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 G_0/G_1 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 was 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 inhibits 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. 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. Different growth factors play an important role in myocardial fibrosis, of which transforming growth factor β1 (TGFβ1) is a crucial cytokine involved. 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. The effects of TGFβ1 on the growth of cell depend on the types of cell subjected to it. Accordingly, TGFβ1 stimulates the growth of fibroblasts but is also a potent inhibitor of vascular smooth muscle cell growth. 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. 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 ACE; 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. AcSDKP is degraded specifically by ACE both in vitro and in vivo, and its plasma level rises substantially during ACE inhibitor therapy. It is known to regulate the proliferation of hematopoietic progenitors and is also shown to reduce the proliferation of hepatocytes and renal fibroblasts. 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. 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 fibroblasts22 and were prepared as described previously.23 All experiments were performed on cells from the second passage. Each preparation contained >95% fibroblasts as determined by their positivity under phase contrast microscopy and immunocytochemical staining with an antibody against cardiac troponin-C and His-52.

Both cardiac fibroblasts and PAC1 cells were cultured in DMEM supplemented with 10% and 5% FBS, respectively, along with 1% L-glutamate, 50 U/mL penicillin, and 0.1 g/L streptomycin, and were incubated at 37 °C in 5% CO₂ atmosphere. Lisinopril (100 nmol/L) was added in the medium to prevent degradation of TGFβ1 (5 ng/mL). Neonatal rat cardiac fibroblasts were grown to confluence in 6-well plates. AcSDKP (0.01 to 100 nmol/L) was added 15 minutes before addition of TGFβ1 (5 ng/mL). Twenty-four hours later, whole cell protein was isolated by adding 100 μL of ice-cold protein lysis buffer (62.5 mM Tris/ HCl, pH 8.0, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 100 mM sodium pyrophosphate). Five hundred microliters of 2x sodium dodecyl sulfate sample buffer (125 mM Tris/ HCl, pH 6.8, 2% sodium dodecyl sulfate) was added and incubated at 100 °C until assayed. Bands on Western blot were visualized by enhanced chemiluminescence (Amersham).

**Immunocytochemical Localization of Smad Proteins**

Immunocytochemistry was performed on the formalin-fixed (3.7%) cells. After washing with PBS, ice-cold methanol (100%) was added for 10 minutes; the cells were washed 3 times with PBS and blocked with 5% horse serum for 60 minutes. Smad2 or Smad7 antibody (1:20 to 1:50 in 0.1% BSA in PBS) were applied for 4 hours at room temperature followed by horseradish peroxidase–labeled streptavidin for 60 minutes. After washing 3 times with 5 minutes in PBS, 3,3′-diaminobenzidine tetrahydrochloride reagent was applied to visualize the reaction.

**Statistical Analysis**

All the data are expressed as mean ± SEM. One-way ANOVA followed by Student-Newman-Keuls method was used for comparing the differences among multiple groups (Instat). Significant differences among the groups were defined by P<0.05.

**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 low-serum medium (0.1% FBS) for 24 hours before the experiment. This protocol was approved by an animal studies review committee (University of Groningen, Haren, The Netherlands). This plasmid contains 4 copies of Smad binding element (SBE) driving the firefly luciferase reporter gene. SBE is composed of the sequence CAGACA in the promoter of the junB gene, which is induced by TGFβ1.24 Renilla luciferase reporter plasmid pRL-CMV, which was used for normalization of transfection efficiency, was purchased from Promega. Cells were transfected with Saint™ (Saint BV) according to the manufacturer’s protocol and lysed with passive lyses buffer (Promega); luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega). The efficiency of transfection reaction was established by comparing the luciferase activity of transfected cells with a dilution series of purified luciferase enzyme.

**Luciferase Assay of TGFβ1-Inducible Smad Binding Element Promoter Activity**

Neonatal rat cardiac fibroblasts were plated at a density of 12,000 cells/well in 24-well clusters. Reporter plasmid pGL3tiSBE(4) was a generous gift from Dr L.J.C. Jonk (Department of Genetics, University of Groningen, Haren, The Netherlands). This plasmid contains 4 copies of Smad binding element (SBE) driving the firefly luciferase reporter gene. SBE is composed of the sequence CAGACA in the
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/G 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

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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 signaling, we used luciferase reporter assays with the pGL3ti(SBE)4 plasmid, which contains a TGFβ1-responsive element, SBE, and luciferase reporter gene. We first transfected pGL3ti(SBE)4 (250 ng/well) together with the control reporter pRL-CMV (25 ng/well) into cardiac fibroblasts and then continued the culture with or without TGFβ1. After 24 hours of treatment with 5 ng/mL TGFβ1, luciferase activity was elevated 6.7-fold compared with that of the control cells (P<0.01). However, cotreatment with AcSDKP (1 nmol/L) decreased the reporter activity by 55.3±9.7% (P=0.01) in TGFβ1-stimulated cells (Figure 3a). This effect was not seen in cells not stimulated by TGFβ1 (data not shown). Renilla luciferase expression was not affected by agonist treatment (control, 0.39 versus TGFβ1, 0.36, and TGFβ1+AcSDKP, 0.35; n=8, P=NS) and could, therefore, be used as an internal standard. The efficiency of the transfection reaction of neonatal rat cardiac fibroblasts was 3 pg luciferase/12 000 cells, indicating a high degree of transfection.

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 3b).
heterologomerize with the Co-Smad4, and these complexes are translocated in to 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.26,27 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,28–30 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,20 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.31 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.20 Undoubtedly, there is cross-talk between the TGFβ1 and ERK signaling cascade. Some
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 neo-intima 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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