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Angiotensin II induces TIMP-1 production in rat heart endothelial cells

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Abstract

Angiotensin II (AII) was found to upregulate tissue inhibitor of metalloproteineses-1 (TIMP-1) gene expression in rat heart endothelial cells in a dose and time-dependent manner. The maximal stimulation of TIMP-1 mRNA was achieved by 2 h after the addition of AII. This effect was blocked by losartan, an AT1 receptor antagonist and by calphostin C, a protein kinase C inhibitor. Addition of cycloheximide superinduced and actinomycin D abolished the induction. These results suggest that AII stimulates TIMP-1 production by a protein kinase C dependent pathway which is dependent upon de novo RNA synthesis. Immunoprecipitation experiment showed an enhanced band of 28 kDa from the conditioned medium of AII-treated cultures. Immunoblot analysis revealed that TIMP-1 was detectable in the conditioned medium 4 h after AII stimulation. Since endothelial cells line the blood vessels and sense the rise in AII associated with hypertension, the TIMP-1 released by these cells may provide an initial trigger leading to cardiac fibrosis in angiotensin-renin dependent hypertension.

Keywords: Angiotensin II; TIMP-I; Endothelial cell; Protein kinase C; (Rat heart)

1. Introduction

The primary purpose of this study is to examine the cellular and molecular basis that leads to cardiac fibrosis in renin-angiotensin dependent hypertension. The progression of the fibrolytic process generally begins in the adventitia of intramyocardial arteries and subsequently extends into the interstitial space [1]. In order to study the initiating events contributing to the overall balance of collagen metabolism in the perivascular fibrosis, endothelial cells derived from rat hearts were utilized as an in vitro model. The objective is to examine the role of nonmuscle cells in the modulation of collagen metabolism in response to angiotensin II (AII).

Matrix metalloproteinases are a family of Zn-containing enzymes responsible for the degradation of most matrix components [2–4]. These enzymes play important roles in inflammation, repair, and tissue remodelling. The activities of these metalloproteinases are partly controlled by the natural inhibitors-tissue inhibitors of metalloproteinases (TIMP) [5]. TIMP-1 was first described by Stricklin and Welgus [6]. It is a 28 kDa glycoprotein which binds to active metalloproteinases in 1:1 stoichiometry. TIMP-1 was shown to have sequence homology with erythropoitin potentiating activity [7]. Various cell types have been shown to express TIMP-1 including fibroblasts [8,9], monocytes or macrophages [10], and chondrocytes [11]. In addition, several endothelial cell types have been known to secrete TIMP-1 constitutively [12,13]. Recently other TIMP family (TIMP-2, TIMP-3) have been cloned [14–17].

The expression of TIMP-1 in various cell types is stimulated by a number of agents such as epidermal growth factor, basic fibroblast growth factor, transforming growth factor-β, phorbol ester [9,18], interleukin 1 [8], interleukin 6 [11], interleukin 11 [19], TNF-α: [20], oncostatin M [21], retinols [22], and dexamethasone [23].

Since circulating AII is known to increase in renovascular hypertension, we explored the action of AII on the expression of TIMP-1 in rat heart endothelial cells. In this study we present evidence that the vasoconstrictor AII is a potent inducer of TIMP-1 in these cells. The physiological significance of our findings is discussed.

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2. Materials and methods

2.1. Cell culture

Rat heart endothelial cells (RHE) were prepared according to Diglio et al. [24]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Biofluids), 2 mM L-glutamine and 25 μg/ml gentamycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were serially subcultured by treatment with trypsin (0.05% trypsin-0.5 mM Versene, pH 7.4) and used for experimentation between passages 10 and 25. These cells showed contact inhibition and typical 'cobblestone' morphology. Immunofluorescence studies showed positive staining for factor VIII and negative for α-actin.

2.2. Northern blot analysis

Cells were seeded into 100 mm dishes containing DMEM and 10% FBS. Near confluent cells were changed to serum-free DMEM supplemented with ITS (1 μM insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenous acid) for 24 h before the addition of testing agents. Total RNA was extracted using the acid guanidinium thiocyanate-phenol-$CHCl_3$ extraction [25]. Fifteen μg of RNA was denatured and separated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde. RNA ladder (BRL) was included as size marker. Following transfer the RNA was covalently bound to Gene Screen (DuPont-New England Nuclear) by UV-crosslinking. Hybridization and washing conditions were carried out according to the method of Church and Gilbert [26]. Mouse TIMP-1 cDNA probe [27] was generously provided by Dr. Mark D. Johnson, Northwestern University. The cDNAs were labeled with [^{32}P]dCTP using random primer synthesis (Amersham). The blots were exposed to XAR films with an intensifying screen. The intensity of the bands was quantitated by Ambis Image Analysis System. Each experiment was routinely performed three to four times and consistent results were obtained.

2.3. Preparation of TIMP-1 polyclonal antibody

Human TIMP-1 was kindly provided by Synergen Corporation (Denver, CO). An aliquot of 100 μg TIMP-1 was emulsified with an equal volume of Freund's complete adjuvant and injected s.c. into rabbits at multiple sites. Booster injections of 50 μg were given every 2 wk. The specificity of the antibody was checked by Western blot analysis [28].

2.4. Immunoprecipitation of TIMP-1

Near confluent RHE cultures in 35 mm dishes were incubated overnight with serum-free DMEM supplemented with ITS (1 μM insulin, 6.25 μg/m transferrin and 6.25 ng/ml selenous acid). The medium was replaced with 1 ml methionine-free DMEM containing 100 μCi [^{35}S]methionine (DuPont-New England Nuclear) in the absence or presence of AII ($1 \cdot 10^{-7}$ M). Cells were labeled at 37°C for 16 h. Control and AII-treated media containing the same amount of radioactivity was immunoprecipitated with rabbit anti-human TIMP-1 antibody. The immunoprecipitates were washed as previously described and displayed on a 10% SDS-PAGE [29].

2.5. Immunoblot analysis of TIMP-1

Near confluent RHE cultures in 100 mm dishes were incubated with 5 ml serum-free DMEM supplemented with ITS (1 ELM insulin, 6,25 μg/ml transferrin and 6.25 ng/ml selenous acid). Cells were treated with or without AII for designated times. Secreted proteins were precipitated from the medium with quinine sulfate-SDS according to the method of Werb et al. [30]. The precipitates were washed with 80% acetone and resuspended in Laemmli gel sample buffer. The proteins were electrophoresed on 8% SDS-PAGE. Immunoblots were carried out with rabbit anti-human TIMP-1 antibody (1:250) at room temperature for 2 h and then with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:10000, Sigma Chemical Co.) for 2 h. Color reaction was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.6. Chemicals

Angiotensin II was a gift from Ciba Pharmaceutical Co., Division of Ciba-Geygi Corp. Losartan (DuP 753) and PD 123319 were provided by E.I. Dupont de Nemours and Company and Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, respectively. Calphostin C, H7, and GF109203X were obtained from Calbiochem, Sigma, and Biomol Research Laboratories, Inc., respectively. AII other molecular biology reagents were of the highest grade available from Promega.

3. Results

Rat heart endothelial cultures were arrested by serum-starvation for 24 h to avoid interference of serum components. Steady-state levels of TIMP-1 mRNA was determined in RHE cultures by Northern blot analysis. Basal level of the TIMP-1 mRNA (1 kb) was low and its level increased rapidly upon the addition of AII. Levels were increased 5-fold at 2 h (Fig. 1, lane 4) and then declined over 6 h (lane 8). The next series of experiments were carried out in RHE cultures treated with a wide range of AII doses ($1 \cdot 10^{-11}$ M–$1 \cdot 10^{-6}$ M) for 2 h. Fig. 2 shows that the optimal induction was obtained with $1 \cdot 10^{-7}$ M AII (lane 6).

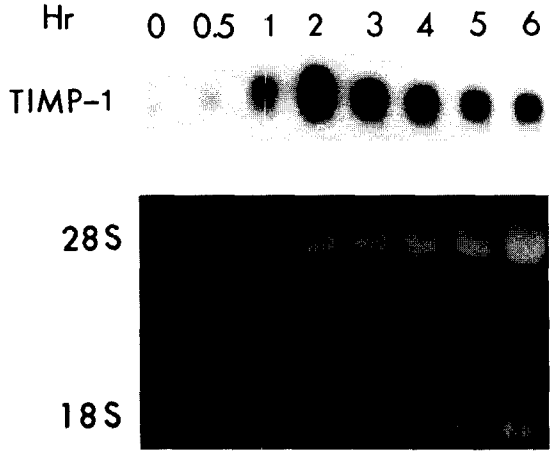

Fig. 1. Time-course of TIMP-1 mRNA induction in RHE by AII. Near confluent RHE cultures were exposed to $1 \cdot 10^{-7}$ M AII for the times indicated. 15 μg of total RNA was hybridized with a mouse TIMP-1 cDNA probe (top panel). The staining pattern of 28 S and 18 S ribosomal RNAs is shown in the bottom panel. Hybridization patterns of cells treated with AII for 0 min (lane 1); 30 min (lane 2); 1 h (lane 3); 2 h (lane 4); 3 h (lane 5); 4 h (lane 6); 5 h (lane 7); 6 h (lane 8); are shown. Figure shown is a representation of three independent experiments.

In order to examine the mechanism of TIMP-1 mRNA induction, inhibitors of protein and RNA synthesis were included singly or in combination with AII. Cycloheximide alone induced TIMP-1 mRNA (Fig. 3, lane 3). In the presence of AII and cycloheximide the induction was higher than AII alone (lane 4), implying that the induction does not require de novo protein synthesis. Addition of actinomycin D abolished the induction (lane 6), indicating that de novo RNA synthesis is required.

Losartan (DuP 753), a specific antagonist for type I

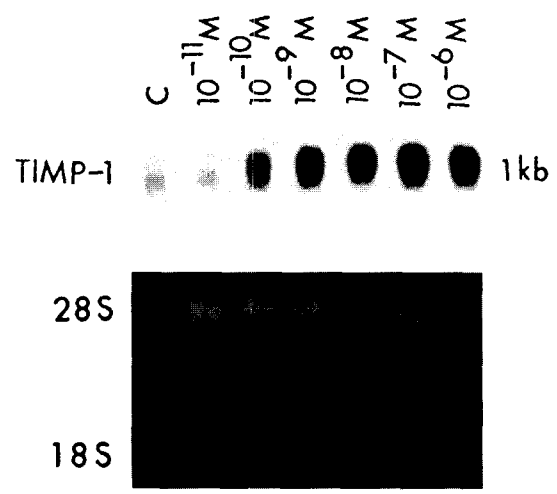

Fig. 2. Induction of TIMP-1 mRNA as a function of AII dose. Near confluent cultures of RHE were treated with $1 \cdot 10^{-11}$ M to $1 \cdot 10^{6}$ M of AII for 2 h. 15 μg of total RNA was hybridized with a cDNA probe for mouse TIMP-1 (top panel). The hybridization patterns of control cells (lane 1), cells treated with $1 \cdot 10^{-11}$ M AII (lane 2); $1 \cdot 10^{-10}$ M AII (lane 3); $1 \cdot 10^{-9}$ M AII (lane 4); $1 \cdot 10^{-8}$ M AII (lane 5); $1 \cdot 10^{-7}$ M AII (lane 6); $1 \cdot 10^{-6}$ M AII (lane 7); are shown.

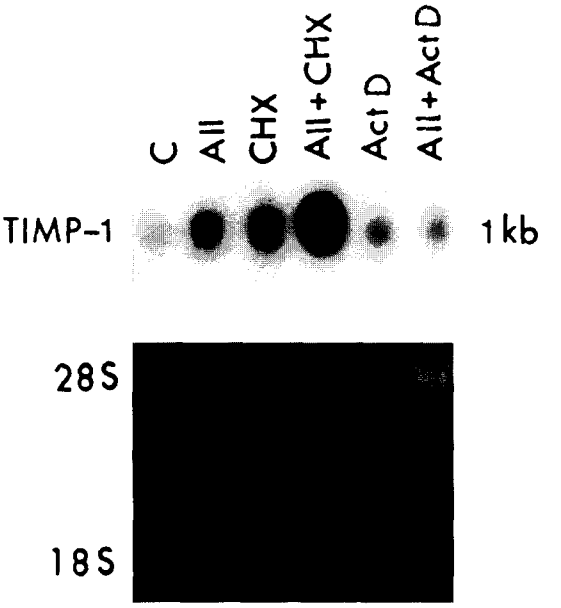

Fig. 3. Effect of cycloheximide (CHX) and actinomycin D (Act D) on the induction of TIMP-1 mRNA by AII. Near confluent cultures of RHE were treated with AII for 2 h in the absence or presence of CHX (20 μg/ml) or Act D (1 μg/ml). Fifteen μg of total RNA was hybridized with a mouse TIMP-1 cDNA probe. Hybridization patterns of control cells (lane 1); cells treated with $1 \cdot 10^{-7}$ M AII (lane 2); CHX (lane 3); AII and CHX (lane 4); Act D (lane 5); AII and Act D (lane 6); are shown.

receptors of AII [31] was included to examine if the effect of AII is mediated by type I receptors. In the presence of $1 \cdot 10^{-6}$ M losartan, the induction of TIMP-1 mRNA was

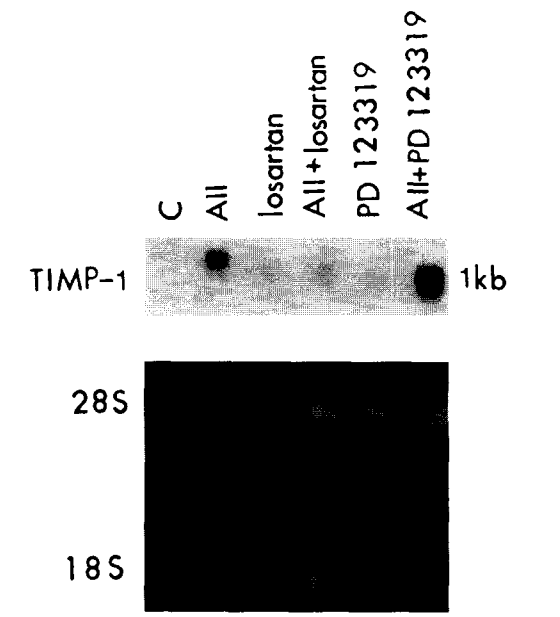

Fig. 4. Effect of losartan and PD 123319 on the induction of TIMP-1 mRNA by AII. Total RNA was isolated from RHE cultures in the absence or presence of test agent(s) for 2 h as indicated. Hybridization patterns of control cells (lane 1); cells treated with $1 \cdot 10^{-7}$ M AII (lane 2); $1 \cdot 10^{-6}$ M losartan (lane 3); AII and losartan (lane 4); $1 \cdot 10^{-6}$ M PD 123319 (lane 5); AII and PD 123319 (lane 6); are shown.

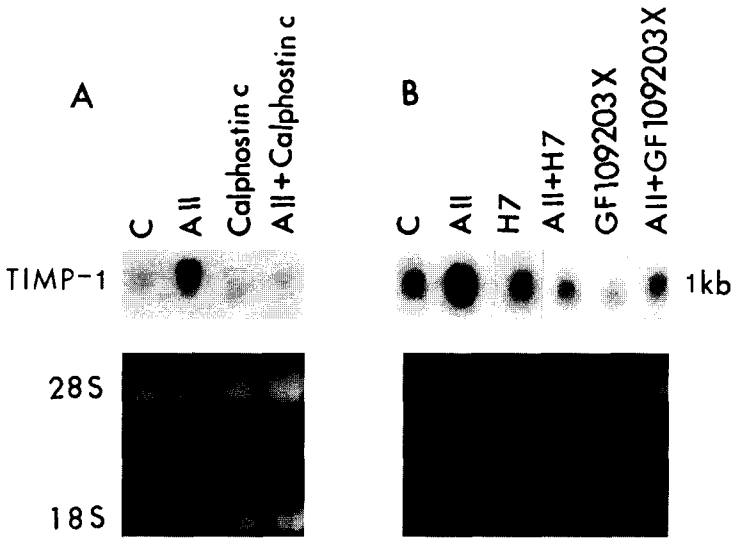

Fig. 5. Effect of protein kinase C inhibitors, calphostin C, H7, and GF109203X, on the induction of TIMP-1 mRNA by AII. Near confluent RHE cultures were pretreated with calphostin C (0.05 μM), H7 (1 μM) or GF109203X (1 μM) for 30 min before the addition of AII for 2 h. Panel A shows the hybridization pattern of control cells (lane 1); cells treated with AII (lane 2); 0.05 μM calphostin C (lane 3); AII and calphostin C (lane 4). Panel B shows the hybridization pattern of control cells (lane 1); AII (lane 2); 1 μM H7 (lane 3); AII and H7 (lane 4); 1 μM GF109203X (lane 5); AII and GF109203X (lane 6).

abolished (Fig. 4, lane 4), indicating that the induction of TIMP-1 mRNA by AII was mediated by type I receptors. In order to examine the involvement of type II receptors of AII, PD 123319 was included [32]. PD 123319 at a concentration of $1 \cdot 10^{-6}$ M did not abolish the induction of TIMP-1 mRNA (Fig. 4, lane 6).

To study if the induction of TIMP-1 mRNA involves a protein kinase C dependent pathway, calphostin C [33] a protein kinase C inhibitor, was included. For these experiments RHE cultures were pretreated with 0.05 μM calphostin C for 30 min prior to the addition of AII for 2 h. Calphostin C was able to block the induction of TIMP-1 mRNA (Fig. 5A, lane 4). Other protein kinase C inhibitors, such as H7 [34] and GF 109203X [35], were also tested and similar results were obtained (Fig. 5B, lanes 4 and 6). Our results suggest that the effect of AII is mediated by a protein kinase C dependent pathway.

Immunoprecipitation experiments were carried out with [^{35}S]methionine labeled cells to study the biosynthesis of TIMP-1 in the absence or presence of AII. Conditioned medium from AII-treated RHE cultures contained a protein of M_r 28 kDa molecular mass upon precipitation with TIMP-1 antibody (Fig. 6, lane 4). This protein was absent when AII-stimulated conditioned medium was immunoprecipitated with preimmune serum (lane 3). A faint band of TIMP-1 could be detected when the control medium was immunoprecipitated with TIMP-1 antibody (lane 2).

The appearance of TIMP-1 in the conditioned medium of AII-stimulated RHE cultures was examined by immunoblot analysis. In the absence of AII stimulation, RHE cells did not secrete any detectable amount of TIMP-1 (Fig. 7, lane 1). A band of M_r 28 kDa TIMP-1 appeared in the medium 4 h after AII-stimulation (lane 4). The level of TIMP-1 increased as time progressed from 6 h to 12 h (lanes 5–7). After 24 h, the basal level of TIMP-1 was elevated possibly due to the accumulation of growth fac-

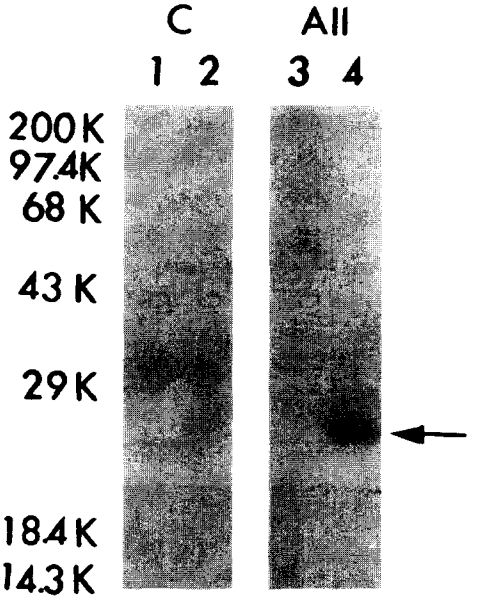

Fig. 6. AII enhanced the synthesis of TIMP-1. Near confluent RHE cultures were incubated overnight with serum-free DMEM supplemented with ITS. The medium was replaced with methionine-free-DMEM containing 100 μCi/ml [^{35}S]methionine in the presence or absence of AII ($1 \cdot 10^{-7}$ M). Cells were labeled at 37°C for 16 h. Immunoprecipitation with rabbit anti-human TIMP1 antibody was carried out as described in Section 2. Immunoprecipitates were displayed on 10% SDS-PAGE. Lanes 1 and 3 show the fluorogram of control and AII-treated medium immunoprecipitated with preimmunne serum, lanes 2 and 4 show the pattern from control and AII-treated medium immunoprecipitated with TIMP-1 antiserum.

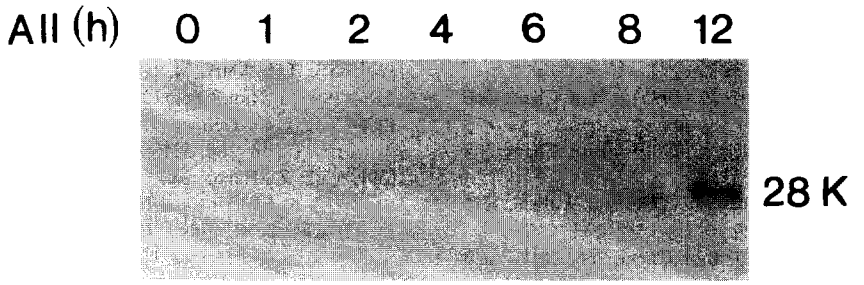

Fig. 7. Appearance of TIMP-1 in the conditioned medium. Near confluent RHE cultures were treated with or without AII for different times. Secreted proteins were precipitated with quinine sulfate-SDS and separated on 8% SDS-PAGE. Immunoblot with human TIMP-1 antibody was carried out as described in Section 2.

tors and cytokines in the conditioned medium (results not shown).

4. Discussion

In this study we found that vasoconstrictor AII could induce TIMP-1 in rat heart endothelial cells in a dose and time dependent manner. The induction requires RNA synthesis. Furthermore the induction appears to be mediated by type I receptors of AII and is dependent on protein kinase C pathway.

It is interesting to note that the level of TIMP-1 mRNA in the presence of cycloheximide alone is higher than the basal level. In addition, TIMP-1 mRNA is superinduced in the presence of cycloheximide and AII. Cycloheximide might inhibit the synthesis of RNases which are involved in the degradation of TIMP-1 mRNA. The increase in TIMP-1 mRNA correlated well with the appearance of TIMP-1 in the conditioned medium. Upregulation of TIMP-1 mRNA peaked at 2 h after AII stimulation, whereas TIMP-1 release was detectable 4 h after the addition of AII.

Our data demonstrated that the induction of TIMP-1 mRNA could be mainly attributed to transcriptional activation via protein kinase C pathway. The elements responsible for this activation are not characterized in the present study. TIMP-1 promoter and its regulatory elements have been studied by a number of investigators [36–38]. The promoter of the TIMP-1 gene contains AP-1 sites which can modulate gene expression. In addition, AII induced the expression of transcription factors c-fos and c-jun in RHE cells (data not shown). These proteins form an AP-1 complex which activates gene transcription. Whether the effect of AII is mediated by the AP-1 element in our cell system is under investigation.

We reported previously that AII could induce TGF-β1 mRNA in RHE cells [39]. TGF-β1 has been shown in several studies to upregulate the expression of TIMP-1 in human fibroblasts [9] and rat bone cells [40]. The induction of TIMP-1 by AII observed in the present study could be a direct effect or is secondary to the effect of TGF-β1 induction. Two lines of evidence tend to favor the first possibility. First of all, the TGF-β1 secreted into the conditioned medium was in a latent form as demonstrated by the bioactivity assays [39]. Secondly, the kinetics of mRNA induction for TGF-β1 and TIMP-1 are quite different. The optimal induction of TGF-β1 mRNA was achieved 6 h after the addition of AII whereas TIMP-1 message level was maximally induced 2 h after AII stimulation.

Previously, we reported that AII induced both TGF-β1 and endothelin-1 message levels in RHE cultures [39,41]. These factors could be utilized by neighboring fibroblasts or smooth muscle cells for the production of extracellular matrix [42,43]. The interplay of AII, TIMP-1, and other humoral factors such as TGF-β1 and endothelin-1 is implicated in the fibrosis of the cardiovascular system.

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