit Akt activation in rat arteries,18 also inhibited ISR significantly (Figure 2). The I/M ratio in the wortmannin-treated animals was reduced to 0.6 ± 0.3, compared with 1.8 ± 0.3 in the stented aortas receiving only DMSO (Figure 2). To investigate whether ROS is the upstream of Akt in stented aortas, we isolated proteins

**Figure 2.** NAC and wortmannin block the neointima formation in the stented rat aortas. The procedures for rat aortic stenting were the same as those in Figure 1 except for the daily administration of NAC or wortmannin. Five animals were used for each group. The aortas harvested from sham, stented plus PBS administration (stent+PBS), and stented plus NAC administration (stent+NAC) groups were retrieved 3 weeks after the surgical procedures, whereas those from stented plus DMSO administration (stent+DMSO) and stented plus wortmannin administration (stent+wortmannin) groups were retrieved 2 weeks post-surgery. I/M ratios of the harvested aortas were calculated and graphed in the lower panel of the representative HE-stained sections. Neointima is indicated by the small arrows, and the stent strut-occupied area is indicated by the arrowheads. *P < 0.05 vs stented vessels without NAC or wortmannin treatment.

**Figure 3.** NAC blocks the stent activation of Akt. A, Protein extracts were obtained from the media of abdominal aortas removed from sham-operated, stented, and stented/NAC-treated rats (n = 5 in each group). The extracts isolated from the pooled aortas were subjected to Western blotting with anti-pAkt(Thr308), anti-pAkt(Ser473), anti-pGSK-3β(Ser9), or anti-Akt. The bands recognized by various antibodies were indicated.
Static Stretch Induces Akt Activation in Cultured VSMCs

Results presented in Figures 1 through 3 indicate that stent implantation can cause Akt activation, which was mediated through ROS and PI3K. To investigate the roles of mechanical distention and longitudinal stretch in stent activation of Akt, rat VSMCs were subjected to equibiaxial static stretch with 5% or 20% area change for 6 hours. In control experiments, cells were kept unstretched for the same duration. Western blotting revealed that either 5% or 20% stretch induced Akt phosphorylation at Thr308 and Ser473 (Figure 4A). The application of static stretch on VSMCs also increased the phosphorylation of GSK-3β at Ser9. The increase in phosphorylation of Akt and GSK-3β was not attributable to changes in the amount of Akt protein, because the anti-Akt antibody revealed the same level of Akt in unstretched and stretched samples. Densitometry results showed that 20% stretch was more potent than 5% stretch in causing the phosphorylation of Akt and GSK-3β (ie, these phosphorylation events depended on the magnitude of the stretch). As shown in Figure 4B, the application of 20% static stretch induced the phosphorylation of Akt and GSK-3β as early as 0.5 hours, which was sustained for at least 6 hours. In separate experiments, we found that the stretch-induced Akt activation and GSK-3β phosphorylation lasted for at least 24 hours (data not shown). As a positive control, cells were treated with PDGF-BB, and the activation of Akt by PDGF-BB was transient (please see the online data supplement).

Static Stretch-Activated Akt Is PI3K-Dependent

To determine whether PI3K regulates the stretch activation of Akt, we examined whether stretch activates PI3K, and if it does, whether blocking PI3K by LY294002 would inhibit the Akt activation in VSMCs in response to stretch. As shown in Figure 5A, the application of 20% stretch to rat VSMCs for 1 minute caused a 3-fold induction of PI3K activity compared with static controls, and this tapered to a 2-fold induction 60 minutes after the application of stretch. To confirm additionally that PI3K regulates the stretch activation of Akt, rat VSMCs were treated with LY294002 before and during stretch experiments. As shown in Figure 5B, LY294002 treatment abolished the Akt phosphorylation at both Thr308 and Ser473, as well as GSK-3β phosphorylation at Ser9, in both A7r5 and primary VSMC cell types subjected to 20% static stretch. Wortmannin had a similar inhibitory effect on Akt (data not shown). To confirm additionally that LY294002 inhibits the stretch activation of Akt, we assessed the Akt kinase activity in VSMCs in the presence or absence of LY294002. LY294002 treatment ablated the stretch-induced Akt kinase activity (please see the online data supplement). These results show that static stretch activates Akt in a PI3K-dependent manner.

NAC Blocks the Static Stretch–Activated Akt

Because of the involvement of ROS in the activation of Akt in the stented vessels, we studied the effects of NAC on the stretch-activated Akt. Consistent with our data from in vivo experiments (Figures 2 and 3), NAC treatment blocked the phosphorylation of Akt and GSK-3β in cultured VSMCs subjected to 20% static stretch (Figure 6).

Discussion

The long-term efficacy of vessel stenting is hampered by ISR, which is an inflammatory/proliferative response of the vascular wall. In this study, we used a rat abdominal aorta model to address the stent activation of Akt, because the in-stent neointima formation in this model is similar to that in human vessels. Our results showed that stent implantation activates the Akt pathway in the media of the stented aortic wall. Akt is activated by its phosphorylation at Thr308 and Ser473. Stenting induced the phosphorylation of Akt at both Thr308 and Ser473. The activation of Akt was additionally demonstrated by the phosphorylation of its endogenous substrate GSK-3β in the stented vessels (Figure 3). Previous studies showed that molecules such as transforming growth factor-β, PDGF, and vascular endothelial growth factor are activated in stented vessels.20,21 These molecules are either upstream signaling molecules or downstream target genes of Akt. Thus, the activation of Akt by stenting would represent an important IRS-related signaling event in the arterial media in vivo. The mechanical forces imposed on the vessel wall by stenting depend on the size of stent and the topology of the vessel. The neointima formation in the stented coronary arteries correlates positively with the stent-to-lumen ratio.2 Using intravascular ultrasound in a 6-month follow-up, Koyama et al2 found that coronary artery stenting induced intimal hyperplasia and that the extent of restenosis was in proportion to the magnitude of vascular stretch. Thicker neointima caused by greater...
degrees of stretch has also been demonstrated in the porcine coronary artery model. Furthermore, angular changes of coronary arteries caused by stent implantation have been shown to be a predictive factor for ISR and major adverse cardiac events, including acute myocardial infarction.

In addition to transverse distention, stent deployment can also cause longitudinal stretch by straightening the vessel wall, especially in regions of artery tortuosity. We used an in vitro stretch device to exert equibiaxial static stretch on cultured VSMCs to explore the effects of mechanical deformation circumferentially and longitudinally. The advantage of this approach is to exclude nonmechanical factors (e.g., platelet activation) associated with stenting in vivo. Our data showed that Akt activation in VSMCs depends on the magnitude of the equibiaxial stretch (Figure 4A). The results of our in vitro stretch experiments are in tune with those from clinical and animal studies, which suggested that the stent-to-artery ratio was an important determinant in causing ISR.

The effects of mechanical stretch on VSMCs have been studied by the use of devices applying cyclic stretch. Cyclic stretch activates many signaling molecules, such as PDGF receptor, integrins, calcium and sodium channels, and G proteins. Cyclic stretch can induce the proliferation and apoptosis of VSMCs, separate processes that can occur concurrently in the vascular neointima after angioplasty. The Akt phosphorylation in VSMCs induced by cyclic stretch is transient, with a peak at 30 minutes, which is similar to its time course in response to PDGF. In contrast to cyclic stretch, the application of static stretch in our study induced a sustained activation of Akt, as demonstrated by increased phosphorylation and kinase activity. Thus, static stretch differs from cyclic stretch in its sustained activation of Akt. It is to be noted that stent implantation leads to a loss of pulsatility, and hence the application of static stretch to VSMCs is relevant.

Multiple lines of evidence suggest that Akt is a downstream target of PI3K in different cell types, including VSMCs. However, other signaling molecules also regulate Akt, depending on the cell types and the imposed stimuli. In glomerular mesangial cells, angiotensin II activates Akt by an arachidonic acid/ROS-dependent pathway. Environmental stresses such as heat shock and hyperosmolarity can activate Akt by pathways independent of PI3K in COS-7 and NIH3T3 cells. In VSMCs, ceramide inhibits Akt phosphorylation.
ylation through PKCζ activation.31 Our results show that mechanical stretch activates PI3K in VSMCs and that the static stretch activation of Akt is PI3K-dependent because it is inhibited by the PI3K-blocker LY294002 (Figure 5). Indeed, the involvement of the PI3K-Akt pathway in ISR was demonstrated by reduced neointima formation in rats receiving Wortmannin (Figure 2).

ROS have been reported to be engaged in the PI3K/Akt pathway.32 ROS involvement in the deformed artery has also been shown in experimental abdominal aortic aneurysms in the rat.33 ROS production was increased in balloon-injured arteries, and the administration of the reducing agent L-cysteine reduced the intimal proliferation.33 In our study, the use of NAC in both in vivo stenting and in vitro stretch experiments inhibited Akt activation under both conditions and decreased the neointima formation after in vivo stenting. These results suggest that mechanical stretch is likely to activate the PI3K-Akt pathway in VSMCs in a redox-sensitive manner. In summary, this study has demonstrated the importance of the choice of the appropriate stent size in relation to the vessel geometry in eliminating Akt activation, which may lead to ISR.

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