Prevention of aortic fibrosis by \(N\)-acetyl-seryl-aspartyl-lysyl-proline in angiotensin II-induced hypertension

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Lin CX, Rhaleb NE, Yang XP, Liao TD, D’Ambrosio MA, Carretero OA. Prevention of aortic fibrosis by \(N\)-acetyl-seryl-aspartyl-lysyl-proline in angiotensin II-induced hypertension. Am J Physiol Heart Circ Physiol 295: H1253–H1261, 2008. First published July 18, 2008; doi:10.1152/ajpheart.00481.2008.—Fibrosis is an important component of large conduit artery disease in hypertension. The endogenous tetrapeptide \(N\)-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) has anti-inflammatory and profibrotic effects in the heart and kidney. However, it is not known whether Ac-SDKP has an anti-inflammatory and profibrotic effect on conduit arteries such as the aorta. We hypothesize that in ANG II-induced hypertension Ac-SDKP prevents aortic fibrosis and that this effect is associated with decreased protein kinase C (PKC) activation, leading to reduced oxidative stress and inflammation and a decrease in the profibrotic cytokine transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) and phosphorylation of its second messenger Smad2. To test this hypothesis we used rats with ANG II-induced hypertension and treated them with either vehicle or Ac-SDKP. In this hypertensive model we found an increased collagen deposition and collagen type I and III mRNA expression in the aorta. These changes were associated with increased PKC activation, oxidative stress, intercellular adhesion molecule (ICAM)-1 mRNA expression, and macrophage infiltration. TGF-\(\beta\)1 expression and Smad2 phosphorylation also increased. Ac-SDKP prevented these effects without decreasing blood pressure or aortic hypertrophy. Ac-SDKP also enhanced expression of inhibitory Smad7. These data indicate that in ANG II-induced hypertension Ac-SDKP has an aortic antifibrotic effect. This effect may be due in part to inhibition of PKC activation, which in turn could reduce oxidative stress, ICAM-1 expression, and macrophage infiltration. Part of the effect of Ac-SDKP could also be due to reduced expression of the profibrotic cytokine TGF-\(\beta\)1 and inhibition of Smad2 phosphorylation.

artherosclerosis; vascular hypertension; inflammation; protein kinase C; transforming growth factor-\(\beta\)/Smad

Hypertension is associated with large conduit artery hypertension and an increase in extracellular matrix (ECM) content, especially collagen. Fibrosis is a major component of hypertensive vascular disease, causing vascular stiffness and arteriosclerosis (20, 37). Angiotensin II (ANG II) contributes to vascular injury by inducing inflammation, oxidative stress, excessive deposition of ECM, and hypertrophy and/or hyperplasia of vascular smooth muscle cells (VSMCs) (10). ANG II via its type 1 (AT\(_1\)) receptor initiates activation of intracellular signaling cascades, including protein kinase C (PKC) activation. PKC-dependent activation of NAD(P)H oxidase leads to increased oxidative stress and inflammation, which play essential roles in vascular disease (1, 10, 19, 36). ANG II also induces vascular fibrosis by stimulating transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1), which mediates its profibrotic effects by activating receptor-associated Smads (R-Smads, including Smad2 and Smad3) (35). Inhibitory Smads (I-Smads, i.e., Smad6 and Smad7) block TGF-\(\beta\)1 signaling by binding to TGF-\(\beta\) receptor type 1 receptors or by competing with activated R-Smad for binding to Smad4 (48).

\(N\)-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is an endogenous tetrapeptide originally described as a natural inhibitor of pluripotent hematopoietic stem cell proliferation (29). In vitro Ac-SDKP prevents collagen synthesis, cell proliferation, and TGF-\(\beta\)1-stimulated Smad2 phosphorylation in cardiac fibroblasts (42, 46). Ac-SDKP appears to act via a receptor that we have recently characterized (62) but not yet cloned. Furthermore, chronic blockade of endogenous Ac-SDKP with a specific prolyl oligopeptidase inhibitor promotes collagen accumulation (7).

Angiotensin-converting enzyme (ACE) inhibitors prevent degradation of endogenous Ac-SDKP, raising its circulating concentrations approximately fivefold (2, 3, 23). In ANG II-induced hypertension Ac-SDKP mediates anti-inflammatory and profibrotic effects of ACE inhibitors (41). In hypertensive rats, long-term treatment with Ac-SDKP decreases inflammatory cell infiltration as well as cardiac and renal fibrosis (40, 41, 47). Furthermore, in rats with ANG II-induced hypertension Ac-SDKP also decreases the number of TGF-\(\beta\)-positive cells and Smad2 activation in the heart (41). Although the anti-inflammatory and profibrotic effects of Ac-SDKP are well documented in the heart and kidney, it is not known whether Ac-SDKP has similar effects on conduit arteries such as the aorta. Thus we hypothesize that in ANG II-induced hypertension Ac-SDKP prevents aortic fibrosis. We also hypothesize that this effect is associated with decreased PKC activation, leading to reduced oxidative stress and inflammation and a decrease in the profibrotic cytokine TGF-\(\beta\)1 and phosphorylation of its second messenger Smad2.

Materials and Methods

Experimental groups. This protocol was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee (IAUC). Male Sprague-Dawley rats weighing 250–300 g (Charles River, Wilmington, MA) were housed under controlled conditions and randomly divided into four groups: 1) vehicle; 2) Ac-SDKP (800 \(\mu\)g·kg\(^{-1}\)·day\(^{-1}\)); 3) ANG II (750 \(\mu\)g·kg\(^{-1}\)·day\(^{-1}\)); and 4) ANG II +...
Ac-SDKP. Ac-SDKP and ANG II were dissolved in 0.01 N acetic acid in saline. The dose of ANG II and Ac-SDKP was selected based on previous studies (21, 28, 40, 45). All treatments were carried out for 7 days by subcutaneous implantation of miniosmotic pumps (Alzet 2ML1). Systolic blood pressure (SBP) was measured by tail cuff (IITC/Life Science) on day 0 (basal) and then at 3 and 6 days after miniosmotic pump implantation.

Morphological analysis. After 7 days of treatment, rats were anesthetized with 50 mg/kg pentobarbital sodium. For histological studies the aortas were perfused via the left ventricle with cold phosphate-buffered saline (PBS, pH 7.4) for 5 min, followed by 10% buffered formalin for 5 min under pressure (150 mmHg). A 6-μm ring from the thoracic aorta obtained halfway between the left subclavian artery and the diaphragm was embedded in paraffin and cut into 6-μm sections. Extreme care was used to ensure that the aorta was not stretched on dissection. Paraffin-embedded sections were stained with hematoxylin and eosin and photographed at 200 magnification with an inverted microscope (1X81, Olympus America). Images were obtained under light microscopy (×200) and quantified with a computerized image analysis system (Microsuite Biological Imaging software, Olympus America, Center Valley, PA) by an investigator unaware of the group assignment (32).

Collagen and elastin deposition. To quantify collagen density, 6-μm sections were stained with a modified picrosirius red method (41). Elastin deposition was determined by staining with Verhoff Van Giesson (56). Images were obtained with Sigma Scan Pro 5.0 software (Jandel Scientific) by an investigator unaware of the group assignment (32).

Immunohistochemistry for lipid oxidation (4-hydroxy-2-nonenal) and macrophage infiltration. Immunohistochemistry was performed as described previously (33). Briefly, paraffin-embedded sections were incubated with primary antibodies against 4-hydroxy-2-nonenal (4-HNE, 1:200, OXIS), nitrotyrosine (1:50, Millipore), or ED-1 (a macrophage marker, 1:50, Novocastra). Immunoreactivity was detected with a Vectastain Elite ABC peroxidase kit (Vector Labs) and 3-aminop-ethylcarbazole solution (AEC, Zymed Labs). A reddish-brown color was considered a positive stain. Sections were counterstained with hematoxylin. Images were captured with an ×400 magnification with an inverted microscope (1X81, Olympus America) and a digital camera (DP70, Olympus America). Quantitative analysis was performed by an investigator unaware of the group assignment (32).

Gene expression of collagen type I, collagen type III, intercellular adhesion molecule-1, and 18S. Another group of rats on each treatment (n = 6) was used for these studies, since formalin-fixed tissues cannot be used for assessing gene and protein expression. The thoracic aorta was immediately excised, rinsed briefly with PBS precooled at 4°C, dissected from adherent fat and connective tissues, and then immediately frozen in liquid nitrogen and stored at −80°C until use. Frozen aortic tissue was homogenized with mortar and pestle under liquid nitrogen. Total RNA was isolated with a RNeasy Fibrous Tissue minikit (Qiagen). Reverse transcription was performed with an Omniscript Reverse Transcription kit (Qiagen). Semi-quantitative polymerase chain reaction (PCR) was performed with the following specific primers: collagen type I: forward 5′-TCACCTACAC-CACGGCTTG-3′, reverse 5′-GTTGTGTTTTCAAGGTTG-3′ (245 bp); collagen type III: forward 5′-ATATCAAACAGCAAGGC-3′, reverse 5′-GATTAAAGCAAGGAGCAAC-3′ (175 bp); intercellular adhesion molecule (ICAM)-1: forward 5′-AGAAAGACTGTGTTGGGGA-3′, reverse 5′-CCTTGCCGTTAATAGGTTG-3′ (332 bp); 18S RNA: forward 5′-AGGAAATGACGGAAGGCCAC-3′, reverse 5′-GTGCAAC-GCCGACATCTAAG-3′ (327 bp). 18S was used as internal control. The 28-cycle PCR program consisted of 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 40 s of extension at 72°C. The PCR products were analyzed on 1.5% agarose gel with ethidium bromide staining. Results are expressed as arbitrary densitometric units relative to 18S.

Western blot for phosphorylated PKC, TGF-β1, phosphorylated Smad2, and Smad7. Aortic protein was extracted from frozen tissue (51) and concentration was determined with a Micro BCA protein assay kit (Pierce). Aliquots were separated by electrophoresis under nonreducing conditions, transferred onto nitrocellulose membranes, and incubated with specific antibodies to phosphorylated (p-)PKC-α/βII (1:1,000, Cell Signal Technology), TGF-β1 (1:1,000, MAB240, R&D Systems), p-Smad2 (1:250, Cell Signal Technology), and Smad7 (1:250, Santa Cruz). Signals were revealed with chemiluminescence (ECL kit, Amersham Pharmacia) and visualized by autoradiography. Membranes were then stripped (Pierce) and reprobed with β-actin (1:1,000, Santa Cruz) or total Smad2 (T-Smad2, 1:500, Cell Signal Technology) to verify equal loading. Optical band density was quantified and expressed as arbitrary units relative to corresponding β-actin or T-Smad2. The molecular masses of positive bands were p-PKC-α/βII, 80 and 82 kDa; TGF-β1, 25 kDa; p-Smad2 and T-Smad2, 60 kDa; and Smad7, 51 kDa.

Statistical analysis. All values are expressed as mean ± SE, with n indicating the number of animals in each group. For all parameters, Student’s two-sample t-test was used to compare differences between groups, such as control vs. ANG II or ANG II vs. ANG II + Ac-SDKP. Hochberg’s method of adjusting the α-level was used to control the possibility of making an incorrect decision across multiple tests. The type I error rate was set as α = 0.05.
RESULTS

**Systolic blood pressure.** Baseline SBP was similar in all groups. At days 3 and 6, SBP increased significantly \( P < 0.0001 \) in rats receiving ANG II compared with vehicle. There was no significant difference in SBP between ANG II and ANG II plus Ac-SDKP, indicating that Ac-SDKP did not alter ANG II-induced hypertension (Fig. 1, top).

**Morphological analysis.** ANG II significantly increased medial cross-sectional area (Fig. 1, bottom) and wall thickness of the thoracic aorta (vehicle 99.81 ± 7.37 vs. ANG II 157.71 ± 7.64 \( \mu \)m, \( P < 0.01 \)). Ac-SDKP had no significant effect on arterial wall morphology.

**Collagen and elastin deposition and collagen mRNA expression.** Picrosirius red staining was performed to assess collagen deposition in the aortic wall. As illustrated in Fig. 2, in ANG II-induced hypertension collagen deposition in the aorta media was significantly increased \( P < 0.001 \), vehicle vs. ANG II). Ac-SDKP prevented these increases \( P < 0.001 \), ANG II vs. ANG II + Ac-SDKP). Similarly, semiquantitative PCR analysis showed that ANG II significantly increased collagen type I and III mRNA expression, which was inhibited by Ac-SDKP (Fig. 3). Verhoeff Van Giesson staining of the aorta revealed increased elastin deposition within the media of ANG II-infused rats \( P < 0.001 \), vehicle vs. ANG II). As shown in Fig. 4, this increase was blocked by coadministration of Ac-SDKP \( P < 0.001 \), ANG II vs. ANG II + Ac-SDKP). Ac-SDKP alone had no effect on elastin density. The ratio of aortic elastin to collagen density (E/C) had a tendency to decrease in the ANG II group and increase in the ANG II plus Ac-SDKP group; however, these differences were not statistically significant (vehicle 2.10 ± 0.31, ANG II 1.78 ± 0.23, and ANG II + Ac-SDKP 2.19 ± 0.40).

**PKC phosphorylation.** ANG II significantly increased PKC phosphorylation \( P < 0.05 \), vehicle vs. ANG II); Ac-SDKP prevented such increase \( P < 0.05 \), ANG II vs. ANG II + Ac-SDKP) (Fig. 5).

**In situ reactive oxygen species detection.** As expected, ANG II-induced hypertension caused a significant increase in oxidative stress as indicated by the immunohistochemical staining for 4-HNE. Staining was present across the media in the ANG II group \( P < 0.01 \), vehicle vs. ANG II (Fig. 6A). Similarly, staining for nitrotyrosine (a marker of peroxynitrite) was also significantly increased in the ANG II group (vehicle vs. ANG II, \( P < 0.05 \) (Fig. 6B). The increase in these oxidative stress markers was prevented by Ac-SDKP (Fig. 6). These data indicate that Ac-SDKP had an inhibitory effect on ANG II-induced oxidative stress.

**Macrophage infiltration and ICAM-1 mRNA expression.** The infiltrating macrophages were measured with ED-1 (a marker for macrophages) immunohistochemical staining and ICAM-1 mRNA expression with semiquantitative PCR. ED-1-positive cells in the aortic wall stained reddish-brown. Specificity of this immunostaining was confirmed by a negative staining in the absence of primary antibody in rabbit serum.
serial sections. Quantitative cell count analysis showed that ANG II significantly increased the number of ED-1-positive cells in the aortic wall (P < 0.01, vehicle vs. ANG II) and Ac-SDKP attenuated this effect (P < 0.05, ANG II vs. ANG II + Ac-SDKP) (Fig. 7). Semiquantitative PCR showed that ANG II produced a twofold increase in aortic ICAM-1 mRNA expression, which was significantly prevented by Ac-SDKP treatment (P < 0.05, ANG II vs. ANG II + Ac-SDKP) (Fig. 8).

\[ TGF-\beta/Smad signaling. \] TGF-\( \beta \)1 expression increased significantly with ANG II administration (P < 0.05, vehicle vs. ANG II). This increase was significantly prevented by Ac-SDKP (P < 0.05, ANG II vs. ANG II + Ac-SDKP) (Fig. 9A). ANG II induced a 5.2-fold increase in Smad2 phosphorylation compared with vehicle (P < 0.01), and Ac-SDKP significantly prevented this increase (P < 0.05, ANG II vs. ANG II + Ac-SDKP) (Fig. 9B). I-Smad7 did not change in ANG II-treated rats compared with vehicle, whereas treatment

Fig. 3. mRNA expression of collagen type I and III in rat aorta by semiquantitative PCR. Rats were treated with vehicle, Ac-SDKP, ANG II, or ANG II + Ac-SDKP. A: collagen type I mRNA expression. B: collagen type III mRNA expression. Bar graphs show mRNA expression of collagen type I or III indicated as the density ratio of collagen I or III to 18S. Data are means ± SE; n = 6 rats/group.

Fig. 4. Effect of Ac-SDKP on ANG II-induced elastin deposition in the aortic media. Top: representative images showing elastic fibers (purple) stained with Verhoef van Giesson in aortas from rats treated with vehicle, Ac-SDKP, ANG II, or ANG II + Ac-SDKP. Magnification ×200. Bottom: quantitative evaluation of elastin density in the aortic media. The stained area is indicated as % of the medial area. n = 8 or 9/group.
with both ANG II and Ac-SDKP resulted in increased aortic I-Smad7 expression ($P < 0.01$, ANG II vs. ANG II + Ac-SDKP) (Fig. 9C).

**DISCUSSION**

Previous studies from our group (40, 41, 47) have found that Ac-SDKP decreases cardiac and renal inflammation and fibrosis in various models of hypertension and in heart failure after myocardial infarction. We have now found that in the aorta of rats with ANG II-induced hypertension Ac-SDKP prevented increases in 1) collagen and elastin deposition and collagen type I and type III mRNA expression, 2) PKC activation, 3) oxidative stress, 4) ICAM-1 mRNA expression and macrophage infiltration, and 5) TGF-β1 expression and Smad2 activation, but it enhanced I-Smad7 expression. The present study provides the first in vivo evidence that Ac-SDKP plays an important role in preventing ANG II-induced aortic fibrosis. This protective effect may be mediated via inhibition of PKC activation that causes a decrease in oxidative stress, aortic inflammation, and activation of the TGF-β1/Smad2 pathway.

Fibrosis in the large-capacitance arteries occurs in hypertension and with aging, and it may contribute to the development of arteriosclerosis and isolated systolic hypertension (9, 22). We evaluated aortic collagen deposition with a histomorphometric approach that closely approximates hydroxyproline concentrations within tissue (4). Consistent with previous reports (6, 58), we found that ANG II increased aortic collagen deposition, associated with marked upregulation of collagen type I and III mRNA expression. The effects of Ac-SDKP occurred without significant changes in blood pressure or aortic hypertrophy (indicated by aortic wall thickness and medial cross-sectional area). In parallel with this, we previously reported (40, 41, 45, 47, 61) that Ac-SDKP, despite decreasing cardiac inflammation and fibrosis, changed neither blood pressure nor left ventricular hypertrophy.
Elastin is a vessel wall component with a low elastic modulus (5 × 10^6 dyn/cm^2) that contributes to aortic distensibility (11). Contradictory results have been reported concerning elastin density in the aorta of hypertensive animals, but such discrepancies may be explained by differences in the models studied. Elastin was reportedly increased in spontaneously hypertensive rats (SHR) (4) and in ANG II-induced hypertension (11, 30). On the other hand, elastin was reportedly decreased in rats with renovascular hypertension, which is a model of high renin (55). In the present study, we observed that in ANG II-induced hypertension elastin levels increased and Ac-SDKP prevented this increase. In agreement with our findings, it has been reported that the ACE inhibitor enalapril (increases plasma Ac-SDKP concentrations) prevented elastin accumulation in young Wistar rats (26). Since elastin provides vascular elasticity while collagen provides tensile strength (11), the ratio of aortic elastin to collagen density (E/C) has been proposed to predict corresponding changes in vessel stiffness in vivo. In the present study, the E/C had a tendency to decrease in the ANG II group and increase in the ANG II plus Ac-SDKP group; however, these differences are not statistically significant. The E/C were reportedly decreased in SHR (31) and renovascular hypertension (55) and normalized by ACE inhibition (which increases Ac-SDKP). However, it must be noted that the hypertension and the treatment were more chronic (months) than in our study (1 wk). Also, the decrease in E/C is not a uniform finding (5, 11, 25).

We also investigated whether Ac-SDKP decreases PKC-α/β phosphorylation in ANG II-induced hypertension. PKC has been implicated in NADPH activation and reactive oxygen species (ROS) generation in ANG II models of hypertension (15, 17, 50). PKC-α selectively regulates lipopolysaccharide-
induced macrophage activation, and PKC-β participates in differentiation of monocytes to macrophages (49, 53). Furthermore, we showed previously (52) that in vitro Ac-SDKP decreases the transformation of monocytes to macrophages and also inhibits macrophage activation. In VSMCs and mesangial cells, PKC has also been implicated in induction of TGF-β gene expression and activity in response to ANG II (12, 59). PKC-β inhibition attenuates macrophage infiltration, interstitial fibrosis, TGF-β expression, and Smad2 phosphorylation in kidneys of diabetic rats (27). In the present study, we found that ANG II induced a significant increase in PKC-β phosphorylation, and Ac-SDKP prevented this increase. Thus it is possible that enhanced aortic PKC activation contributes to ANG II-induced oxidative stress, macrophage infiltration, and activation of the TGF-β1/Smad2 pathway, which in turn stimulates collagen production.

Evidence supports the association between oxidative stress and fibrosis. ROS may stimulate ECM deposition (collagen and elastin) by increasing proinflammatory and profibrotic cytokines such as TGF-β1 that, via the Smad system, induce collagen synthesis (43). In the present model of ANG II-induced hypertension, we explored whether Ac-SDKP affects arterial oxidative stress. Consistent with other reports (8, 44, 57), we found that ANG II induced an increase in oxidative stress in the aorta as evidenced by increased 4-HNE (a product of lipid oxidation by H2O2, OONO−, and OH−) and nitrotyrosine expression, and that Ac-SDKP prevented these increases. It is also known that hypertension can induce oxidative stress through its effect on vessel wall stretching (18); however, in the present study Ac-SDKP did not lower blood pressure in ANG II-induced hypertension. Thus it could be proposed that the inhibitory effect of Ac-SDKP on arterial oxidative stress is independent of changes in blood pressure.

Although we previously reported that Ac-SDKP inhibited macrophage infiltration in the heart and in the kidney, it is not certain whether the same effect will occur in the aorta (39, 41, 45, 61). Indeed, it has been recognized that macrophages isolated from different anatomic sites display a diversity of phenotypes and capabilities. Because macrophage function is dependent in part on signals received from the immediate microenvironment (tissues), it is suggested that macrophage heterogeneity may arise from unique conditions within specific tissues (13, 14, 54). Studies have suggested a proinflammatory role for ROS in the vasculature, including increased adhesion molecule expression, release of cytokines, and chemotraction of leukocytes to the aorta at sites of endothelial damage (16, 34). Thus in the present study, we measured ICAM mRNA expression and macrophage infiltration as indicators of inflammation. Consistent with previous reports that Ac-SDKP decreases cardiac and renal inflammatory cell infiltration in hypertensive rats (40, 41, 47), here we found that in the aorta of rats with ANG II-induced hypertension Ac-SDKP signifi-
cantly prevented both ICAM-1 mRNA expression and the increase in ED-1-positive cells (macrophages) in the aorta. Macrophages have been identified as a major source of the profibrotic growth factor TGF-β1. Macrophages have been identified as a major source of the profibrotic growth factor TGF-β1. Consistent with our previous reports that Ac-SDKP prevents ANG II-induced aortic fibrosis, we found that Ac-SDKP significantly attenuated ANG II-induced aortic TGF-β1 expression and Smad2 phosphorylation. Interestingly, Ac-SDKP increased I-Smad7 expression in aortas of ANG II hypertensive rats, whereas Ac-SDKP or ANG II alone did not alter I-Smad7. It could be that I-Smad7 partially accounts for the inhibitory effect of Ac-SDKP on ANG II-induced Smad2 activation. In agreement with our findings, Kandasari et al. (24) demonstrated that in human mesangial cells Ac-SDKP inhibits TGF-β signal transduction through suppression of R-Smad activation via nuclear export of I-Smad7. However, in contrast to our present findings in the vasculature, we previously reported that Ac-SDKP did not affect I-Smad7 expression in the heart after 4 wk of ANG II infusion, possibly because of low cardiac expression of I-Smad7.

Taken together, our data indicate that Ac-SDKP has an antifibrotic effect on the aorta of ANG II hypertensive rats. This effect may be mediated by inhibiting aortic PKC activation, causing a decrease in oxidative stress, inflammation, and the TGF-β1/Smad2 pathway (Fig. 10).

**Perspectives**

Increased ECM deposition is an important feature of target organ damage in hypertension. In the present study, we found that Ac-SDKP attenuates aortic fibrosis in ANG II-induced hypertension, possibly by preventing PKC activation, oxidative stress, inflammation, and TGF-β1/Smad2 activation. These findings will help us understand some of the mechanisms by which vascular fibrosis develops in hypertension and arteriosclerosis and also in aging subjects. Design of nonpeptidic analogs of Ac-SDKP would be useful for treating fibrosis in large vessels.

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