

## Thrombospondin 1 mediates angiotensin II induction of TGF- $\beta$ activation by cardiac and renal cells under both high and low glucose conditions

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### Abstract

Renal and cardiac fibrosis leading to organ failure are complications of both diabetes and hypertension. These disease processes, when combined, exacerbate development of fibrotic complications. Control of latent transforming growth factor (TGF)- $\beta$  activation is a potential determinant of fibrotic progression. Both glucose and angiotensin II (Ang II) upregulate thrombospondin-1 (TSP1), a major activator of latent TGF- $\beta$ , and stimulate increased TGF- $\beta$  activity. We previously showed that high glucose stimulated TSP1-dependent TGF- $\beta$  activation in rat mesangial cells (RMCs). In this paper, we examined whether Ang II similarly upregulates TSP1 production and TSP1-dependent TGF- $\beta$  activation alone or in combination with high glucose concentrations. Ang II and high glucose stimulated increases in TSP1 protein levels in the conditioned media of both rat cardiac fibroblasts (RCFs) and rat mesangial cells (RMCs). Meanwhile, Ang II stimulated increases in both TGF- $\beta$  activity and protein by RMCs, whereas, RCFs responded to both Ang II and high glucose with increased TGF- $\beta$  activity in the absence of altered TGF- $\beta$  protein levels. A combination of Ang II and high glucose induced synergistic TGF- $\beta$  activation by RCFs. Moreover, Ang II induction of TSP1 and increased TGF- $\beta$  activity were blocked by losartan, an antagonist of the Ang II type 1 (AT1) receptor. The increase in TSP1 expression leads to increased TGF- $\beta$  activity upon Ang II and/or glucose treatment, since peptide antagonists of TSP1-mediated TGF- $\beta$  activation blocked Ang II and glucose-induced TGF- $\beta$  activation. Our data support a role for TSP1 in the development and progression of renal and cardiac fibrosis in hypertension and diabetes.

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Activation of the renin–angiotensin system (RAS) is critical for development of cardiovascular and renal fibrosis in hypertension and diabetes. Angiotensin II (Ang II) is a major effector molecule produced by the RAS. Accumulated evidence suggests that Ang II plays a role in the development of fibrotic diseases. In the heart, Ang II promotes fibrotic changes by inducing proliferation of fibroblasts and increasing synthesis and secretion of extracellular matrix (ECM) proteins [1]. Animal studies showed that a long-term infusion of Ang II into rats resulted in the devel-

opment of cardiac fibrosis in vivo [2]. The combination of hypertension and hyperglycemia induced more pronounced tissue fibrosis than either hypertension or diabetes alone [3]. In addition, severe and often fatal cardiomyopathy with interstitial fibrosis occurred in diabetic animals when hypertension was induced [4]. These results suggest that both hypertension and hyperglycemia are associated with accelerated fibrotic progression in the heart. Angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor blockers reduce systemic and glomerular hypertension and attenuate the development of renal and cardiac fibrosis [5,6]. However, the molecular mechanisms underlying Ang II and glucose induction of fibrosis are incompletely understood.

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Chronic uncontrolled activation of RAS in hypertension and diabetes causes cardiovascular and renal damage through mechanisms that involve TGF- $\beta$  [7]. In a streptozotocin (STZ)-induced animal model of diabetes, increases in TGF- $\beta$  production correlate with increases in fibronectin, biglycan, and tenascin in glomeruli [8]. Overexpression of TGF- $\beta$  induces glomerulosclerosis in rat kidneys [9], whereas, treatment with anti-TGF- $\beta$  antibodies attenuates glomerular hypertrophy and inhibits mesangial matrix protein synthesis in diabetic mice [10]. Ang II stimulates mesangial cells to release TGF- $\beta$  which results in the development of glomerular fibrosis [11]. Regulation of TGF- $\beta$  expression and activity in the combined presence of both Ang II and glucose is not well studied. Weigert et al. [12] have reported that co-incubation of Ang II and high glucose in mesangial cells had no additive effect on TGF- $\beta$ 1 promoter activity. However, the effect of combined Ang II and high glucose on TGF- $\beta$  activity in cardiac fibroblasts has not been addressed.

TGF- $\beta$  is synthesized as an inactive latent complex. A major regulatory step in determining appropriate levels of TGF- $\beta$  is bioactivation of the latent complex [13]. We previously established that the matricellular protein TSP1 is a physiologic regulator of latent TGF- $\beta$  activation in vitro and in a number of homeostatic and pathologic conditions in vivo [14–18]. We and others showed that high glucose stimulates TSP1 expression in both human and rat mesangial cells [17,18]. The increases in TSP1 expression result in enhanced TGF- $\beta$  activation and increased synthesis of extracellular matrix proteins. These data suggest that TSP1 plays an important role in the development of renal fibrosis under diabetic conditions.

In this study, we determined whether TSP1 is also involved in regulation of TGF- $\beta$  activation by cardiac fibroblasts in response to high glucose conditions or Ang II stimulation. Recently, Naito et al. [11] showed that Ang II induced both time- and dose-dependent increases in TSP1 in human mesangial cells. However, it is unknown whether Ang II induction of TSP1 results in increased activation of TGF- $\beta$ . In addition, we examined the role of TSP1 regulation of TGF- $\beta$  activation by rat mesangial cells (RMCs) and rat cardiac fibroblasts (RCFs) in response to Ang II when combined with high glucose conditions. Our data indicate that TSP1 is a key mediator of Ang II-induced TGF- $\beta$  activation in both cell types. It suggests a pivotal role for TSP1 in the development of cardiac and glomerular fibrosis in hypertension and diabetes.

## Materials and methods

**Peptides, antibodies, and other reagents.** Peptides were synthesized by AnaSpec (San Jose, CA). The GGWSHW peptides mimic the W<sub>xx</sub>W motif in the type I repeats of TSP1 which acts as a “docking site” to orient the TSP1 molecule correctly and facilitate its interaction with latent TGF- $\beta$  [15]. The LSKL peptides competitively inhibit binding of TSP1 to latent TGF- $\beta$  at the L<sub>54</sub>SKL<sub>58</sub> site of latency-associated protein (LAP) [15]. The GGASHA (or GGYSHW) peptides and the SLLK peptides are control peptides for the GGWSHW peptides and the LSKL peptides, respectively.

Ang II, aprotinin, transferrin, and sodium selenium were purchased from Sigma (St. Louis, MO). Losartan was purchased from Merck (Whitehouse Station, NJ). Monoclonal antibody Mab133 raised against human platelet TSP1 stripped of TGF- $\beta$  activity was developed as described previously [14].

**Cells, cell culture, and treatment.** RMCs were a generous gift from Dr. Anne Woods, University of Alabama at Birmingham. Cells were cultured according to published protocols [17]. RCFs were isolated from 1-day to 1-week-old WKY rats according to established procedures [19]. RCFs were used prior to the third passage. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cells were allowed to grow until confluent (for RMCs) or 80–90% confluent (for RCFs) in 6-well plates and then were made quiescent by culturing in serum- and insulin-free RPMI media (for RMCs) or DMEM (for RCFs) containing 5 mM glucose, 10  $\mu$ g/ml transferrin, and 5 ng/ml sodium selenium for 48 h. Treatments of cells were carried out in serum-free media containing 5 or 30 mM glucose in the presence or absence of Ang II for 24 h. For some cells, antibodies, peptides, losartan or aprotinin were added as indicated in figure legends.

**Preparation of conditioned media and bioassay of TGF- $\beta$  activity.** Culture supernatants were harvested in siliconized tubes on ice and centrifuged at 1000 rpm for 10 min at 4 °C. Cell-free conditioned media were stored at –80 °C until used.

Bioassay of TGF- $\beta$  activity was performed using either the NRK colony formation assay [14,17] or the PAI-1 promoter luciferase reporter (PAIL) assay [20]. For measurement of active TGF- $\beta$ , 0.5 ml conditioned media were directly added to each well. For assay of total TGF- $\beta$  with the PAIL assay, 0.05 ml conditioned media were first heat-activated for 3 min at 100 °C, and then mixed with 0.45 ml serum-free media and a final volume of 0.5 ml media was incubated with reporter cells. For total TGF- $\beta$  assay using the NRK soft agar assay, conditioned media were heat-activated for 3 min at 100 °C [17]. All assays were performed in triplicate.

**Western blot analysis.** Protein concentration in the conditioned media was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Conditioned media containing equal amounts of protein were loaded onto 8% SDS-polyacrylamide gels under reducing conditions. Equal loading and transfer of protein samples were verified by staining the blots with Ponceau S. Membranes were incubated with 0.2  $\mu$ g/ml Mab133 for 18 h at 4 °C. After extensive washing, membranes were incubated with 0.1  $\mu$ g/ml peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunodetection was carried out by chemiluminescence with Western Lighting (Perkin-Elmer, Boston, MA).

**Densitometry and statistical analysis.** Immunoblots were scanned and bands were quantified using Scanalytic One-Dscan, version 1.31. Statistical differences among treatment conditions were determined using one-way analysis of variance (ANOVA) followed by a Dunnett's *t* test. The analysis was performed with SigmaStat 3.0 software (SPSS, Chicago, IL). Values of *p* < 0.05 or *p* < 0.01 were considered significant.

## Results

### *Ang II stimulates TGF- $\beta$ activation by RMCs and RCFs*

To determine whether Ang II has a stimulatory effect on TGF- $\beta$  activation and production in RMCs and RCFs, cells were treated with increasing concentrations of Ang II (0–1  $\mu$ M) for 0–48 h. TGF- $\beta$  production was evaluated by measurement of total (active plus latent) TGF- $\beta$  activity in heat-activated conditioned media.

Stimulation with Ang II induced a dose-dependent upregulation of both active and total TGF- $\beta$  activity by RMCs (Fig. 1A). The percent TGF- $\beta$  in the active state increased from 41% to 69% when Ang II was added. These results indicate that Ang II increased not only TGF- $\beta$

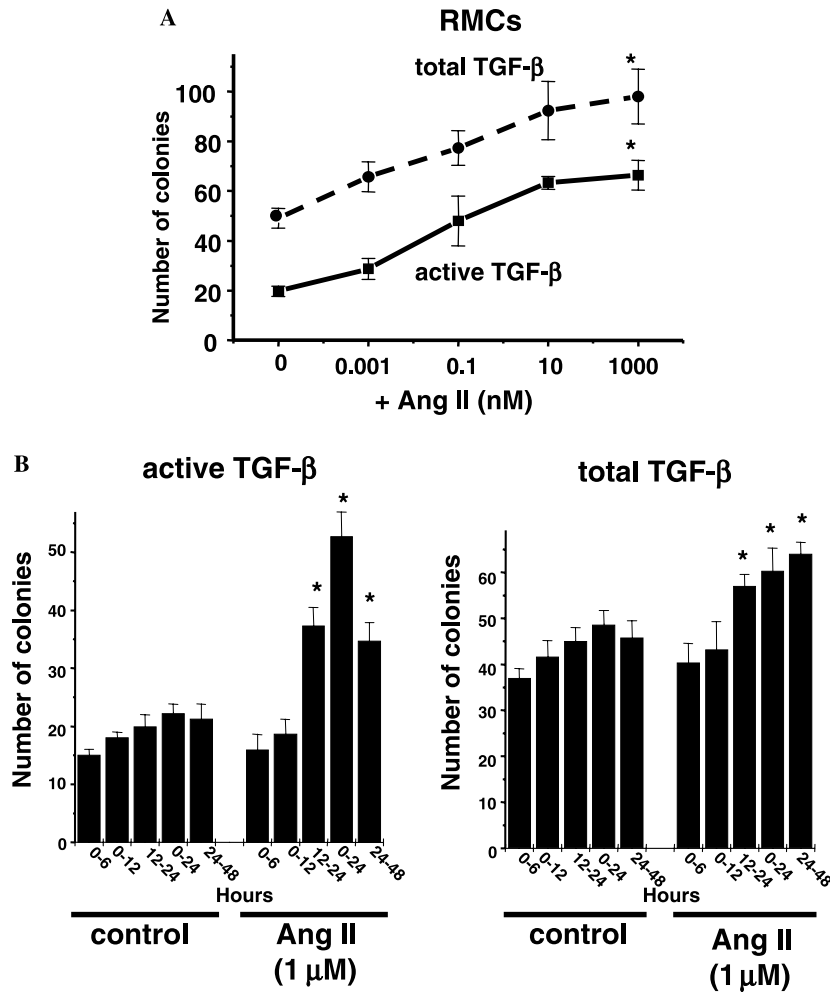


Fig. 1. Ang II stimulates increased TGF-β activity by RMCs. (A) After quiescence, RMCs were stimulated with or without 0–10<sup>3</sup> nM Ang II for 24 h. Conditioned media were harvested and assayed for active and total TGF-β activity using the NRK colony formation assay. (B) Quiescent RMCs were treated with or without 1 μM Ang II for the time periods indicated. Active and total TGF-β activity from conditioned media were assayed. Experiments were performed in triplicate on three separate occasions. Results are shown as means ± SD. \**p* < 0.01 for TGF-β activity under indicated conditions vs. TGF-β at basal levels.

production, but also increased the proportion of TGF-β that is in the biologically active form. In addition, Ang II stimulates TGF-β activity and protein production by RMCs in a time-dependent manner, peaking within the first 24 h of stimulation with 1 μM Ang II (Fig. 1B). Active TGF-β was most significantly increased between 12 and 24 h of Ang II stimulation, whereas the increase in total TGF-β protein persisted for over 12–48 h.

Cardiac fibroblasts also respond to 24 h of Ang II stimulation with increases in active TGF-β (Fig. 2). However, in contrast to the RMCs, TGF-β protein expression was not stimulated by Ang II as total TGF-β levels were not changed by Ang II (Fig. 2). The two cell types also exhibited different sensitivities to Ang II concentration. In RCFs, Ang II stimulation of TGF-β was maximal at 100 nM Ang II and decreased at higher Ang II conditions (Fig. 2), whereas, 10–1000 nM Ang II stimulated maximal levels of TGF-β activity in RMCs (Fig. 1A).

Addition of losartan, an AT1 receptor antagonist, completely blocked Ang II-induced increases in active TGF-β

(Fig. 2), suggesting that Ang II-induced TGF-β activation by RCFs is AT1 dependent. Losartan had no effect on total TGF-β levels (Fig. 2), indicating that Ang II does not regulate TGF-β synthesis in cardiac fibroblasts, consistent with the lack of TGF-β protein induction by Ang II in these cells.

*Ang II-stimulated TGF-β activation is further enhanced under high glucose conditions in RCFs*

Previously, we showed that high glucose levels stimulate increases in TGF-β activity and protein in RMCs [17]. In the current study, we determined whether high glucose also stimulates TGF-β activity by RCFs and the possible effect of high glucose on Ang II-induced TGF-β activation in RCFs. Treatment of RCFs with increasing concentrations of glucose (5–40 mM) induced a dose-dependent increase in active TGF-β (Fig. 3A). In contrast to RMCs, total TGF-β in RCF cultures was unchanged by glucose. A combination of Ang II with high glucose caused further increases in

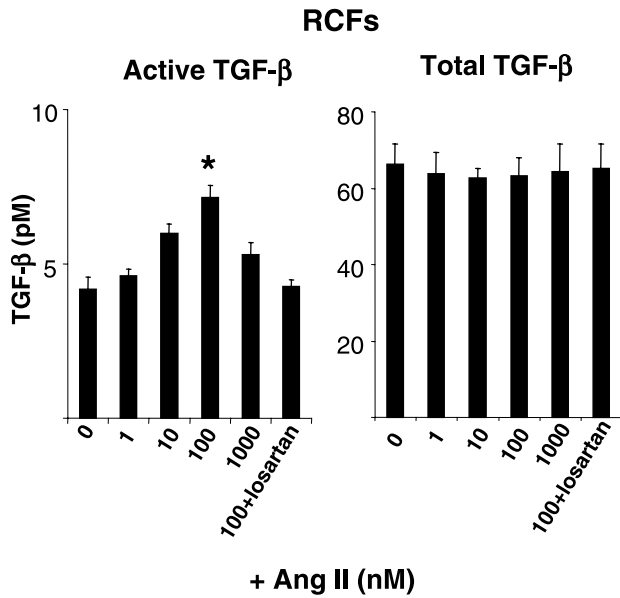


Fig. 2. Ang II stimulates AT1-dependent TGF-β activation by RCFs. Quiescent RCFs were treated with 0–10<sup>3</sup> nM Ang II for 24 h. Some cells were treated with 100 nM losartan in combination with 100 nM Ang II. Active and total TGF-β in the conditioned media were determined by PAIL assay. Experiments were performed in triplicate on three separate occasions. Results are shown as means ± SD. \**p* < 0.01 for basal active TGF-β vs. 100 nM Ang II-induced TGF-β.

active TGF-β (4.6 pM). The increase in TGF-β activity was significantly higher than the sum (2.9 pM) of the increases induced by either Ang II (1.5 pM) or high glucose (1.4 pM) alone (*p* < 0.01), suggesting that the combined action of Ang II and glucose on TGF-β activity is synergistic (Fig. 3B). In contrast, Ang II in combination with high glucose failed to stimulate total TGF-β protein. Losartan decreased the stimulation of active TGF-β induced by the combination of Ang II and high glucose to levels equivalent to those induced by glucose alone. As expected, losartan had no effect on glucose induction of TGF-β activity.

*Ang II and high glucose upregulate expression of soluble TSP1 in the conditioned media from RMCs and RCFs*

Ang II has been shown to induce a rapid (within 2 h) increase of TSP1 in heart endothelial cells [21,22]. In our previous studies, we showed that high glucose conditions stimulate increases in soluble TSP1 in RMCs and that this increased TSP1 increases TGF-β activation [17]. To determine whether Ang II/high glucose upregulates TSP1 by RCFs and RMCs, we measured levels of TSP1 protein in the conditioned media from RMCs and RCFs cultured in the presence or absence of Ang II and/or a high concentration of glucose. Immunoblot analysis showed that Ang II induced a dose-dependent increase in TSP1 by RMCs with significant increases observed with 100 pM to 1 μM Ang II (Fig. 4A). Induction of TSP1 was abolished by losartan (Fig. 4A). Moreover, significant increases in TSP1 levels were observed in the first 24-h period of Ang II stimulation,

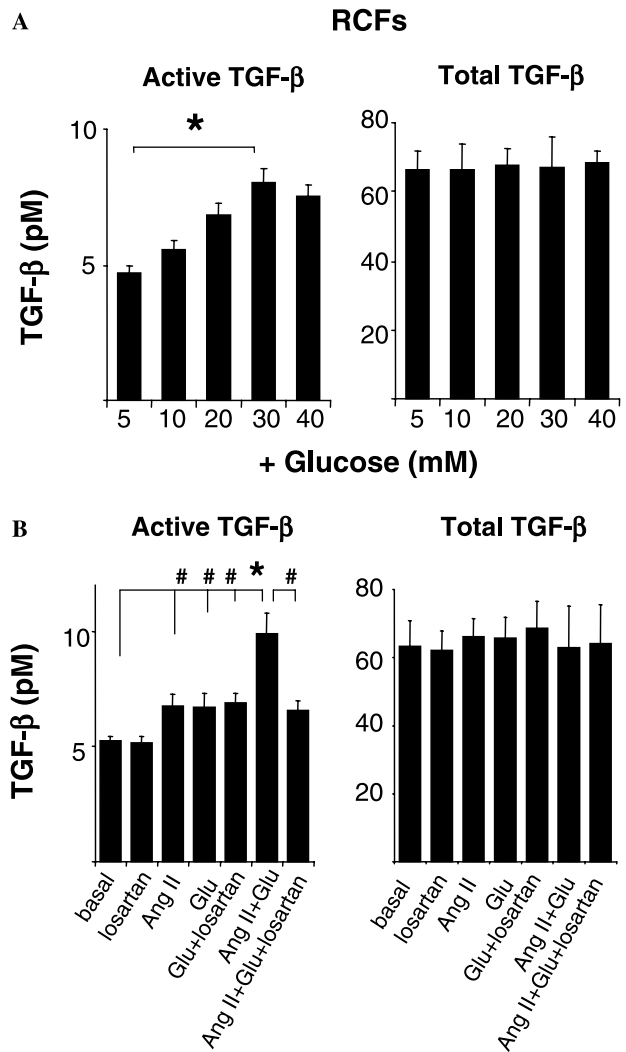


Fig. 3. Ang II-induced TGF-β activation is further enhanced by high glucose in RCFs. (A) Quiescent RCFs were treated with 5–40 mM glucose. Conditioned media were harvested after 24 h. Active and total TGF-β levels were determined using the PAIL assay. (B) Quiescent cells were treated with 30 mM glucose in the presence or absence of 100 nM Ang II or 100 nM losartan as indicated for 24 h. Active and total TGF-β in the conditioned media were determined using the PAIL assay. Experiments were performed in triplicate on three separate occasions. Results are shown as means ± SD. \**p* < 0.01 and #*p* < 0.05 for comparisons as indicated.

with a 2.7-fold and a 4.3-fold increase at 12–24 h and 0–24 h of Ang II stimulation, respectively (Fig. 4B). The kinetics of TSP1 stimulation are consistent with the kinetics of Ang II-induced increases in active TGF-β, suggesting a possible role for TSP1 in Ang II-induced TGF-β activation. These findings are consistent with a recent study from Naito et al. [11] who showed that 1–100 nM Ang II induced AT1-dependent TSP1 expression in human mesangial cells.

Consistent with previous observations in RMCs, high glucose stimulated a significant increase in TSP1 protein by RCFs, which was unaffected by losartan (Fig. 5). Ang II induced an increase in TSP1 and the induction was completely blocked by addition of losartan, suggesting that the

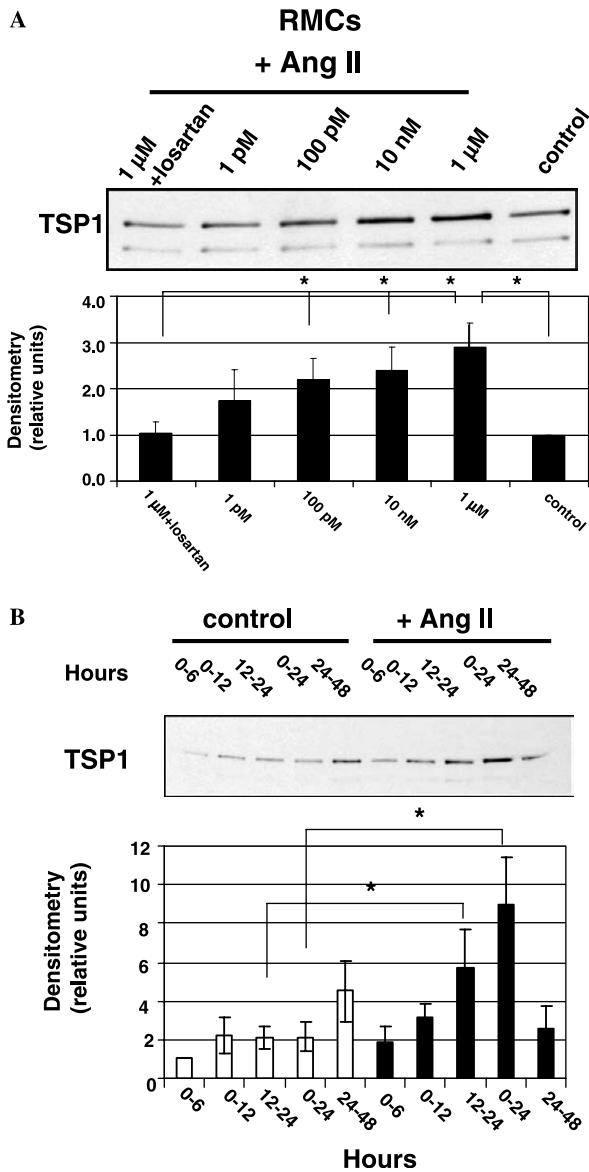


Fig. 4. Ang II stimulates AT1-dependent expression of soluble TSP1 protein by RMCs. (A) Quiescent cells were treated with 1 pM–1 μM Ang II or 1 μM Ang II in combination with 100 nM losartan for 24 h. Conditioned media were harvested and equal amounts of protein were subjected to electrophoresis on 8% SDS–PAGE under reducing conditions. Protein levels of TSP1 were detected by immunoblotting with Mab133 anti-TSP1 antibody. Relative TSP1 protein levels were determined by scanning densitometry of the blots. The level of TSP1 expressed in cells without Ang II treatment was set to 1. (B) Quiescent cells were treated with or without 1 μM Ang II for the time periods as indicated. Levels of TSP1 protein from conditioned media were determined as described above. The level of TSP1 from cells incubated with serum-free media for 6 h was set to 1. Results are means of three independent experiments ± SD. \**p* < 0.01 for comparisons as indicated.

Ang II-induced TSP1 expression is mediated through AT1 receptors. In addition, a synergistic increase in TSP1 was observed when RCFs were treated with a combination of Ang II and high glucose (Fig. 5), indicating a possible role for TSP1 in the synergistic stimulation of TGF-β activation by Ang II under high glucose conditions (Fig. 3B).

*TSP1 mediates Ang II/high glucose-induced TGF-β activation by RMCs and RCFs*

Previously we showed that treatment of RMC with peptide antagonists of TSP1-dependent TGF-β activation blocked glucose-stimulated increases in TGF-β activity [17]. In the present study, we examined whether antagonist peptides could similarly block increases in TGF-β activity stimulated by Ang II. Treatment of RMCs with LSKL and WSHW peptides, which antagonize TSP1-mediated TGF-β activation, inhibited Ang II-induced increases in active TGF-β (Fig. 6A), whereas treatment with control peptides, SLLK and GGYSHW, did not. As expected, total TGF-β levels were not affected by the peptide antagonists and the antagonist peptides did not reduce basal TGF-β activity. These data suggest that TSP1 mediates the increase in TGF-β activity in response to Ang II treatment. Cultures treated with Ang II in the presence of aprotinin, a serine protease inhibitor, showed no reduction in TGF-β activity, suggesting that plasmin does not play a role in mediating TGF-β activation under these conditions.

The involvement of TSP1 in Ang II-stimulated TGF-β activity is not limited to RMCs, since treatment of RCF cultures with either anti-TSP1 neutralizing antibody (Mab133) or the antagonist peptide LSKL totally blocked TGF-β activation by RCFs in response to Ang II (Fig. 6C). The antagonist peptide GGWSHW also partially inhibited the induction of TGF-β activation (Fig. 6C). Levels of total TGF-β were not affected by the TSP1 antagonists (Fig. 6C). In contrast, neither Mab133 nor the antagonist peptides affected levels of active or total TGF-β activity in the conditioned media of unstimulated RCFs (Fig. 6B). These agents (Mab133 antibody, LSKL peptide or GGWSHW peptide) also abrogated glucose-induced TGF-β activation in RCF cultures (Fig. 7A). Addition of aprotinin did not inhibit either Ang II or glucose-induced TGF-β activation in RCFs. Our data suggest that TSP1 is a major activator of TGF-β by RCFs in response to Ang II or glucose stimulation.

Finally, we determined the effects of the TSP1 antibody and the antagonist peptides on TGF-β activation in response to stimulation with the combination of Ang II and glucose. When RCFs were stimulated with Ang II and high glucose in combination, in the presence of 50 μg/ml Mab133, 2 μM LSKL or 40 μM GGWSHW, the induction of TGF-β activation was significantly inhibited (Fig. 7B). The control IgG, control peptides, and aprotinin had no inhibitory effect on the stimulation of TGF-β activity. The results suggest that TSP1 mediates TGF-β activation by RCFs in response to Ang II under high glucose conditions.

**Discussion**

The development of interstitial and replacement fibrosis is exacerbated in diabetes with hypertension [4]. Hypertension in diabetic models is associated with hyperglycemia-

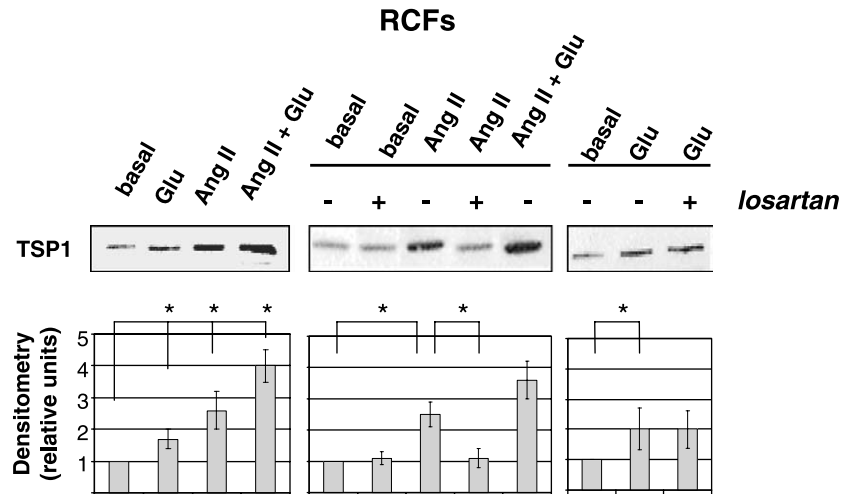


Fig. 5. Ang II and high glucose stimulate expression of soluble TSP1 by RCFs. Quiescent RCFs were treated with 30 mM glucose, 100 nM Ang II or 100 nM Ang II in combination with 30 mM glucose in the presence or absence of 100 nM losartan for 24 h. Conditioned media were harvested. Levels of TSP1 in the conditioned media were determined by immunoblot. Relative TSP1 protein levels were determined by scanning densitometry of the blots. The level of TSP1 expressed in cells incubated with serum-free media was set to 1. Results are means of three independent experiments  $\pm$  SD. \* $p$  < 0.01 for comparisons as indicated.

induced stimulation of RAS and resultant increases in Ang II [23]. In the present study, treatment with either high glucose or Ang II alone is able to induce TGF- $\beta$  activation by RCFs. Moreover, Ang II and high glucose in combination induced a synergistic stimulation of active TGF- $\beta$ . Although the mechanisms underlying the synergistic TGF- $\beta$  activation are currently unknown, the synthesis of TGF- $\beta$  protein is not required for increased active TGF- $\beta$  since increases in latent TGF- $\beta$  production in the conditioned media were not observed. These results suggest that the exacerbation of cardiomyopathy in hypertensive diabetics may be associated with modulation of TGF- $\beta$  activation by high glucose and Ang II. Previously, we showed that high glucose stimulated increases in both TGF- $\beta$  production and activation in RMCs [17]. However, in contrast to the RCFs, further increases in TGF- $\beta$  production and activation were not observed in RMCs treated with a combination of Ang II and high glucose (data not shown). The results are consistent with those from Weigert et al. [12] who showed that stimulation with both high glucose and Ang II did not induce further increase in either nuclear protein binding to the TGF- $\beta$  promoter or activation of the p38 MAPK pathway in mesangial cells. It is possible that glucose and Ang II may employ a common pathway(s) for regulation of TGF- $\beta$  in mesangial cells.

TGF- $\beta$  is initially synthesized as a biologically latent molecule. Sime et al. [24] have shown that constitutively active TGF- $\beta$ , but not latent TGF- $\beta$ , delivered by adenovirus, induces severe and persistent lung fibrosis in mice. This finding suggests that regulation of TGF- $\beta$  activity at the level of its activation could be a more critical determinant of its fibrogenic potential than regulation of the growth factor at the level of protein expression. Our observations that Ang II does not stimulate synthesis of the latent

TGF- $\beta$  by RCFs, but increases the level of active TGF- $\beta$  are supportive of this hypothesis.

Increases in TSP1 levels are associated with diabetic and hypertensive complications. Patients with type 1 diabetes show elevated levels of TSP1 in plasma [25]. Increased expression of TSP1 is present in fibrotic areas in diabetic glomerulonephritis [26]. Long-term treatment of human mesangial cells with pathologic levels of glucose in vitro caused increases in TSP1 mRNA levels [27]. Animal studies showed that induction of hypertension in rats resulted in increased renal expression of TSP, whereas, treatment with the Ang II receptor antagonist lowered blood pressure and abrogated TSP upregulation [28]. We and others previously determined that high glucose upregulates TSP1 in RMCs, resulting in subsequent activation of latent TGF- $\beta$  and synthesis of matrix proteins [17,18]. These studies suggest that increases in TSP1 in diabetic conditions could result in excessive TGF- $\beta$  activation which exacerbates fibrotic complications. To date, the role of TSP1 in hypertensive diabetic cardiomyopathy has not been defined. In the present study, we show that Ang II or high glucose alone stimulates expression of TSP1 and TGF- $\beta$  activation by RCFs. Addition of anti-TSP1 neutralizing antibody or antagonist peptides abrogates Ang II- and high glucose-stimulated TGF- $\beta$  activation. Our data suggest that TSP1 is a key factor in Ang II and high glucose induction of TGF- $\beta$  activation by RCFs. In addition, a combination of glucose and Ang II stimulates synergistic increases in TSP1 by RCFs. Both anti-TSP1 neutralizing antibody and antagonist peptides are able to inhibit the stimulated synergistic increases in TGF- $\beta$  activity. Interestingly, approximately 27–43% of the stimulated TGF- $\beta$  activity was not inhibited by the TSP1 antagonists, even when higher concentrations of peptides or antibodies were tested (data not shown). While it is

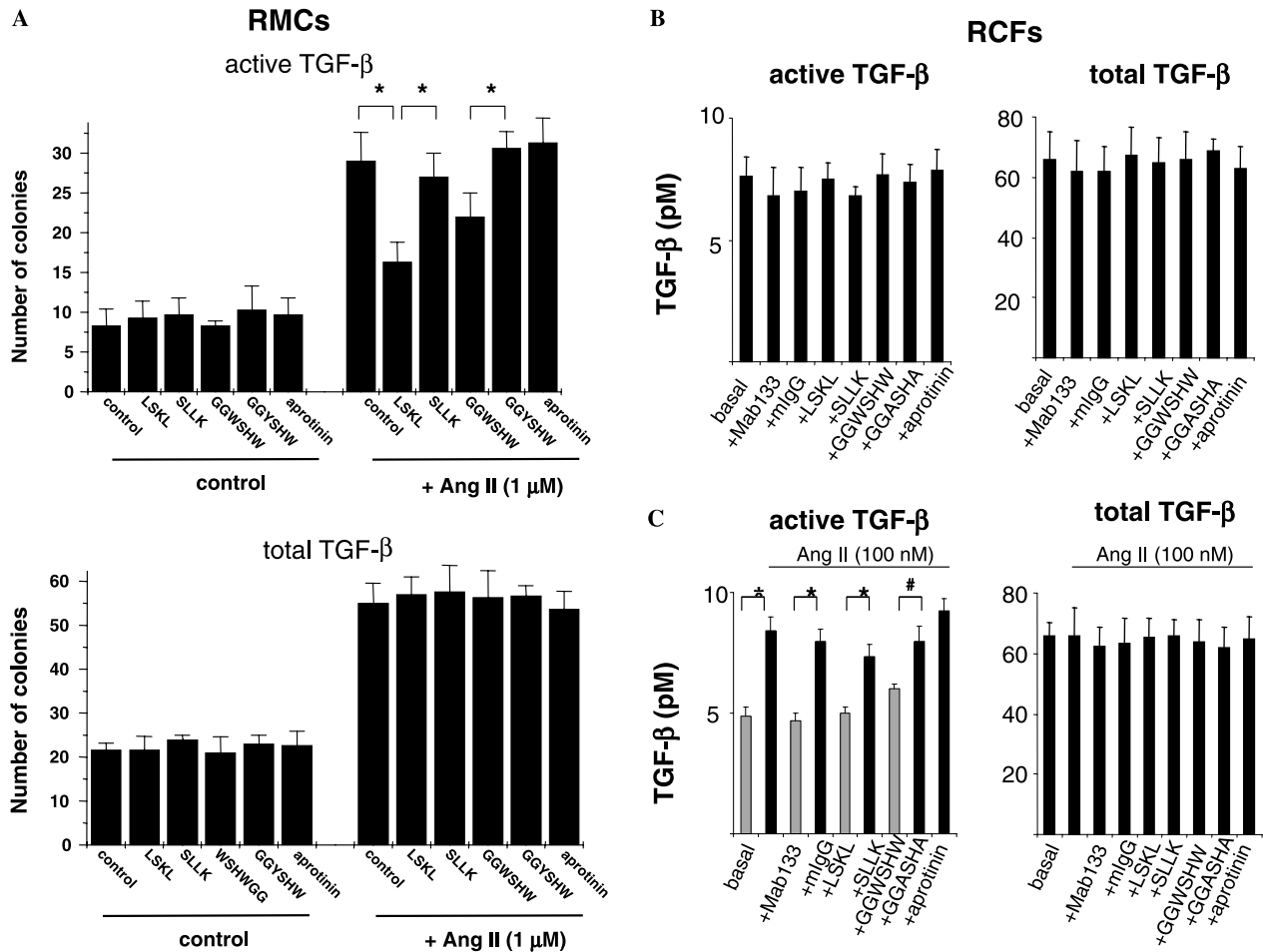


Fig. 6. TSP1 mediates Ang II-induced TGF-β activation by RMCs and RCFs. (A) Quiescent RMCs were treated with or without 1 M Ang II in the presence of 1 μM LSKL, 1 μM SLLK, 10 μM WSHW, 10 μM YSHW, or 200 μg/ml aprotinin for 24 h. Conditioned media were harvested. Active and total TGF-β activity was determined by NRK colony formation assay. (B) Quiescent RCFs were treated with 25 μg/ml non-immune mouse IgG (mIgG), 25 μg/ml Mab133, and peptides or aprotinin as described in (A) for 24 h. Conditioned media were collected. Active and total TGF-β were determined with the PAEL assay. (C) Quiescent RCFs were treated with 100 nM Ang II in the presence or absence of IgGs, peptides, and aprotinin as described in (A) and (B) for 24 h. Conditioned media were harvested and assayed for active and total TGF-β activity. Results are means of three separate experiments ± SD, each performed in triplicate. \**p* < 0.01 and #*p* < 0.05 for comparisons as indicated.

clear that the TSP1-dependent TGF-β activation is the primary mechanism of activation in the presence of Ang II and glucose, it is likely that other activation mechanisms also play a minor role. Serine protease activity, however, is unlikely to be involved since addition of aprotinin did not have any significant inhibitory effect. This result is consistent with the finding from Kagami et al. [29] who showed that Ang II inhibited plasmin activation by both upregulating plasminogen activator inhibitor (PAI-1) and downregulating plasminogen activator. It is not clear whether integrin-dependent activation could be playing a role, since there is no evidence to date showing that integrins expressed on fibroblasts are capable of activating latent TGF-β. Integrin αvβ6 expression and activation is limited to stimulated epithelial cells [30]. Integrin αvβ8-mediated activation has been reported in epithelial cells and perivascular astrocytes [31,32]. To the best of our knowledge, cardiac fibroblast expression of integrin αvβ8 has not been

reported. It remains to be seen whether integrins can regulate TGF-β activation by Ang II-stimulated fibroblasts.

Consistent with our observations showing that losartan blocks Ang II induction of TSP1 by both RCFs and RMCs, Naito et al. [11] showed that the upregulation of TSP1 expression by human mesangial cells is mediated by AT1 receptors. In heart endothelial cells, Ang II regulated AT1-dependent expression of TSP1 occurs via PKC [21]. Alternately, Fischer et al. [22] reported that Ang II-induced TSP1 expression in both macro- and micro-vascular endothelial cells is AT2 receptor-dependent. These results suggest that the involvement of Ang II receptors in regulation of TSP1 expression is complex with possible cell-type specificity. It is not known which signaling pathway(s) downstream of the AT2 receptor mediates TSP1 upregulation. However, PD123319, an AT2 receptor antagonist, produced a dose-dependent antagonism of Ang II-induced activation of PKC in cardiomyocytes

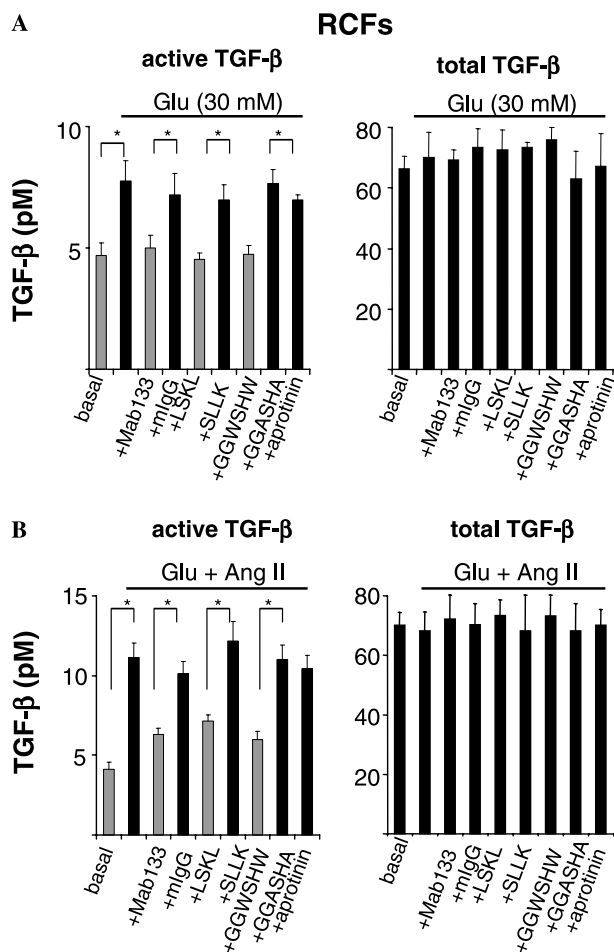


Fig. 7. TSP1 mediates glucose-induced TGF- $\beta$  activation by RCFs. (A) Quiescent cells were treated with 30 mM glucose in the presence or absence of 25  $\mu$ g/ml non-immune mouse IgG (mIgG), 25  $\mu$ g/ml Mab133, 1  $\mu$ M LSKL peptide, 1  $\mu$ M SLLK control peptide, 20  $\mu$ M GGWSHW peptide, 20  $\mu$ M GGASHA control peptide, or 200  $\mu$ g/ml aprotinin for 24 h. Conditioned media were collected. Active and total TGF- $\beta$  were determined with the PAIL assay. (B) Quiescent cells were treated with a combination of 100 nM Ang II and 30 mM glucose in the presence or absence of 50  $\mu$ g/ml of non-immune mIgG or Mab133, 2  $\mu$ M LSKL or SLLK peptides, 40  $\mu$ M GGWSHW or GGASHA peptides and 200  $\mu$ g/ml aprotinin as described in (A) for 24 h. Conditioned media were harvested and assayed for active and total TGF- $\beta$  activity. Results are means of three separate experiments  $\pm$  SD, each performed in triplicate. \* $p$  < 0.01 and # $p$  < 0.05 for comparisons as indicated.

[33]. Thus, it is possible that both types of Ang II receptors use the same signaling pathway to mediate TSP1 regulation.

In summary, Ang II and high glucose alone or in combination stimulate TGF- $\beta$  bioactivation by mesangial cells and cardiac fibroblasts through upregulation of TSP1. Our data suggest that TSP1-induced activation of TGF- $\beta$  may be an important mechanism involved in the development and progression of renal and cardiac fibrosis.

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