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UNIVERSITÀ DEGLI STUDI DI TORINO

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**Angiotensin II induces Tumor Necrosis Factor- $\alpha$  expression and release from cultured human podocytes**

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**Running title:** Effects of Ang II on TNF- $\alpha$  production by human podocytes

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## **ABSTRACT**

*Objective* High level of both Angiotensin (Ang) II and Tumor Necrosis Factor (TNF)- $\alpha$  have been implicated in the pathogenesis of glomerular injury by affecting podocytes. The aim of this study was to investigate the Ang II-TNF- $\alpha$  relationship in human podocytes.

*Methods* Immortalized podocytes were exposed to Ang II for 6 days in the absence or presence of either losartan or PD123,319 (both at 100 nM), AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists, respectively.

*Results* Ang II, after at least 72 h of repeated treatment, increased basal *TNFA* expression and cytokine release with a biphasic pattern and the maximum response at 10 nM. Losartan dampened the Ang II effects on TNF- $\alpha$  production throughout all the experimental period, demonstrating an AT<sub>1</sub> contribution. PD123,319 affected the second TNF- $\alpha$  production peak showing even an AT<sub>2</sub> receptor contribution. Moreover, Ang II causes TNFR1 and TNFR2 over-expression in a time-dependent manner. The functional interaction between Ang II and TNF- $\alpha$  has been demonstrated when the pro-proliferative effect of Ang II was antagonized by a neutralizing TNF- $\alpha$  antibody.

*Conclusions* In conclusion, our results point out a functional interaction between Ang II and TNF- $\alpha$  and indicate this cytokine as a mediator in Ang II long-term pathoadaptive podocytes changes.

**Keywords** podocytes, Tumor Necrosis Factor- $\alpha$ , angiotensin II

Clinical studies have shown that the angiotensin-converting-enzyme inhibitors (ACEi) and the angiotensin II-receptor blockers (ARB) exert significantly nephroprotective effects by decreasing proteinuria and disease progression both in patients affected by diabetic and non diabetic nephropathies [1, 2]. These data suggest a relevant contribution of the angiotensin (Ang) II signal to the development of glomerular dysfunction. Podocytes are terminally differentiated epithelial cells [3] and constituents of the glomerular filtration barrier (GFB). Their dysfunction and loss have been implicated in the onset and progression of glomerular diseases. These cells express all the components of the renin-angiotensin system (RAS) and are a major source of intraglomerular Ang II [4]. Ang II production results in being enhanced in podocytes following their exposure to mechanical stretch and hyperglycemia [5, 6]. Moreover, the expression of both AT<sub>1</sub> and AT<sub>2</sub> Ang II receptors on podocyte plasma membrane suggests that **could be targeted by Ang II** [7]. It has been demonstrated in podocytes that Ang II promotes actin rearrangement, tight junction protein ZO-1 and nephrin protein down-regulation, thus leading to proteinuria, as well as an increase of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-synthesis and, finally, it triggers apoptosis [8, 9]. Since podocyte response to Ang II may be a complex mixture of both direct and indirect effects, a further elucidation is warranted, particularly by testing the effects of a prolonged cell exposure to the vaso peptide with the aim **of mimicking** the clinical condition of a chronic exposure to high level of Ang II.

The proinflammatory cytokine tumor necrosis factor (TNF)- $\alpha$  has been reported to participate in the pathogenesis of glomerular injury. **In particular, high levels of TNF- $\alpha$  mRNA were found in the glomeruli of rodents and patients with diabetic nephropathy [10, 11], and this overexpression has been associated with the development of renal injury, leading to microalbuminuria [12, 13]. TNF- $\alpha$  is expressed and released not only by infiltrating leukocytes but also by resident cells, including endothelial, mesangial and tubular epithelial cells [14]. Among the many deleterious effects, TNF- $\alpha$  in the kidney promotes an inflammatory response and the accumulation of extracellular matrix thus**

reducing the glomerular blood flow and damaging the GFB [15]. Recently, Bruggeman et al. (2011) have demonstrated that TNF- $\alpha$ , through the TNFR2-NF- $\kappa$ B pathway, contributes to the proliferative and pro-inflammatory phenotype of diseased podocytes [16]. The detrimental role of TNF- $\alpha$  in the pathogenesis of glomerular injury has been confirmed by the attempt to use TNF- $\alpha$  antagonists to treat glomerular diseases [17]. Infliximab, a chimeric monoclonal antibody against TNF- $\alpha$ , has been reported to significantly decrease albuminuria in a rat model of diabetic nephropathy [18].

*In vitro* studies suggest that a functional linkage between Ang II and TNF- $\alpha$  may exist, having been demonstrated that Ang II induces TNF- $\alpha$  expression in endothelial and mesangial cells [19, 20]. However, at the best of our knowledge, whether Ang II could promote TNF- $\alpha$  production by podocytes remains still an unsolved question. As the high level of both Ang II and TNF- $\alpha$  have been reported in chronic progressive glomerular diseases, where podocytes dysfunction plays a critical role (i.e., hypertensive nephropathy, diabetic nephropathy, classic focal segmental glomerulosclerosis) [15, 21-23], the aim of this study was to investigate the Ang II-TNF- $\alpha$  relationship in human podocytes.

## **MATERIALS AND METHODS**

### **Drugs and chemicals**

Ang II, TNF- $\alpha$ , losartan, PD123,329 and all other reagents were from Sigma-Aldrich (Milan, Italy). Ang II receptor antagonists were dissolved in dimethylsulfoxide, TNF- $\alpha$  in water, as indicated by manufacturers' instructions. Final drug concentrations were obtained by dilution of stock solutions in the experimental buffers. The final concentration of organic solvent was less than 0.1%, which has no effect on cell viability. In all experiments un-stimulated cells served as controls.

### **Cell cultures**

Primary cultures of human podocytes were kindly provided by Prof. G. Camussi (Department of Internal Medicine and Center for Molecular Biotechnology, University of Turin, Turin, Italy). Podocytes were characterized and cultured as previously reported [24, 25].

### **Cytokine release**

TNF- $\alpha$  release was evaluated by measuring cytokine concentration in the experimental medium by the Instant ELISA system (Bender MedSystems, GmbH, W., Austria) following the manufacturers' instructions. All experiments were performed with  $30 \times 10^3$  cells/well as initial cell density and results were corrected for the number of cells (cell growth) and are expressed as percent change over the control.

### **RT-PCR and quantitative real-time PCR analysis**

The analyses were performed as previously described [25, 26]. Briefly, RT-PCR amplifications were performed in 25  $\mu$ l reaction mixtures containing 1.0  $\mu$ l of cDNA, 2.5  $\mu$ l of 10 $\times$  buffer, 1.0  $\mu$ l of 50 mM MgCl<sub>2</sub>, 0.20  $\mu$ l of 25 mM dNTPs mix, 0.05 U of Euro*Taq* DNA polymerase (Euroclone,

Milan, Italy), and 2.5 nM of each primer. PCR amplicons were resolved in an ethidium bromide-stained agarose gel (2%-3%) by electrophoresis, and signals were quantified using NIH Image J 1.41 software.  $\beta$ -actin expression was used as an internal control (Table 1). Real-time PCR experiments were performed in 25  $\mu$ l reaction mixtures containing 10 ng of cDNA template, the Power SYBR® Green PCR Master Mix and the AmpliTaq Gold® DNA Polymerase LD (Applied Biosystems). Relative quantization of the products was performed using a 48-well StepOne™ Real Time System (Applied Biosystems). For all real-time PCR analyses,  $\beta$ -actin mRNA was used to normalize RNA inputs (Table 1).

### **Western blot analysis**

Cell culture dishes were washed with phosphate-buffered saline (PBS) before adding ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, 1  $\mu$ l/ml protease inhibitors, 0.1 mM ZnCl<sub>2</sub>, and 1 mM Ph-Me-Sul-Flu). Cell lysates were processed to determine protein concentrations using a BCA protein assay (Pierce Biotechnology Inc. Rockford, IL, USA) following the manufacturers' instruction. Samples containing 20  $\mu$ g of protein were subjected to SDS-PAGE using a 10% gel. Proteins were transferred to a PVDF membrane (Millipore, Bradford, USA), which was incubated with Super Block blocking buffer (Pierce Biotechnology Inc.). AT<sub>1</sub>R, TNFR1 and TNFR2 were detected following incubation with mouse monoclonal antibodies (Santa Cruz Biotechnology, CA, USA). ATR2 was detected following incubation with rabbit polyclonal antibody (Santa Cruz Biotechnology), according to the manufacturer's instructions. To confirm the homogeneity of the proteins loaded, the membranes were stripped and incubated with an anti- $\beta$ -actin monoclonal antibody (Sigma-Aldrich). The membranes were overlaid with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Science, Norwalk, CT, USA) and then exposed to Hyperfilm ECL film (Amersham Biosciences, Piscataway, NJ, USA).



## Cell proliferation assay

Podocytes (3000 cells/well) were exposed to Ang II 10 nM for 6 days according to an everyday medium changing protocol. In some experiments, at the day 3, a polyclonal goat anti-human IgG blocking TNF- $\alpha$  or a nonspecific polyclonal goat IgG (Santa Cruz Biotechnology) were added. The proliferative response was determined by counting the viable cells in a hemacytometer by the trypan blue exclusion test, blind by an observer.

## Data analysis

Results are expressed as means  $\pm$  SEM of at least three experiments. One-way analysis of variance (ANOVA) followed by the post hoc Dunnet's multiple comparison and Student –Newman-Keuls tests were used to determine significant differences between means. Differences were considered statistically significant when  $P < 0.05$ .

## RESULTS

### Effects of Ang II on TNF- $\alpha$ production by human podocytes

Our cell line posses both AT<sub>1</sub> and AT<sub>2</sub> receptors, as measured at both mRNA and protein level (Figure 1A and 1B). Their **activation** by Ang II stimulates TNF- $\alpha$  production as shown in Figure 2A and 2B. Cells were treated with Ang II (10 nM, 1-6 days) according to a **daily** exposure protocol and both *TNFA* expression and TNF- $\alpha$  release were measured (see materials and methods). The cytokine release showed a biphasic pattern with a first peak at the forth day and a second one at the sixth: medium TNF- $\alpha$  concentration was 14.9 $\pm$ 3.0 pg/ml at day 0, 37.9 $\pm$ 3.7 pg/ml at the forth day (about +150%;  $P < 0.01$ ) and 49.2 $\pm$ 5.2 pg/ml at the sixth day (about +230%;  $P < 0.01$  vs both day 0 and forth day). The same pattern was displayed by *TNFA* but with a ~24 h time-lag (Figure 2A).

Ang II concentration-dependent curve (0.03-100 nM) disclosed a sigmoid shape with the maximum effect achieved from 10 nM and the EC<sub>50</sub> at 0.43 $\pm$ 0.02 nM (Figure 2B). These results demonstrate

that Ang II modulates TNF- $\alpha$  release by human podocytes in a time- and concentration-dependent manner. These effects are regulated at the gene level.

### **Effects of losartan and PD123,319 on cytokine production induced by Ang II**

To evaluate whether AT<sub>1</sub> and AT<sub>2</sub> receptors contribute differently to Ang II response, podocytes were treated with the vasoactive peptide (10 nM, 1-6 days) in the presence or absence of selective AT<sub>1</sub> or AT<sub>2</sub> receptor antagonists, losartan (AT<sub>1</sub>) or PD123,319 (AT<sub>2</sub>), both at 100 nM (Figure 3). Only losartan was able to abolish Ang II effect on TNF- $\alpha$  release throughout all the experimental period. On the contrary, PD123,319 did not affect the first peak, but antagonized the second one (Figure 3). From these data it appears that Ang II response is differently and selectively mediated by both Ang II receptors. The same results were recorded by testing gene expression (data not shown).

### **Effects of Ang II on TNF- $\alpha$ receptor expression**

A further link between Ang II and TNF- $\alpha$  in human podocytes was discovered when the vasoactive peptide effect on TNF- $\alpha$  receptor expression was investigated at both mRNA and protein level. As previously, cells were exposed to 10 nM Ang II up to 6 days (Figure 4A and 4B). Ang II upregulated TNF- $\alpha$  receptors expression in a time-dependent manner starting respectively from the first day for TNFR2 and the third one for TNFR1.

### **Effects of Ang II on human podocytes proliferation**

To evaluate whether the stimulatory agonism of Ang II on TNF- $\alpha$  production by human podocytes may have functional effects, the proliferative podocyte response to Ang II was tested. In comparison to medium alone, Ang II daily exposure increased cell growth rate: the cell number was significantly from the fourth day (Figure 5A). This effect was blunted by an  $\alpha$ TNF- $\alpha$  neutralizing

antibody (at the third day; Figure 5B), thus suggesting that the Ang II induced proliferative response is mediated by TNF- $\alpha$ .

## DISCUSSION

In this study we reported, in a concentration- and time-dependent manner, that Ang II induces TNF- $\alpha$  production by human podocytes. These results suggest that Ang II could activate a paracrine/autocrine loop centred on TNF- $\alpha$ . A similar positive effect of Ang II has been described in other kidney cells, such as cells from isolated tubules of rat medullary thick ascending limb and mesangial cells [19, 20], extending the evidence to the human podocytes. In our experiments Ang II, after at least 72 h of repeated treatment, increased basal *TNFA* expression and cytokine release with a biphasic pattern. Ang II-induced TNF- $\alpha$  release required a repeated exposure to the vasoactive peptide and it is related to the gene transcription. The analysis of the two kinetic profiles highlights a ~24 h-delay of release in comparison to gene expression, likely due to the multi-step process leading to mature TNF- $\alpha$  secretion [27]. Ang II up-regulates TNF- $\alpha$  in a concentration-dependent manner ( $EC_{50}$  0.43 $\pm$ 0.02 nM) with the maximum response at 10 nM. Notably, these concentrations are within the intrarenal range reported to be ~one thousand times higher than the circulating one [28, 29]. Furthermore, our protocol, consisting of repeated stimulations, intended to mimic a chronic *in vivo* podocyte exposure to the vasoactive peptide. The everyday replacement of the medium with or without Ang II was planned to overcome Ang II catabolism [30]. Velez et al. (2007) have demonstrated that podocytes not only synthesize and release Ang II, but also convert it into the bioactive Ang (1-7) and Ang III [31]. The formation of these active metabolites has to be considered while discussing the data. However, as losartan, a selective AT<sub>1</sub> antagonist, abolished the Ang II effects, the contribution of Ang (1-7), acting on the mas receptor [32], can be excluded. Even Ang III contribution can be rejected, because, even if it activates AT<sub>1</sub> and AT<sub>2</sub> receptors with

an affinity quite close to Ang II [32], its half life is ~5 fold shorter than the one of the parent peptide [31]. Thus, we may conclude that the observed effects are mainly due to Ang II. Taken these data together, our experimental conditions might be relevant to elucidate at least one of the podocytes pathoadaptive changes, TNF- $\alpha$  up-regulation, caused by a prolonged exposure to high concentration of Ang II, in the same way as it happens in chronic progressive glomerular disease where podocyte dysfunction plays a critical role (i.e., hypertensive nephropathy, diabetic nephropathy, classic focal segmental glomerulosclerosis) [15, 21-23].

Previous findings indicate that in rat renal cortex [22, 33] and in human renal proximal tubular epithelial cells [34] the interaction between Ang II and TNF- $\alpha$  is mediated by AT<sub>1</sub> receptors. These evidences have been confirmed by using a selective AT<sub>1</sub> antagonist, such as losartan. Drug treatment dampened all the Ang II effects on TNF- $\alpha$  gene expression and release, throughout the entire experimental period. However, our results show that even AT<sub>2</sub> receptors contribute to evoke the effects measured in the last period of the experimental observation, thus indicating a delayed involvement of theirs. This suggests that while the first peak reflects the AT<sub>1</sub> receptor contribution on cytokine production, the second one seems to be driven by a more complex signaling.

Another original result of this study is that Ang II, besides evoking TNF- $\alpha$  production, causes TNFR1 and TNFR2 over-expression, which might underlie an increased cell responsiveness to the cytokine. Actually, a functional interaction between Ang II and TNF- $\alpha$  has been demonstrated in our study when the pro-proliferative effect of Ang II was antagonized by a neutralizing TNF- $\alpha$  antibody. This experiment has been planned only to ascertain the cytokine role, being not able to elucidate the relative contribution of the two TNF- $\alpha$  receptor types. However, the kinetic data depicted in figure 4 indicate a more rapid and more intense modulation by Ang II of the TNFR2 expression in comparison to that of TNFR1. Interestingly, while TNFR1 has been implicated in cell death processes [14, 27], recent data indicate the TNF- $\alpha$ -TNFR2 axis as a candidate pathway contributing to the development of a proliferative and pro-inflammatory podocyte phenotype,

associated with proliferative podocytopathies [16]. Our data are derived from cultured immortalized human podocytes that are proliferating cells. Therefore these observations are not in keeping with that podocytes are postmitotic terminally differentiated cells with little or no capacity for regenerative replication [35]. However, proliferating podocytes have been observed in experimental models of glomerular injury [36], which may be explained admitting that some podocytes reengage the cell cycle as an adaptive response to injury in an attempt to mitigate their loss [37]. Further, indirect evidence for podocyte proliferation in human diabetes stems from the increased numbers of podocytes that are detected in the urine long before any reduction in glomerular podocyte numbers [38, 39]. In our study, we have demonstrated that our model leads to an Ang II induced proliferation of human podocytes. A critical aspect of our model is the use of immortalized podocytes, that are a cell type which is able to proliferate by itself. While recognizing this experimental bias, we have to stress that an increase in proliferation rate was observed in the presence of Ang II, as well as its reduction after  $\alpha$ TNF- $\alpha$ . Our results, consistently with previous data indicate that TNF- $\alpha$  could act as a mitogenic stimulus on podocytes [16] and suggest that the TNF- $\alpha$  release and the TNFRs expression, could mediate the proliferative effects induced by Ang II on podocytes.

In conclusion, our results point out a functional interaction between Ang II and TNF- $\alpha$  and indicate this cytokine as a mediator in Ang II long-term pathoadaptive podocytes changes. These results get more insight into the pathophysiological Ang II role in podocytes dysfunction leading to glomerular disease.

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Part of this study was presented at the 16<sup>th</sup> World Congress on Basic and Clinical Pharmacology, July 17-23, 2010, Copenhagen, Denmark; and was published in abstract form on *Basic Clin Pharmacol Toxicol*, 2010, 107 (Suppl. 1), 548.

The specific contributions to the work is listed as follow: performed the research A.C.R., L.R. and G.M.; designed the research study A.C.R. and R.F.; analyzed the data A.C.R., L.R., M.C. and G.M.; wrote the paper A.C.R., G.M. and R.F.

## **CONFLICT OF INTEREST**

None.

## TITLES AND LEGENDS

**Figure 1.** Basal AT<sub>1</sub> and AT<sub>2</sub> receptor expression by human podocytes. Representative pictures of *AGTR1* and *AGTR2* mRNAs detected by RT-PCR (A) or AT<sub>1</sub> and AT<sub>2</sub> proteins by Western blot analysis (B).

**Figure 2.** Effects of Ang II on TNF- $\alpha$  production by human podocytes. (A) Podocytes were exposed to Ang II. Experimental medium and Ang II were replaced every day. Cells were processed for quantitative real-time PCR analysis and cytokine concentration in the supernatants were measured by ELISA. Data are expressed as percent change over the control (untreated cells; day 0). (B) Podocytes were exposed to increasing Ang II concentrations (0.03 – 100 nM) for 4 days and TNF- $\alpha$  release was measured by ELISA. Data are expressed as percentage of the maximal effect. Data are the mean  $\pm$  SEM of three independent experiments performed in duplicate. \* $P$ <0.01 vs. control and # $P$ <0.01 vs. first peak.

**Figure 3.** Effects of losartan and PD123,319 on TNF- $\alpha$  production induced by Ang II. Podocytes were exposed to Ang II (10 nM, 1-6 days) in presence of losartan (100 nM) or PD123,319 (100 nM). Experimental medium, Ang II and antagonists were replaced every day. Cytokine concentration in the supernatants was measured by ELISA. Results are expressed as percent change over the control (untreated cells; day 0). Data are the mean  $\pm$  SEM of three independent experiments performed in duplicate. \* $P$ <0.01 vs. control.

**Figure 4.** Effects of Ang II on TNFR1 and TNFR2 receptor expression. Podocytes were exposed to Ang II. Experimental medium and Ang II were replaced every day and cells were processed for quantitative real-time PCR analysis (A) or Western blot analysis, pictures shown are representative

of three independent experiments (B). Results are expressed as percent change over the control (untreated cells; day 0). Data are the mean  $\pm$  SEM of three independent experiments performed in duplicate. \* $P$ <0.01 vs. control.

**Figure 5.** Effects of Ang II on proliferative response of human podocyte. (A) Cells were cultured in the absence or presence of Ang II (10 nM, 1-6 days) Experimental medium and Ang II were replaced every day. (B) Cells were cultured for 4 days, on the day 3  $\alpha$ TNF- $\alpha$  or nonspecific polyclonal goat IgG were added. Results are expressed as cells/well. Data are the mean  $\pm$  SEM of three independent experiments performed in triplicate. \* $P$ <0.01 vs. control and # $P$ <0.01 vs.  $\alpha$ TNF- $\alpha$ .

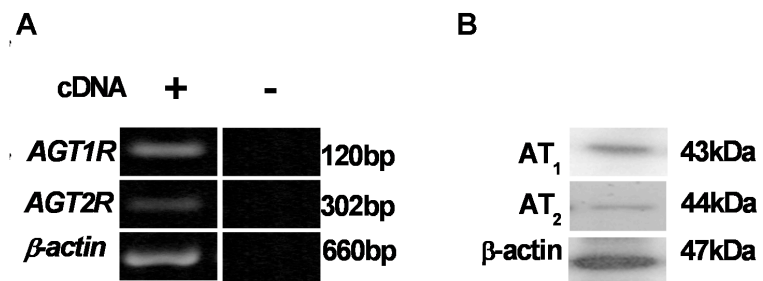


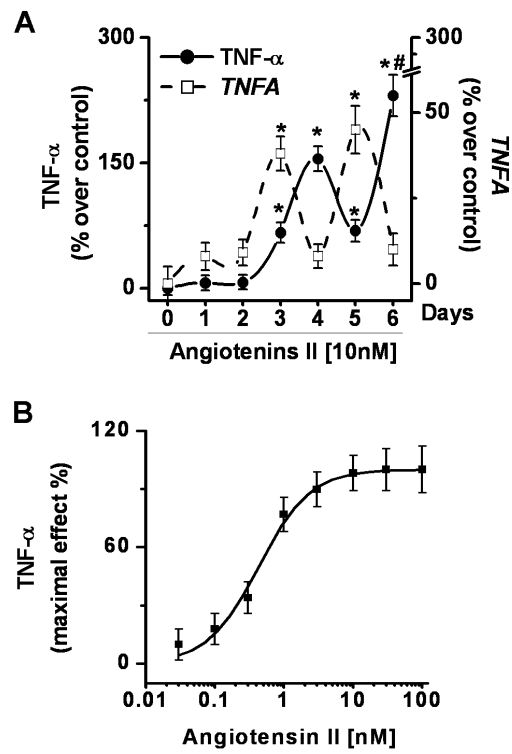
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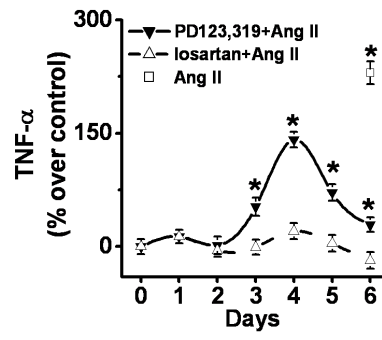
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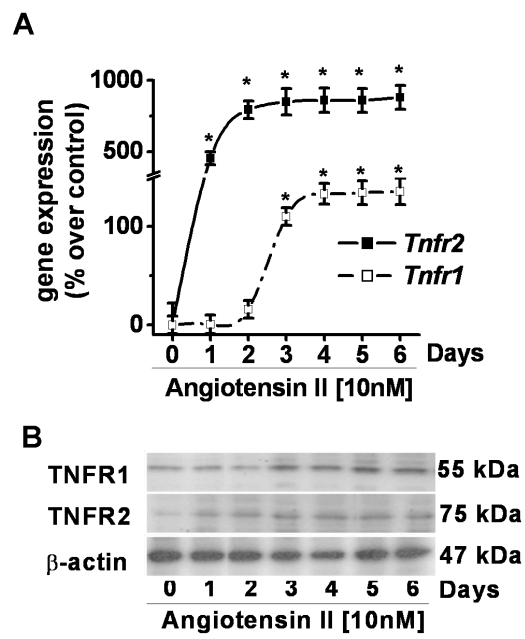
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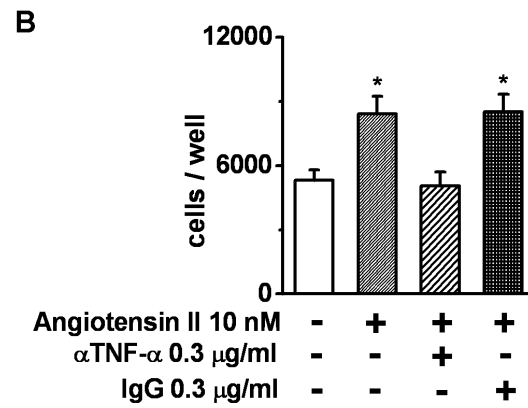
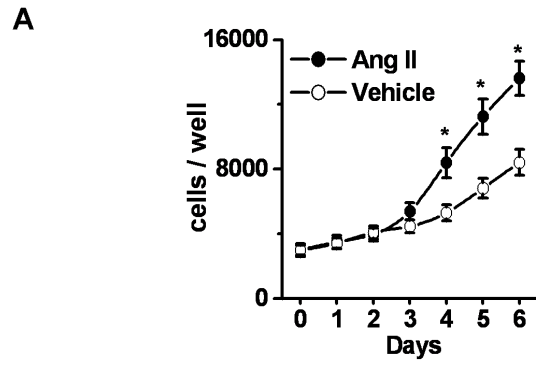
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**Table 1 Supplemental material**

Oligonucleotides and PCR conditions used in this study

PCR primers		Amplicon size (bp)	Denaturation	Annealing
<i>TNFA</i>	F: 5' -CAATGGCGTGGAGCTGAGAGA-3' R: 5' -CCAAAGTAGACCTGCCCAGAC-3'	344	95°C, 60 s	62°C, 6
<i>Tnfr1</i>	F: 5' -TCTATGCCCCGAGTCTCAACC-3' R: 5' -GGTGAGGGACCAGTCCAATA-3'	244	94°C, 30 s	55°C, 3
<i>Tnfr2</i>	F: 5' -AACTGGGTCCCCGAGTGCTTG-3' R: 5' -AGTGCTGGGTCTGGAGTTGG-3'	629	94°C, 30 s	64°C, 3
<i>AGTR1</i>	F: 5' -AGCCAAATCCCACTCAAACCT-3' R: 5' -TCGAACATGTCACTCAACCTCA-3'	120	94°C, 30 s	55°C, 3
<i>AGTR2</i>	F: 5' -AAGAAGAAATCCCTGGCAAGC-3' R: 5' -CCACGGCCTTGCTCTTGTT-3'	302	94°C, 30 s	55°C, 3
$\beta$ -actin	F: 5' -TGACGGGGTCACCCACACTGTGCCCATCTA-3' R: 5' -CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'	660	95°C, 45 s	60°C, 4