N-Acetyl-Ser-Asp-Lys-Pro Inhibits Phosphorylation of Smad2 in Cardiac Fibroblasts

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Abstract—N-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) is a specific substrate for the N-terminal site of ACE and increases 5-fold during ACE inhibitor therapy. It is known to inhibit the proliferation of hematopoietic stem cells and has also recently been reported to inhibit the growth of cardiac fibroblasts. We investigated its mode of action in cardiac fibroblasts by assessing its influence on transforming growth factor β1 (TGFβ1)–mediated Smad signaling. AcSDKP inhibited the proliferation of isolated cardiac fibroblasts (P<0.05) but significantly stimulated the proliferation of vascular smooth muscle cells. Flow cytometry of rat cardiac fibroblasts treated with AcSDKP showed significant inhibition of the progression of cells from G0/G1 phase to S phase of the cell cycle. In cardiac fibroblasts transfected with a Smad-sensitive luciferase reporter construct, AcSDKP decreased luciferase activity by 55±9.7% (P=0.01). Moreover, phosphorylation and nuclear translocation of Smad2 were decreased in cardiac fibroblasts treated with AcSDKP. To conclude, AcSDKP inhibits the growth of cardiac fibroblasts and also inhibits TGFβ1-stimulated phosphorylation of Smad2. Because AcSDKP increases substantially during ACE inhibitor therapy, this suggests a novel pathway independent of angiotensin II, by which ACE inhibitors can inhibit cardiac fibrosis. (Hypertension. 2002;40:155-161.)

Key Words: angiotensin inhibitors fibroblasts transforming growth factors myocardium

Although some attempts have been made to understand the biological behavior of cardiac fibroblasts, far more studies have been done on cardiomyocytes. However, fibroblasts are the cells that comprise more than two thirds of the total myocardial cell volume.1 It has been shown that stimulation of (myo)fibroblast activity leads to the excessive myocardial fibrosis, which is implicated in the decompensation of left ventricular function.2,3 Different growth factors play an important role in myocardial fibrosis, of which transforming growth factor β1 (TGFβ1) is a crucial cytokine involved.4,5 TGFβ signals via plasma membrane serine/threonine kinase receptors followed by the activation of cytoplasmic effectors such as Smad proteins. Phosphorylation of Smad2 and its subsequent translocation to the nucleus are the critical steps in cell signaling through this pathway. Inhibitory Smads, Smad6 and Smad7, antagonize these processes.6,7 The effects of TGFβ1 on the growth of cell depend on the type of cell subjected to it. Accordingly, TGFβ1 stimulates the growth of fibroblasts but is also a potent inhibitor of vascular smooth muscle cell growth.9 Therefore, any factor that inhibits TGFβ1 signaling would be expected to have opposite effects, depending on the type of cell studied.

Upregulation of ACE has been suggested to induce cardiac fibrosis by increasing the formation of angiotensin (Ang) II, and ACE inhibitors effectively inhibit this response. However, ACE inhibitors have been shown to reduce cardiac fibrosis even when the plasma levels of Ang II were not reduced, indicating that Ang II is not the only factor that relates ACE to cardiac fibrosis.10 ACE can metabolize a number of substrates, resulting into either activation or inactivation of their function. N-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) is one of the most recently identified peptides that are inactivated by ACE11; however, the role of AcSDKP in the cardiovascular system was not clearly established until recently.

AcSDKP is an endogenous tetrapeptide secreted by bone marrow and is ubiquitously found in plasma and various tissues.12–14 AcSDKP is degraded specifically by ACE both in vitro and in vivo, and its plasma level rises substantially during ACE inhibitor therapy.15–17 It is known to regulate the proliferation of hematopoietic progenitors17 and is also shown to reduce the proliferation of hepatocytes and renal fibroblasts.18,19 Studies indicate that an increase in AcSDKP may inhibit the growth of different types of cells. Recent studies have shown that AcSDKP attenuates the synthesis of DNA and collagen by cardiac fibroblasts both in vivo and in vitro.20,21 Considering the pivotal role of TGFβ1 in cardiac fibrosis, we hypothesized that AcSDKP may interfere with TGFβ1 signaling and thereby exert antifibrotic effects.
Therefore, we examined whether AcSDKP modulates the TGFβ1-induced signaling pathway in cardiac fibroblasts.

Methods

Reagents
AcSDKP was purchased from Bachem. Lisinopril was purchased from Sigma Zwijndrecht. Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), L-glutamate, penicillin, and streptomycin were purchased from Gibco BRL. Culture plates were obtained from Costar. StreptABC complex/horseradish peroxidase kit (ABC) and 3,3’-diaminobenzidine tetrahydrochloride were purchased from DAKO.

Cell Culture
The rat aortic smooth muscle cell line (PAC1 cell) was a kind gift from Dr G. van Eys (Department of Molecular Genetics, University of Maastricht, The Netherlands). Cardiac fibroblasts were isolated from 2-day-old neonatal Sprague-Dawley rats. Cultured neonatal rat ventricular nonmyocytes have been shown to be predominantly fibroblasts and were prepared as described previously. All experiments were performed on cells from the second passage. Each preparation contained >95% fibroblasts as determined by their morphology.

Effects of AcSDKP on Serum and TGFβ1

Cardiac fibroblasts (plating density, 120 cells/mm²) were grown until 70% to 80% confluent and were synchronized in medium containing 0.1% FBS, and then were stimulated to grow synchronously by adding either 10% FBS or 1 ng/mL TGFβ1 to the culture medium. After 24 hours of treatment of cells with and without AcSDKP, MTS reagent was added directly to the culture wells as described by the manufacturer and incubated for 4 hours; the absorbance was recorded at 490 nm. Background absorbance was subtracted from the absorbance of MTS.

Results

Effects of AcSDKP on Serum and TGFβ1-Stimulated Proliferation of Neonatal Rat Cardiac Fibroblasts

Cardiac fibroblasts (plating density, 120 cells/mm²) were grown until 70% to 80% confluent and were synchronized in medium containing 0.1% FBS, and then were stimulated to grow synchronously by adding either 10% FBS or 1 ng/mL TGFβ1 to the culture medium. After 24 hours of treatment of cells with and without AcSDKP, MTS reagent was added directly to the culture wells as described by the manufacturer and incubated for 4 hours; the absorbance was recorded at 490 nm. Background absorbance was subtracted from the absorbance obtained.

Addition of 10% FBS increased absorbance from 0.389 ± 0.008 to 0.445 ± 0.009 (Figure 1a), whereas TGFβ1 increased the absorbance from 0.333 ± 0.012 (control) to 0.393 ± 0.009 (Figure 1b). In serum-stimulated cells, co-treatment with AcSDKP (0.01 and 1 nmol/L) significantly reduced the absorbance to 0.379 ± 0.009 and 0.371 ± 0.005
In the TGFβ1-stimulated cells, addition of 1 nmol/L concentration of AcSDKP decreased the absorbance to 0.358 ± 0.012, whereas AcSDKP at 0.01 nmol/L did not show any effect (Figure 1b).

To determine whether the absorbance correlates with the numbers of cells in culture, various numbers of cardiac fibroblasts were plated in 96-well plate in the medium supplemented with 10% FBS. After an hour of equilibration, MTS reagent was added directly into the culture wells. Four hours later, absorbance was recorded at 490 nm using an enzyme-linked immunosorbent assay plate reader. The correlation coefficient was 0.96, indicating that there was a linear relationship between cell number and absorbance. Background absorbance shown at 0 cells/well was subtracted from these data (Figure 1c).

Effect of AcSDKP on Transition from G1 to S Phase by Neonatal Rat Cardiac Fibroblasts

To display the fluorescence signal only from living cells, scatter-gated fluorescence analysis was performed. Neonatal rat cardiac fibroblasts, either treated or untreated with AcSDKP, were detached from culture dishes by trypsin, washed with PBS, and incubated with propidium iodide (50 μg/mL in 0.1% [w/v] sodium citrate/0.1% [v/v] triton X-100) for 15 minutes at room temperature. Flow cytometry was performed to determine cell cycle status. In the control group, 85.3 ± 0.59% of the cells were at G0/G phase. After 24 hours of serum stimulation, the percentage of cells in G0/G1 phase decreased to 75 ± 1.4%, but when cotreated with AcSDKP (0.01 and 1 nmol/L), the percentage of cells in G0/G phase remained 82.4 ± 0.4% and 83.7 ± 0.5% (*P*<0.05; *n*=5), respectively (Figure 2). Lisinopril (100 nm/L) did not have any
Effect on the cell cycle of cardiac fibroblast (75.9 ± 1.4% versus 75.4 ± 1.2% G0/G1 cells in 10% FBS). Amount of debris present was < 1% in each group (Figure 2).

Effect of AcSDKP on TGFβ1-Inducible SBE Promoter Activity

To examine the effects of AcSDKP on TGFβ1-induced Smad binding element promoter activity, cardiac fibroblasts were transfected with pGL3ti(SBE)4 reporter construct, then treated with TGFβ1 24 hours later. AcSDKP was added 15 minutes before TGFβ1. Luciferase activity was measured after 24 hours. The data obtained were expressed as the ratio of pGL3ti(SBE)4 to internal control (pRL-CMV) (mean ± SEM; n = 8). AcSDKP inhibited phospho-Smad2. Cardiac fibroblasts were treated with TGFβ1 and AcSDKP. After 24 hours, whole cell lysate was prepared. Western blotting was performed with a phospho-Smad2 specific antibody (mean ± SEM; n = 3 to 4). Representative Western blot showing the effect of AcSDKP on phospho-Smad2 levels. Lane 1 indicates control; lane 2, TGFβ1; lane 3, TGFβ1 + lisinopril; lane 4, TGFβ1 + lisinopril + 0.01 nmol/L AcSDKP; lane 5, TGFβ1 + lisinopril + 1 nmol/L AcSDKP; and lane 6, TGFβ1 + lisinopril + 100 nmol/L AcSDKP. d. Effect of AcSDKP on nuclear translocation of Smad2. Cardiac fibroblasts were treated with TGFβ1 and AcSDKP. After 24 hours, cells were fixed, and immunostaining was performed with Smad2 antibody. (mean ± SEM; n = 3). Numbers of cells positive for nuclear staining were counted per 100 cells per slide. Data represents mean ± SEM; n = 3. #P < 0.08, *P = 0.05 vs TGFβ1. e. Representative cells showing the effect of AcSDKP on nuclear translocation of Smad2. Immunostaining was performed on 50% confluent cells. e1 indicates control; e2, TGFβ1; e3, TGFβ1 + 1 nmol/L AcSDKP; and e4, TGFβ1 + 100 nmol/L AcSDKP.

AcSDKP Inhibited Phosphorylation of Smad2 in TGFβ1-Stimulated Neonatal Rat Cardiac Fibroblasts

Western blots were performed to determine the protein concentration of Smad2, phospho-Smad2, Smad4, and Smad7 derived from cardiac fibroblasts treated with or without AcSDKP. Total Smad2 and Smad4 levels were comparable in all groups (data not shown). TGFβ1 increased phospho-Smad2 levels 5.6-fold compared with that of untreated cells (P < 0.01). AcSDKP did not affect total Smad2 and Smad4 proteins (data not shown) but consistently decreased phospho-Smad2 levels in a dose-dependent manner (Figure
Effects of AcSDKP on Nuclear Translocation of Smad2

Cardiac fibroblasts were subjected to different doses of AcSDKP and were stimulated either with or without TGFβ1 (5 ng/mL) for 24 hours. Immunostaining revealed nuclear translocation of Smad2 only when the cells were 50% to 60% confluent and stimulated with TGFβ1. Numbers of positively stained nuclei were counted per 100 cells in 20 to 25 random fields per slide at 40× magnification. In control cells, no nuclei were stained, so these were not quantified. Positive nuclear staining was seen in 72±4.9% in TGFβ1-stimulated cells. When cotreated with AcSDKP (1 and 100 nmol/L), the number of nuclear staining positive cells decreased to 58±3.4% (P=0.08) and 56±4.04% (P=0.05), respectively (n=3) (Figure 3d and 3e). AcSDKP at 0.01 nmol/L concentration did not show any effect.

Effects of AcSDKP on the Growth of Rat Aortic Smooth Muscle Cells

PAC1 cells were cultured and treated similar to the cardiac fibroblasts. After 24 hours of serum deprivation, cells were stimulated to grow by supplementing them with 5% FBS with or without AcSDKP. Cells were counted 24 hours later. Addition of 5% serum caused an increase in the numbers from 24 495±3 329 (control) to 42 375±7 638 cells/mL. Addition of AcSDKP (1 nmol/L) caused an even greater increase rising to 79 240±3 488 cells/mL. AcSDKP at 0.01 nmol/L also tended to increase the cell number, but the difference was not statistically significant (Figure 4).

Discussion

AcSDKP is a tetrapeptide known to inhibit the proliferation of different cell types, including cardiac fibroblasts and reduce cardiac fibrosis. Considering the important role of TGFβ1 in the induction of cardiac fibrosis, we hypothesized that AcSDKP might inhibit fibrosis via modulation of the TGFβ1-signaling cascade. We demonstrated for the first time that AcSDKP inhibits the phosphorylation and nuclear translocation of Smad2. This provides an important mechanism by which AcSDKP can inhibit TGFβ signaling and thereby exert broad effects on cardiovascular cells. We also showed that AcSDKP inhibits the proliferation of cardiac fibroblasts by increasing these cells in G1/G0 phase of the cell cycle, an effect that does not depend on TGFβ stimulation. Taken together, this provides a novel mechanism by which AcSDKP can inhibit both cardiac fibroblasts growth and cardiac fibroblast activity, explaining its effects on cardiac fibrosis. Given that AcSDKP inactivation depends exquisitely on ACE, it suggests that the level of activity of ACE might affect TGFβ signaling via altered AcSDKP levels.

TGFβ1 is a cytokine that evokes its biological effects by signaling through 2 different receptor serine/threonine kinases, TGFβ receptor type I (TβR-I) and TβR-II, that form a tetrameric complex after binding of TβR-I and TβR-II. TβR-II activates TβR-I after phosphorylation of serine residue. This in turn leads to the activation of R-Smad. Activated R-Smads heteroligomerize with the Co-Smad4, and these complexes are translocated into the nucleus. Once in the nucleus, the activated Smad complex binds to the promoter region of the respective gene that initiates gene transcription. Smad6 and Smad7 are classified as inhibitory Smads, which prevent R-Smad activation, thereby downregulating the TGFβ signaling.

Thus, phosphorylation of Smad2 and its subsequent translocation to the nucleus is a critical step in the modulation of cell signaling by this pathway. AcSDKP inhibited SBE promoter activity, which indicated interference in Smad complex–mediated gene activation. This is most likely owing to either inhibition of Smad protein synthesis or inhibition of its activation and subsequent nuclear translocation, but cannot definitely prove that AcSDKP inhibits phosphorylation of Smad2.

The effects of TGFβ1 on proliferation are cell-type dependent. Although TGFβ1 has been reported to arrest epithelial and endothelial cells in G1 phase, its direct effects on the cell cycle of cardiac fibroblasts are still controversial. We observed induction of proliferation of cardiac fibroblasts when they were stimulated with TGFβ1. Although AcSDKP blunted the growth-stimulatory effect of TGFβ1, this effect could not be totally explained by inhibition of Smad2 phosphorylation. As reported previously, we noted a biphasic response of cell growth with a decrease in the antiproliferative effects at higher doses. On the other hand, inhibition of Smad2 phosphorylation was dose dependent, showing maximum inhibition at the highest dose we used. To explain this bell-shaped curve, we postulated recruitment of inhibitory Smad at the higher dose of AcSDKP (100 nmol/L). Compared with Smad6, Smad7 is shown to be more sensitive to TGFβ1 stimulation. We evaluated whether AcSDKP induces Smad7 but were unable to detect any signal for Smad7, neither by Western blot nor by immunocytochemistry. This might indicate that Smad7 expression in neonatal rat cardiac fibroblast is very low and is not robustly induced by AcSDKP.

It has already been shown that AcSDKP inhibits the activation of extracellular signal regulated kinase (ERK) in cardiac fibroblasts, and we observed inhibition of serum stimulated growth of cardiac fibroblasts at doses shown to inhibit ERK activation. Undoubtedly, there is cross-talk between the TGFβ1 and ERK signaling cascade. Some

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Effect of AcSDKP on growth of PAC1 cells. Cells were made quiescent by serum starvation for 24 hours. They were then stimulated to grow by supplemental 5% FBS with or without addition of AcSDKP and counted 24 hours later. AcSDKP (0.01 to 1 nmol/L) increased growth of PAC1 cells beyond the effect of 5% FBS (mean±SEM; n=5 to 6).
studies report a positive relation between TGFβ1 and the ERK signaling pathway. Others debate this finding and point out that TGFβ1 stimulates the phosphorylation of ERK, and activated ERK phosphorylates the linker region of Smad2, thereby preventing its nuclear translocation. Collectively, this suggests that AcSDKP might modify different cellular mechanisms involved in cell growth and proliferation, with the net effect depending on the interaction between these altered cellular pathways.

The concentrations at which AcSDKP inhibits Smad2 activation are very likely to be of clinical relevance. The level of AcSDKP can increase up to 20 nmol/L during ACE inhibitor therapy, at which we would expect a clear inhibition of Smad2 phosphorylation (Figure 3b).

TGFβ1 is known to exert an opposite effect on vascular smooth muscle cells as it inhibits their growth. Therefore, we postulated that if AcSDKP is indeed an important inhibitor of TGFβ1 signaling, AcSDKP should have an opposite effect on the growth of smooth muscle cells. Indeed, we observed a significant induction of growth of vascular smooth muscle cells when treated with AcSDKP. This is relevant because it suggests that even though AcSDKP may beneficially inhibit cardiac fibrosis, it may have less desirable growth stimulatory effects on vascular smooth muscle cells. This would suggest that the net effect of ACE inhibition on vascular smooth muscle cells might be influenced by a growth-promoting effect of AcSDKP. This could explain the poorly understood finding that ACE inhibition seemed to increase neointima formation in stented arteries. It is clear that this effect would be most prominent in situations characterized by pure smooth muscle cell proliferation, such as in-stent restenosis.

Our findings might explain the fact that ACE inhibition attenuates cardiac fibrosis even when decreased formation of Ang II does not seem to be involved. We recently found spontaneous cardiac fibrosis in transgenic rats with increased ACE activity. Surprisingly, in these animals, the local cardiac concentration of Ang II was not raised despite a 40-fold increase in ACE.

This study suggested that a substrate of ACE other than Ang II could be instrumental in such condition, and our findings together with previous reports indicate that high ACE activity might induce excessive breakdown of AcSDKP.

Perspectives

In conclusion, the major finding of this study is that AcSDKP inhibits the phosphorylation and subsequent nuclear translocation of Smad2. This antagonism of TGFβ signaling provides a novel pathway by which a rise in AcSDKP (as occurs during ACE inhibition) can exert long-term structural effects.

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