BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Regulation of thrombospondin-1 production by angiotensin II in rat heart endothelial cells

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Received 8 January 1997; accepted 6 February 1997

Abstract

Thrombospondin-1 (TSP-1) is synthesized, secreted, and incorporated into the extracellular matrix by a variety of cells, including the endothelial cells. Addition of angiotensin II (AII) significantly induced TSP-1 mRNA in rat heart-derived endothelial cells. TSP-1 mRNA levels reached a plateau within 2 h after the addition of AII and decreased after 5 h. The induction was superinduced by cycloheximide and blocked by actinomycin D. Losartan, an AT1 receptor antagonist, could abolish the induction of TSP-1 mRNA by AII. Phorbol 12-myristate 13-acetate (TPA) was found to enhance TSP-1 mRNA level whereas a protein kinase C inhibitor, H7, was shown to block the induction. Immunoblot analysis revealed that TSP-1 was detectable in the medium 4 h after AII stimulation. Our results suggest that the upregulation of TSP-1 by AII represents an important mechanism leading to perivascular fibrosis in the heart.

Keywords: Angiotensin II; Thrombospondin-1; Heart endothelial cell; Protein kinase C; (Rat)

1. Introduction

Thrombospondin (TSP) was first identified as a thrombin-sensitive protein which was released upon the activation of human platelets [1]. It is a multimodular glycoprotein made up of three identical chains linked by disulfide bonds. TSP can adhere to many matrix macromolecules including heparan sulfate proteoglycans, fibronectin, laminin, and types I and V collagens. It can also bind to plasma proteins such as fibrinogen and plasminogen, calcium ions, and cell surface receptors. TSP has been implicated in a number of biological processes such as hemostasis, cell adhesion, proliferation, differentiation, wound healing, and angiogenesis [2–5].

It is now recognized that TSP is comprised of a family of related proteins, TSP-1 to TSP-5. TSP-1 and TSP-2 are both trimeric and similar in structure [3]. These two genes have different tissue distribution and are expressed differently in the developing mouse [6]. TSP-3, TSP-4, and TSP-5 are all pentameric molecules [3]. They resemble one another in structure, but differ from TSP-1 and TSP-2.

TSP-1 was synthesized and secreted by a number of cultured cells including fibroblasts, smooth muscle cells, epithelial cells, macrophages and endothelial

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PII S0167-4889(97)00029-3

cells [7]. Several growth factors have been demonstrated to induce the expression of TSP-1. For example, serum and platelet-derived growth factor were found to upregulate TSP-1 gene expression in smooth muscle cells [8,9]. Transforming growth factor-β1 could induce TSP-1 mRNA in 3T3 cells [10], smooth muscle cells [11], and adrenocortical cells [12]. In addition, basic fibroblast growth factor was shown to stimulate TSP-1 mRNA and protein in 3T3 cells [13].

Endothelial cells are found to secrete TSP-1 and possess TSP-1 receptors [14–16]. TSP-1 inhibits the proliferation and modulates the motility of these cells [17–19]. Previously we demonstrated that AII induced the gene expression of endothelin-1 [20], TGF-β1 [21], and tissue inhibitor of metalloproteinases (TIMP-1) [22]. These proteins are known to be directly or indirectly involved in the synthesis of extracellular matrix. In this study we examined the effect of AII on the modulation on another matrix protein TSP-1 and found that AII is a strong inducer of TSP-1.

2. Materials and methods

2.1. Cell culture and materials

Rat heart-derived endothelial cells (RHE) were provided by Diglio et al. [23]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS), 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 in 0.5 mM Versene) and were used for experimentation between passages 12 and 25. These cells exhibited typical 'cobblestone' morphology devoid of any smooth muscle cells. H7 and phorbol 12-myristate 13-acetate (TPA) were obtained from Sigma Chemical Company. [^{32}P]dCTP was obtained from ICN. ITS was purchased from Collaborative Biomedical (Bedford, MA, USA). Fetal bovine serum was obtained from Biofluids. Human TSP-1 was purchased from Life Technologies. All other reagents used were of the highest grade commercially available.

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 test agents. After the designated time, total RNA was extracted using the acid guanidinium thiocyanate-phenol-$CHCl_3$ extraction method [24]. Fifteen micrograms of RNA were denatured and separated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde. Following transfer, the RNA was covalently bound to GeneScreen (DuPont-New England Nuclear) by UV-crosslinking. Hybridization and washing conditions were carried out according to the method of Church and Gilbert [25]. A human TSP-1 cDNA probe, a 1100 bp *Eco*RI insert in pGEM-2 [26], was provided by Dr Jack Lawler, Harvard University. The cDNA was labeled with [^{32}P]dCTP using random primer synthesis. The blots were exposed to XAR films with intensifying screens. The intensity of the bands was quantitated by Digital Imaging System (Alpha Innotech Corp.). Experiments were repeated at least three times and representative data were shown.

2.3. Immunoblot analysis of TSP-1

Near confluent RHE cultures in 100 mm dishes were incubated with serum-free DMEM supplemented with ITS. Cells were treated with or without AII for designated times. Conditioned media were collected in the presence of protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride). Secreted proteins in the conditioned medium were precipitated with quinine sulfate-SDS according to the method of Werb et al. [27]. The precipitates were resuspended in Laemmli SDS gel sample buffer and electrophoresed on 6% SDS-PAGE. Human TSP-1 (2 μg) was included as standard. Immunoblots were carried out with rabbit anti-bovine TSP-1 antibodies (1:500) (kindly provided by Dr Daniel Walz, Wayne State University) at room temperature for 1.5 h and then with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:10 000, Sigma Chemical Company) for 1 h. Color reaction was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

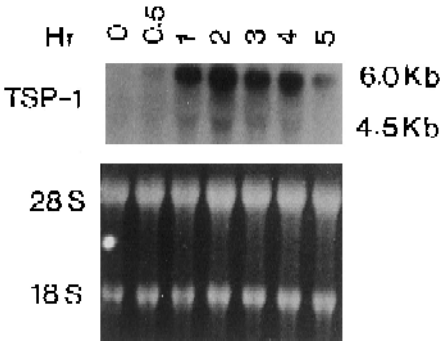

Fig. 1. Time course of TSP-1 mRNA induction in rat heart endothelial cells by AII. Near confluent cultures were exposed to AII (10^{-7} M) for the times indicated. Fifteen μg of total RNA were hybridized with a cDNA probe for human TSP-1. Hybridization patterns of cells treated with AII for 0 min (lane 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); are shown.

3. Results

Steady-state levels of TSP-1 mRNA were determined in RHE cultures by Northern blot analysis. Two specific transcripts of TSP-1, 6.0 kb and 4.5 kb were observed. The basal level of TSP-1 mRNA was low. The mRNA level increased rapidly upon the addition of AII. Levels of TSP-1 mRNA were increased 5-fold at 2 h (Fig. 1, lane 4) and then declined over 5 h (lane 7). As shown in Fig. 2, TSP-1 mRNA responded to AII in a dose-dependent manner.

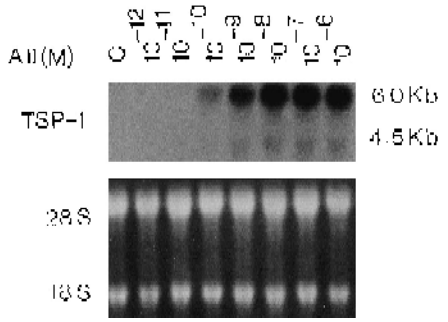

Fig. 2. Dose-dependent induction of TSP-1 mRNA by AII. Near confluent cultures of rat heart endothelial cells were treated with 10^{-12} M to 10^{-6} M of AII for 2 h. Fifteen μg of total RNA was hybridized with a cDNA probe for human TSP-1. Hybridization patterns of control cells (lane 1); cells treated with 10^{-12} M AII (lane 2); 10^{-11} M AII (lane 3); 10^{-10} M AII (lane 4); 10^{-9} M AII (lane 5); 10^{-8} M AII (lane 6); 10^{-7} M AII (lane 7); 10^{-6} M AII (lane 8); are shown.

The next series of experiments were performed to examine the mechanism of TSP-1 mRNA induction. Inhibitors of protein and RNA synthesis were included singly or in combination with AII. Cycloheximide alone induced TSP-1 mRNA (Fig. 3, lane 3). In the presence of AII and cycloheximide (lane 4) the induction was higher than AII alone (lane 2), implying that the induction does not require de novo protein synthesis. In the presence of actinomycin D, the induction was abolished (lane 6), indicating that de novo RNA synthesis is required.

Losartan, a specific antagonist for AT-1 receptors [28] was included to examine whether the effect of AII was mediated by AT1 receptors. In the presence of 1 μM losartan, the induction of TSP-1 mRNA decreased drastically (Fig. 4, lane 4), suggesting that the induction of TSP-1 mRNA might be mediated by AT1 receptors. Involvement of AT2 receptors was investigated by including PD 123319, an AT2 receptor antagonist [29]. PD 123319 at a concentration of 1 μM did not abolish the induction of TSP-1 mRNA (lane 6), suggesting that AT2 receptors are not involved.

The promoter of the TSP-1 gene has been shown to contain AP-1 sites [30]. We next investigated if AII exerted its effect through a protein kinase C-dependent pathway. H7, a protein kinase C inhibitor [31], was included. In these experiments, cells were pretreated with 1 μM H7 for 30 min prior to the

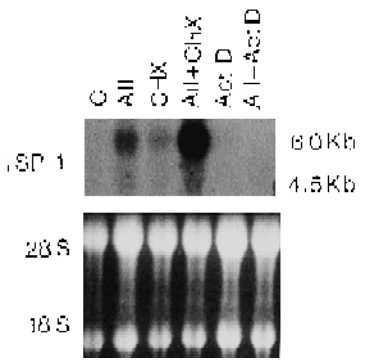

Fig. 3. Effect of cycloheximide (CHX) and actinomycin D (Act D) on the induction of TSP-1 mRNA by AII. Near confluent cultures of rat heart endothelial cells were treated with AII for 2 h in the absence or presence of CHX (20 μg/ml) or Act D (1 μg/ml). Fifteen μg of total RNA was hybridized with a human TSP-1 cDNA probe. Hybridization patterns of control cells (lane 1); cells treated with 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.

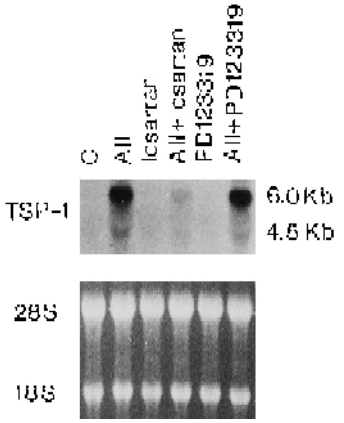

Fig. 4. Effect of AII receptor antagonists losartan and PD 123319 on the induction of TSP-1 mRNA. Total RNA was isolated from rat heart endothelial cell cultures in the absence or presence of the antagonist and AII. Hybridization patterns of control cells (lane 1); cells treated with 10^{-7} M AII (lane 2); 1 μM losartan (lane 3); 1 μM losartan and AII (lane 4); 1 μM PD 123319 (lane 5); 1 μM PD 123319 and AII (lane 6); are shown.

addition of AII. H7 was able to block the induction of TSP-1 mRNA (Fig. 5A, lane 4). Treatment of cells with TPA (100 ng/ml) for 2 h was found to stimulate the TSP-1 mRNA level (Fig. 5A, lane 2). Our results suggest that the effect of AII is mediated by a protein kinase C-dependent pathway.

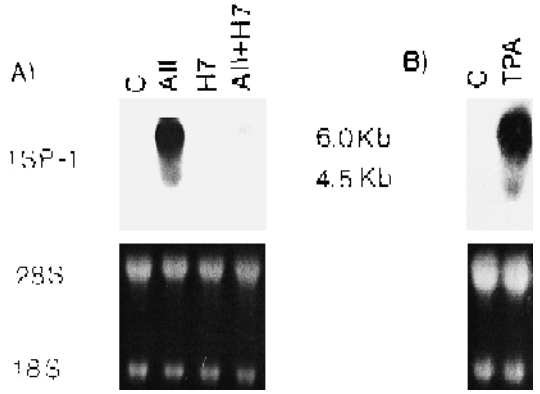

Fig. 5. Effect of protein kinase C inhibitor and activator on the induction of TSP-1 mRNA by AII. Panel A: near confluent rat heart endothelial cells were pretreated with a protein kinase C inhibitor, H7, for 30 min before the addition of AII for 2 h. Hybridization patterns of control cells (lane 1); cells treated with 10^{-7} M AII (lane 2); 1 μM H7 (lane 3); AII and H7 (lane 4); are shown. Panel B: near confluent cells were treated with or without TPA (100 ng/ml) for 2 h. Hybridization patterns of control cells (lane 1) and cells treated with TPA (lane 2), are shown.

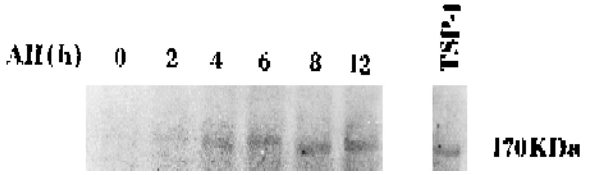

Fig. 6. Appearance of TSP-1 in the conditioned medium. Near confluent rat heart endothelial cells were treated with or without AII for different times. Secreted proteins in the conditioned medium were precipitated with quinine sulfate-SDS and separated on 6% SDS-PAGE. Immunoblot was carried out with rabbit anti-bovine TSP-1 antibodies as described in Section 2. The immunostaining patterns of control cells (lane 1); cells treated with AII for 2 h (lane 2); 4 h (lane 3); 6 h (lane 4), 8 h (lane 5), and 12 h (lane 6); are shown. Human TSP-1 (2 μg) was included as standard (lane 7).

In order to examine if the increase of TSP-1 mRNA correlates well with the appearance of TSP-1 in the conditioned medium, immunoblot analysis was carried out. The conditioned media of control and AII-treated cells were precipitated by quinine-sulfate-SDS. In the absence of AII, RHE cells secreted only trace amount of TSP-1 (Fig. 6, lane 1). A band of M_r 170 000 appeared in the medium 2 h after the addition of AII (lane 2). The amount of TSP-1 increased from 4 to 6 h (lanes 3,4) and stayed about the same after 8 to 12 h of AII treatment (lanes 5,6).

4. Discussion

The present study demonstrated that AII acts on rat heart endothelial cells to induce TSP-1 mRNA in a dose and time dependent manner. The induction is mediated by AT1 receptors and involves a protein kinase C-dependent pathway. In addition, the increase of TSP-1 mRNA correlated well with the release of TSP-1 into the conditioned medium.

Our data demonstrated that the induction of TSP-1 mRNA by AII could be mainly attributed to transcriptional activation. Although the elements responsible for this activation were not characterized in the present study, the promoter of the TSP-1 gene has been shown to contain AP-1 sites [30]. Whether the effect of AII is mediated by the AP-1 elements in our system is under investigation. It is interesting to note that TSP-1 mRNA is ‘superinduced’ in the presence of cycloheximide and AII. The level of TSP-1 mRNA

in the presence of cycloheximide alone is higher than the basal level. Cycloheximide might inhibit the synthesis of either labile ribonucleases that are involved in degrading TSP-1 mRNA or repressor protein(s) involved in the regulation of TSP-1 gene expression [32].

The effect of TSP-1 varies with different cell types. For example, TSP-1 has been shown to be an autocrine growth factor for smooth muscle cells. It can augment the mitogenic effect of epidermal growth factor on these cells [33,34]. On the other hand, TSP-1 inhibits the proliferation of endothelial cells [17,18]. The production of TSP-1 in AII-treated rat heart endothelial cultures in the present study may provide a mechanism for the homeostatic regulation of proliferation in these cells.

We previously reported that AII induced TGF-β1 mRNA in rat heart endothelial cells [21]. Since TGF-β1 has been demonstrated to induce TSP-1 in several cell types [10–12], a question arises as to whether the increase of TSP-1 level observed in this study is a result of the secondary effect of TGF-β1. Two lines of evidence appear to negate this possibility. First, the kinetics of induction of TSP-1 mRNA precedes that of TGF-β1. Secondly, the released TGF-β1 was found to be in the latent form by bioassays [21].

TSP-1 was shown to activate the latent TGF-β1 in bovine aortic endothelial cells [35,36]. The sequence responsible for the activation has been localized to the unique amino acids 412–415 found between the first and the second repeats of TSP-1 [37]. In our preliminary experiment, TGF-β1 in the medium could be converted to the active form when exogenous TSP-1 (20 μg/ml) was added to rat heart endothelial cells (results not shown). In the present study, the TSP-1 in the conditioned medium is probably too dilute to activate the latent TGF-β1. However, high TSP-1 concentration may be achievable in a localized microenvironment in vivo.

It has been well documented that cardiac fibrosis occurs in renin-angiotensin-dependent hypertension. The pattern of fibrosis generally progresses in the adventia of intramyocardial arteries and finally extends into the interstitial space [38]. In addition to TSP-1, AII could induce endothelin-1, TGF-β1, and tissue inhibitor of metalloproteinases-1 (TIMP-1) in rat heart endothelial cultures [20–22]. These proteins could modulate collagen metabolism of adjacent smooth muscle cells or fibroblasts [39,40]. Hence, the enhanced production of TSP-1, TGF-β1, and TIMP-1 in response to the rise of AII by heart endothelial cells during the development of hypertension may be the initiating events that lead to perivascular fibrosis in renovascular hypertension.

Acknowledgements

This work was supported in part by NIH grant HL 56340 and a fund by Cecile Cox Quillen Chair of Geriatrics. We are indebted to Dr Clement A. Diglio and Dr Daniel Walz, Wayne State University, for providing rat heart endothelial cells and TSP-1 antibodies, respectively. We thank Dr Jack Lawler, Harvard University, for providing a human TSP-1 cDNA probe. We thank Dr Ronald D. Smith, E.I. Du Pont de Nemours and Company for losartan and Dr Jack Hodges, Parke-Davis Pharmaceutical Research Division for PD 123319.

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