Wall Stiffness Suppresses Akt/eNOS and Cytoprotection in Pulse-Perfused Endothelium

Xinqi Peng, Saptarsi Haldar, Shailesh Deshpande, Kaikobad Irani, David A. Kass

Abstract—Increased steady shear stress stimulates nitric oxide synthase (eNOS) in part by Akt-dependent phosphorylation. Arteries in vivo are exposed to pulse perfusion (PP) combining phasic shear with stretch. In compliant vessels, enhancing PP lowers vascular tone by stimulating eNOS; whereas in aged, stiff arteries, flow-mediated dilation declines and PP is a prominent risk factor. Here, we tested the hypothesis that reduced wall distensibility alters PP-induced eNOS/Akt mecano-signalization. Bovine aortic endothelial cells cultured within distensible tubes were exposed to physiological nonreversing steady or PP (7 dynes/cm² mean shear, pulse pressure 0 or 90 mm Hg×2 hours) in a custom servo-system. In compliant tubes, PP doubled Akt phosphorylation above nonpulsatile flow levels, whereas P-Akt declined to static levels from PP in stiffer tubes. eNOS phosphorylation (S-1179) similarly increased with PP in compliant tubes but was nearly undetectable with increased PP in stiffer tubes. After PP, brief exposure of cells to ultraviolet irradiation (oxidant stress) and subsequent culture revealed cytoprotection in compliant tubes but diffuse cytotoxicity and cell detachment in stiffer tubes. NOS inhibition by L-NAME converted compliant-tube post-UV behavior to that of stiffer tubes. These data provide novel evidence that wall compliance can directionally mediate endothelial Akt/eNOS phosphorylation and mecano-signalization. This may help explain increased vascular risks resulting from artery stiffening. (Hypertension. 2003;41:378-381.)

Key Words: endothelium ■ pulse ■ arteries ■ shear stress ■ nitric oxide synthase ■ Akt ■ compliance

The vascular endothelium responds to mechanical stimuli to regulate vessel biology. Among the more prominent signaling cascades triggered in response to steady shear is activation of phosphatidylinositol-OH kinase (PI-3K), which in turn phosphorylates Akt kinase. Akt plays a central role in vascular homeostasis, regulating cell survival, migration, and angiogenesis, glucose metabolism, protein synthesis, and cytoprotection.1-2 Shear stress also modulates vascular tone, in large part, by serine phosphorylation (S1179) of endothelial Akt and eNOS coupled to Akt activation3-5 and PKA stimulation.6 However, prior studies have solely investigated steady, nonpulsatile shear, whereas perfusion in vivo is pulsatile, combining both phasic shear with stretch. The latter itself can stimulate eNOS7 and PI-3K/Akt.8 Furthermore, enhancing pulse perfusion (PP) of compliant coronary and skeletal arteries in vivo triggers vasodilation with increased eNOS activity.9-11 Higher perfusion pulsatility also occurs in aged, less compliant vessels. Yet, in this instance, flow-mediated dilation declines12 and pulse pressure becomes a dominant vascular risk factor.13

One potential explanation for seemingly opposing influences of PP in normal versus stiffer vessels is that wall compliance itself can modify endothelial mecano-biochemical signaling. The present study tested the hypothesis that endothelial Akt and eNOS responses to PP are potently altered by reduction of wall compliance. We further examined whether differential mecano-signalizing coupled to wall compliance affords or compromises cytoprotection to oxidant stress. These questions were addressed using a custom-designed novel in vitro servo-controlled perfusion system with which endothelial cells cultured in distensible tubes could be subjected to physiological nonreversing pulsatile flow and pressure waveforms.14

Methods

Bovine aortic endothelial cells (Coriell Cell Repositories, passage 4 to 8) were grown to confluence in silastic tubes (inner diameter: 4 mm; length: 18 cm; Specialty Manufacturing) precoated with 0.01% fibronectin and exposed to physiological pressure-flow waveforms (Figure 1A) using a custom-designed servo-motor apparatus.14 Compliant tube distensibility was similar to a normal carotid artery (7% radial stretch at 90 mm Hg pulse pressure), whereas stiffer tubes had 85% lower distensibility. Tubes were perfused with DMEM (37°C, physiological pH, O₂, and CO₂, no antibiotics), at a mean flow rate of 250 mL/min (7 dynes/cm² mean shear stress) and mean pressure of 90 mm Hg—under nonpulsatile or pulsatile conditions—for 2 hours (time of maximal P-Akt from constant shear¹⁵). In additional studies, the PI-3K inhibitor Wortmannin (20 nmol/L; Calbiochem) or eNOS inhibitor L-NAME (1 mmol/L, Sigma) were added to the culture medium during PP exposure. Immediately following perfusion study (with or without pulsatility), tubes were removed from the apparatus and cells examined under light microscopy to confirm maintenance of confluent attach-
ment. Culture media was replaced with iced PBS buffer, followed by lysis buffer (200 to 250 μL), and incubated on ice for 10 minutes. Lysate was manually squeezed from the tubes and centrifuged at 15,000 g for 20 minutes. The supernatant was removed and frozen at −80°C until protein assays were performed. In some studies, a portion of the tube was first removed for subsequent histology analysis. Protein quantitation was performed by bicinchoninic acid (BCA) assay (BioRad). Western blotting was performed as previously described, with 25 μL protein loading per lane, and polyclonal antibodies for either P-Akt (S473; 1:1000) or P-eNOS (S1179, 1:1000) (Cell Signaling Technology).

To assess whether PP conferred differential cytoprotection in compliant versus stiff tubes, additional studies were performed in which tubes were rapidly removed following perfusion exposure (2-hour, 90 mm Hg pulse-pressure), the medium replaced by physiological buffer, and cells transiently irradiated by 90 to 100 J/m² ultraviolet light (20 sec). After refilling with DMEM, cells were incubated for an additional 18 hours and subsequently imaged to assess morphology and attachment.

All protocols and conditions were performed in quadruplicate, and each gel run in duplicate, with analysis performed on the average result. Western blots were analyzed by first normalizing band density to the average value from lanes reflecting nonpulsatile shear (PP=0 in figures). These results were then subjected to 1- or 2-way ANOVA, with pulse pressure serving as a categorical variable and wall compliance (distensible or stiff) as another. Analyses with 2 comparisons were made using a nonpaired t test. Results are expressed as mean±SD.

Figure 1. A, Pressure, flow, and diameter waveforms in pulse-perfused distensible tubes lined with bovine aortic endothelial cells. Pressure was generated by a servo-controlled linear motor to match a prerecorded in vivo waveform. Phasic flow and wall diameter resulted from the interaction between pressure and the servo-perfusion system. Pulse pressure and flow were similar with both compliant and stiff tubes, whereas wall distension was markedly lower in the latter. B, In compliant tubes (n=5), P-Akt increased with nonpulsatile flow over static (incubator) controls and further rose another 100% with PP. C indicates static control; NP, nonpulsatile flow; and PP, pulse perfusion (pulse pressure=90 mm Hg). C, In stiff tubes (n=4), P-Akt rose similarly with nonpulsatile flow but fell to static-control levels with PP. D, Inhibition of PI-3K by Wortmannin prevented PP-dependent P-Akt increase in compliant tubes (n=8). E, Effect of wall compliance on P-eNOS and total eNOS (n=4). P-eNOS was low in nonstimulated controls (C) in either type of tube (combined data shown). In compliant tubes, PP (90 mm Hg pulse-pressure) resulted in elevated P-eNOS, whereas in stiff tubes this declined to nearly undetectable levels. Total eNOS protein was unaltered by these interventions.

Results

Compared with static (no-flow, incubator only) conditions (C), steady shear (PP=0) increased Akt phosphorylation by +70±42% (P<0.01). This response was similar in both compliant and stiff tubes as perfusion was nonpulsatile. However, PP induced directionally opposite changes in P-Akt depending on tube distensibility. In compliant tubes, P-Akt levels doubled over constant shear at 90 mm Hg PP (F-actin shown as control for protein loading), whereas in stiff tubes, PP reduced P-Akt to static-control levels (Figure 1C). The doublet band noted at higher PP in the compliant tubes was observed particularly at high levels of Akt phosphorylation. This may have reflected detection of both single- and dual-phosphorylation forms of Akt. To test whether the P-Akt increase in compliant tubes was related to PI-3K activation, additional studies were performed with 20 nmol/L Wortmannin added to the perfusate to inhibit PI-3K. Wortmannin had a modest effect on inhibiting P-Akt increase with nonpulsatile flow but markedly limited P-Akt increase with PP (Figure 1D). This supports a role of PI-3K activation to pulse-stretch enhanced P-Akt in compliant tubes.

We next assessed whether wall distension similarly differentially influenced eNOS S-1179 phosphorylation. Nonstimulated cells (Figure 1E, far left lanes) displayed a
low-basal level of S-1179 P-eNOS, whereas nonpulsatile flow increased levels (not shown) in both compliant and stiff tubes, in concordance with prior data. However, there were marked disparities in P-eNOS depending on tube distensibility when cells were exposed to 90 mm Hg PP. P-eNOS remained elevated in compliant tubes, but fell to undetectable levels in stiff tubes (Figure 1E). Total eNOS protein was unchanged between conditions.

The marked disparity in P-Akt and P-eNOS between compliant and stiff tubes suggested that tube distensibility might alter endothelial cell adhesion and survival, particularly if cells were subsequently stressed. To test this, pulse-perfused cells in both compliant (Figure 2A) and stiff (Figure 2C) tubes were exposed to brief UV radiation to stimulate cell death and detachment. In compliant tubes, UV had little effect, with a large proportion of the cells remaining adhered to the tubes and maintaining their endothelial morphology (Figure 2B). In stiff tubes, however, UV led to morphological changes, with cells assuming an elongated stellate shape (Figure 2D). In other stiff tubes, near total cell detachment was observed, whereas this was not produced with compliant tubes (data not shown). The examples reflect representative responses of at least 4 separate experiments under each condition.

To further test for a role for PP-dependent eNOS activation to the cytoprotection of endothelial cells in compliant tubes exposed to UV-irradiation, cells were coincubated with the NOS inhibitor L-NAME (1 mmol/L) during 90 mm Hg PP. L-NAME exposure and acute UV-irradiation themselves did not result in cell detachment or morphological changes (Figure 2E). However, subsequent incubation now yielded diffuse cell damage and detachment similar to that in stiff tubes (Figure 2F).

**Discussion**

We provide the first demonstration that wall compliance and, thus, magnitude of endothelial cell stretch with PP directionally determines both Akt and eNOS phosphorylation and thereby promotes endothelial cell survival and adhesion. This suggests a novel mechanism whereby loss of vascular distensibility (as with aging) might itself modify endothelial mechanosignaling compromising vasodilation and increasing cellular vulnerability to stress. Such a mechanism would explain why pulse pressure contributes to vascular risk in the aged.

Prior studies have shown that activation of PI-3K and Akt are important to endothelial cell survival and growth and regulation of vascular tone via eNOS phosphorylation. Activation of eNOS by Akt has also been implicated in cytoprotection of endothelial cells from corticosteroids and in angiogenesis stimulated by HMG-CoA-reductase inhibition. Phosphorylation of eNOS at S1179 (S1177 in human) greatly enhances NO synthesis at low calcium, whereas transfection of a S1179-inactivated eNOS (alanine for serine) into eNOS-null mice depresses both basal NO release and endothelial-dependent vasomotor response. Furthermore, inhibition of P-Akt by dominant negative Akt mutants prevents enhanced NO synthesis by shear stress. Although many of the aforementioned studies have established the role of constant shear stress and PI-3K/Akt/eNOS signaling, physiological shear is pulsatile. Our findings show that in the context of such physiological pulsatile perfusion, concomitant cell stretch determined by vessel compliance potently contributes to such signaling, in concordance with in vivo evidence for enhanced NOS activity from PP.

The most striking and novel finding of the present study was not that eNOS and Akt phosphorylation can be enhanced by pulsatile perfusion, but rather that these changes were reversed to static (no-shear) levels by diminishing concomitant cell stretch. The profound magnitude of reduced P-Akt and P-eNOS by PP in stiff tubes suggested cells might be more vulnerable to stress. The UV-irradiation protocol was designed to capture potential disparities in cytoprotective signaling mechanisms activated after 2-hour PP, and the results confirmed this hypothesis. It is important that cellular attachment and shape were normal under all conditions immediately following PP and UV irradiation (Figures 2A and 2C). Thus, changes at later times reflect a lack of induction of cytoprotective mechanisms in cells in stiff tubes that are recruited in cells cultured in compliant tubes.

The pulsatile shear employed in the present experiments was nonreversing (Figure 1A), typical of most in vivo flow. This is important, because fully reversing (oscillatory) shear inhibits eNOS activation and NO release and enhances oxidative stress signaling. In the present studies, pulse shear generated different responses in P-eNOS and P-Akt versus constant flow, but the extent of concomitant cell stretch rather than pulse shear itself primarily determined the response.
Perspectives
The present data showing that wall compliance plays a key role in directionally modifying endothelial mechanosignaling and cytotoxicity to oxidant stress in the presence of PP has potential importance to vascular biology, particularly the pathophysiology of arterial stiffening. Normal elevation of pulse pressure with exercise may enhance vasodilator responses and stimulate angiogenesis, in part via enhanced P-Akt signaling. However, a decline in both Akt and eNOS phosphorylation by PP in stiffer vessels would suggest a novel mechanism whereby vessel stiffening, as with aging, may offset normal vasomotor responses and cell protections triggered by luminal PP and thus contribute to vascular risk. This suggests that endothelial function cannot only contribute to arterial stiffening but is itself influenced by the distensibility of the vascular wall, providing an additional mechanism for benefits of enhanced arterial compliance. Future studies will test these mechanisms in arterial segments and intact human vasculatures, and if confirmed, could lead to novel therapies to restore endothelial responses to those of compliant arteries.

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