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Angiotensin II and TRPC6 activation stimulate release of a STAT3-activating factor from mouse podocytes

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Running title: Angiotensin and podocyte STAT3 signaling

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Angiotensin II (Ang II); calmodulin–dependent protein kinase II (CaMKII); diacylglycerol (DAG); focal and segmental glomerulosclerosis (FSGS); glycoprotein 130 (gp130); HIV-associated nephropathy (HIVAN); inositol triphosphate (IP_3); interlkeukin-6 (IL-6); janus kinase (JAK); macrophage colony-stimulating factor-1 (M-CSF); mammalian target of rapamycin (mTOR); 1-oleoyl-2-acetyl-sn-glycerol (OAG); monocyte chemoattractant protein-1 (MCP-1); NADPH oxidase (NOX2); phospholipase C (PLC); reactive oxygen species (ROS); signal transducer and activator of transcription-3 (STAT3); Transient receptor potential channels 6 channels (TRPC6); tumor necrosis factor-α (TNFα); vascular endothelial growth factor (VEGF)

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Abstract

Previous studies have shown that the transcription factor signal transducer and activator of transcription-3 (STAT3) in podocytes plays an important role in progression of HIV nephropathy and in collapsing forms of glomerulonephritis. Here we have observed that application of 100 nM angiotensin II (Ang II) to cultured podocytes for 6-24 hr causes a marked increase in the phosphorylation of STAT3 on tyrosine Y705 but has no effect on phosphorylation at serine S727. By contrast, Ang II treatment for short periods (20-60 min) caused a small but consistent suppression of tyrosine phosphylation of STAT3. A similar biphasic effect was seen after treatment with the diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG), an agent that causes activation of Ca^{2+}-permeable anonical transient receptor potential-6 (TRPC6) channels in podocytes. The stimulatory effects of Ang II on STAT3 phosphorylation were abolished by siRNA knockdown of TRPC6 and also by inhibitors of the Ca^{2+}-dependent downstream enzymes calcineurin and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII). The stimulatory effects of Ang II appear to be mediated by secretion and accumulation of an unknown factor into the surrounding medium, as they are no longer detected when medium is replaced every two hours, even if Ang II is continuously present. By contrast, the inhibitory effect of Ang II on STAT3 phosphorylation persists with frequent medium changes. Experiments with neutralizing and inhibitory antibodies suggest that the STAT3 stimulatory factor secreted from podocytes is not interleukin-6 (IL-6), but also suggest that this factor exerts its actions through a receptor system that requires glycoprotein 130 (gp130).

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Introduction

A subset of glomerular diseases including HIV-associated nephropathy (HIVAN), collapsing glomerulopathy and crescentic glomerulonephritis are characterized by de-differentiation, cell cycle dysregulation, and proliferation of podocytes (Schwimmer et al., 2003; Bariety et al., 1998; Barisoni et al., 1999). In these diseases, podocytes detach from the glomerular basement membrane, cease to express proteins characteristic of their fully differentiated state, and inappropriately re-enter the cell cycle. The resulting proliferation of epithelial cells fills Bowman's space resulting in collapse of the capillary tuft (Bariety et al., 1998; Barisoni et al., 1999). Proliferative podocyte diseases are exacerbated by activation of STAT3 (Dai et al., 2013, Gu et al., 2013, Feng et al., 2009). STAT3 is a widely-expressed cytoplasmic transcription factor engaged in signal transduction cascades, including those evoked by binding of IL-6 and related growth factors to their receptors. Tyrosine phosphorylation of STAT3 at residue 705 causes it to homodimerize and translocate into the nucleus. Additional phosphorylation at serine 727 allows for full transcriptional activity (Wegenka et al., 1993; Schindler and Darnell, 1995). Active STAT3 regulates the expression of genes involved in cell proliferation, apoptosis, and inflammation (Darnell et al., 1994; Darnell, 1997; Aaronson et al., 2002). The degree of STAT3 activation correlates with glomerular cell proliferation in glomerulonephritis (Arakwa et al., 2008). In mice, podocyte-specific deletion of STAT3 reduces the glomerular and tubulointerstitial pathology seen in transgenic models of HIVAN (Feng et al., 2009) and in a mouse model of crescentic glomerulonephritis (Dai et al., 2013).

TRPC6 channels have also been implicated in the pathophysiology of glomerular diseases (Dryer and Reiser, 2010; Reiser et al., 2005). TRPC6 are Ca^{2+}–permeable cationic

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channels that become active in phospholipase C (PLC) signaling cascades that cause breakdown of phosphoinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) (Dryer and Reiser, 2010). TRPC6 channels can also be activated by mechanical stimuli in podocytes (Anderson et al., 2013), and their expression on the podocyte cell surface is increased by oxidative stress (Kim et al., 2010, 2012; Liu et al., 2013). Activation of TRPC6 channels leads to the influx of Ca^{2+} and other cations, which in turn causes activation of a variety of downstream effectors including calcineurin, RhoA, and Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII) (Schlöndorff et al., 2009; Tian et al., 2010; Nijenhuis et al., 2011). TRPC6 channels expressed in podocyte foot processes occur in a complex with nephrin, podocin and other slit diaphragm molecules (Dryer and Reiser, 2010; Reiser et al., 2005). However, TRPC6 channels are also expressed in other parts of the cell, including major processes and the cell body, and the gating of TRPC6 in those locations may be regulated somewhat differently (Anderson et al., 2013; Dryer and Reiser, 2010).

Gain-of-function mutations in the gene encoding TRPC6 channels result in focal segmental glomerulosclerosis (FSGS) in humans, and podocyte-specific over-expression of mutant or wild type TRPC6 channels in transgenic mice results in proteinuria, foot process effacement, and glomerulosclerosis (Krall et al., 2010). Moreover, podocyte TRPC6 channel expression is increased in certain acquired proteinuric diseases such as membranous glomerulonephritis, FSGS, and minimal change disease, as well as in related rodent models of these diseases (Moller et al., 2007). The endogenous TRPC6 channels of podocytes are activated by Ang II (Anderson et al., 2014), and this appears to play a role in glomerular pathology caused by sustained elevation of Ang II (Ruster and Wolf, 2006; Eckel et al., 2011).

Previous studies suggest that Ang II contributes to podocyte injury in a transgenic mouse model of HIVAN (Ideura et al., 2007), and blockade of Ang II receptors reduces the progression of HIVAN in these mice (Hiramatsu et al, 2007). Given these observations, we hypothesized that Ang II might activate STAT3 in podocytes as a result of its signaling through TRPC6 channels. We now report that several hours of continuous exposure of cultured podocytes to Ang II, or a membrane-permeable DAG analog, results in increased STAT3 phosphorylation at tyrosine 705. Ang II-evoked STAT3 activation requires TRPC6 channels and their downstream effectors including calcineurin and CaMKII. However, Ang II-evoked STAT3 activation in podocytes appears to be an indirect process mediated by secretion or shedding of an autocrine/paracrine factor that can activate STAT3 signaling owing to activation of a receptor complex containing gp130. The identity of this secreted factor is not known, but it cannot be absorbed by antibodies that neutralize IL-6, suggesting that this effect is mediated by secretion of some other cytokine.

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Materials & Methods

Cell culture protocols, transfection, and chemicals. Cell culture protocols have been described previously (Kim et al., 2008, 2009). Mouse podocyte cell lines (MPC-5 cells) were obtained from Dr. Peter Mundel of Harvard Medical School and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.2% plasmocin. Undifferentiated cells were propagated in the presence of recombinant mouse interferon-γ at 33°C. In order to differentiate cells, mouse interferon was removed and cells were incubated at 37 °C for two weeks. Differentiated podocytes were treated with 100 nM Ang II or 100 µM OAG for various lengths of time. In some experiments, this was done in the presence of inhibitors, or after siRNA knockdown. For this, a specific siRNA directed against TRPC6, as well as non-targeted siRNAs for use in control experiments, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and transfected into podocytes using Lipofectamine™ (Invitrogen) in serum-reduced medium according to the manufacturer's directions and as previously described (Kim et al., 2011; Anderson et al., 2013; Roshanravan and Dryer, 2014). Analysis was carried out 48 hr after transfection with siRNA targeting TRPC6 or control siRNA. Ang II, OAG, and rapamycin were obtained from Sigma (St. Louis, MO). SKF-96365 was obtained from EMD Millipore (Billerica, MA). Cyclosporine, KN-93, and AG490 were from Tocris Bioscience (Minneapolis, MN). Recombinant mouse IL-6 was obtained from BioLegend (San Diego, CA). Chelerythrine was purchased from RBI, (Natick, MA). Anti-gp130 and anti-IL-6 neutralizing antibodies were from R&D Systems and were used at concentrations that produce maximum inhibition based on data supplied by the manufacturer (Minneapolis, MN).

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Immunoblot analysis. These were carried out by standard methods as described previously (Kim et al., 2010). Briefly, prior to lysis, podocytes were rinsed with ice-cold phosphate buffered saline (PBS). Total lysate was prepared using protein lysis buffer M-PER (Thermo Scientific) and centrifuged at 12,500 g for 10 min at 4°C. Equal amount of samples were separated on 10% SDS– PAGE. The gels were then blotted onto nitrocellulose membrane and blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20, pH 7.6 at 4 °C for 4 hr. The membrane was probed with a 1:1,000 dilution one of the following primary antibodies: anti-STAT3 (Cell Signaling Technology, Danvers MA); anti-phospho-STAT3 Y705 or anti-phospho-STAT3 S727 (GeneTex, Irvine, CA); anti phospho-mTOR, anti-mTOR, or anti-β-actin (Cell Signaling Technology); and anti-nephrin (Abcam, Cambridge, MA). The membranes were then probed with a 1:10,000 dilution of corresponding HRP-conjugated secondary antibody (Cell Signaling Technology). Signals were visualized using chemiluminescence from Thermo Scientific. Densitometry analysis was performed using Image J (Ver. 1.46). The signals obtained for each protein were normalized to β-actin and plotted as mean ± SEM for at least three independent experiments.

Conditioned medium and IL-6 or gp130 neutralization. Podocytes were treated with RPMI medium containing 100 nM Ang II or with control RPMI medium for 8 hr. In one set of cells these media were left undisturbed for the entire 8 hr, so as to allow any secreted factors to accumulate in the media. In another set the two types of media were replaced at 2 hr intervals during the 8 hr duration of the experiment. After 8 hr, cells were lysed and STAT3 activation was measured by immunoblot. In another set of experiments, podocytes were treated with 100

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nM Ang II for 24 hr in the presence or absence of IL-6 or gp130 neutralizing antibodies (R&D Systems, Minneapolis, MN) and STAT3 phosphorylation was measured by immunoblot.

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Results

In a recent study we demonstrated that Ang II evokes concentration-dependent activation of podocyte TRPC6 channels with maximal responses occurring at 100 nM (Anderson et al., 2014). In the initial experiments of this study, we treated differentiated cells of an immortalized mouse podocyte cell line with 100 nM Ang II for various periods of time. We then quantified STAT3 phosphorylation at tyrosine-705 and serine-727 by immunoblot using antibodies specific for the phosphorylation state of the protein (Fig. 1). Ang II had no discernible consistent effect on STAT3 phosphorylation at serine-727 at any time point. It should be noted that oladaic acid can increase phosphorylation at serine-727 (supplemental figure S1). It also had no effect on total levels of STAT3 (measured using an antibody insensitive to phosphorylation state). However, Ang II consistently evoked a biphasic effect on phosphorylation of tyrosine-705, and the rest of the experiments in this study focused on modification at that residue. Hereafter we will refer to this phenomenon simply as "STAT3 phosphorylation". We observed a small but quite reproducible reduction in STAT3 phosphorylation at early time points, after 20-60 min of continuous exposure to Ang II. With longer exposures (≥6 hr), we observed a marked increase in STAT3 phosphorylation that occurred with some variation from experiment to experiment, but which was generally maximal after 12-24 hr of continuous exposure to Ang II (Fig. 1). There are a variety of transduction pathways whereby growth factors signal to STAT3. One of these is through the mammalian target of rapamycin (mTOR) pathway (Yokogami et al., 2000). In podocytes we observed that Ang II also evoked an increase in serine phosphorylation of mTOR, but this response was monophasic and was easily seen with as little as 20 min exposure to Ang II (Fig. 2). In these experiments we also noted that Ang II treatment for 20 min to 24 hr had no

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consistent effect on total nephrin expression, indicating that the podocytes were well differentiated and could survive sustained Ang II treatment (Fig. 2).

In order to address whether TRPC6 channels might be involved in the response to Ang II, we treated podocytes with 100 µM OAG, a membrane-permeable analog of DAG that evokes robust activation of TRPC6 channels in podocytes (Anderson et al., 2013; Kim et al., 2013). OAG evoked the same biphasic response as Ang II. Specifically we observed a small decrease in STAT3 phosphorylation with 40-60 min of OAG exposure, and this was followed by a robust increase in STAT3 phosphorylation that was maximal with 12-24 hr of exposure, but no effect on total STAT3 (Fig. 3). Consistent with this, we observed that transient siRNA knockdown of TRPC6 expression in podocytes eliminated the increase in STAT3 phosphorylation evoked by 24 hr exposure to either 100 nM Ang II or 100 µM OAG compared to cells treated with a non-coding siRNA (Fig. 4). We also reduced Ang II-evoked STAT3 phosphorylation by treating cells with 10 µM SKF-96365, a non-specific inhibitor of most TRP superfamily channels including TRPC6 (supplemental figure S2). These data suggest that Ca^{2+} influx through TRPC6 is part of the pathway leading from AT_1 receptors to STAT3. In support of this theory, we also observed that Ang II-evoked phosphorylation of STAT3 was completely blocked by treating cells with cyclosporine (20 µM) to inhibit calcineurin, or with the CaM kinase II inhibitor KN-93 (10 µM) (Fig. 5A, B). This response was also blocked by the janus kinase (JAK) inhibitor AG490 (20 µM) (Fig. 5A, B). By contrast, we observed that treatment with the pan-protein kinase C inhibitor chelerythrine (1 µM) had no effect on Ang II-evoked STAT3 phosphorylation, even though DAG is produced during Ang II signaling. In addition, the mTOR inhibitor rapamycin (10 nM) had no

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effect on Ang II-evoked STAT3 phosphorylation (Fig. 5B) even though mTOR phosphorylation is increased in podocytes by Ang II (Fig. 2).

We were surprised by the slow time course of Ang II-evoked STAT3 phosphorylation and also by the biphasic effect. In several other cell types Ang II has been reported to stimulate secretion of pro-inflammatory cytokines such as IL-6, which activate STAT3 signaling pathways (Sano et al., 2000; Skurk et al., 2004). Therefore we considered the possibility that Ang II stimulates the secretion or shedding of some other factor that accumulates in the medium over time. To test this hypothesis, we carried out a two-factor experiment on STAT3 phosphorylation in which podocytes were cultured for 8 hr (Fig. 6). The first independent variable was whether or not 100 nM Ang II was present. The second independent variable was whether or not the medium was changed during the 8 hr stimulation period. In one group, the medium was replaced every two hours in order to prevent accumulation of any secreted factors. In the other group, the medium was undisturbed for the entire 8 hr. Ang II was always present in the treated cells, even in the group in which the medium was changed. We observed that Ang II was able to evoke robust STAT3 activation only when the medium was undisturbed. By contrast, when medium was changed every 2 hr, continuous exposure to Ang II actually caused a reduction of STAT3 phosphorylation. From this we conclude that Ang II stimulates the secretion or shedding of some factor into the medium that is capable of activating STAT3 signaling pathways. The slower onset of STAT3 activation could then reflect time required for sufficient amounts of this material to accumulate in surrounding medium. A similar pattern was seen in response to 100 µm OAG (supplemental figure S3). Independent of this effect, Ang II and OAG appear to cause a modest suppression of STAT3 signaling that is more significant at

earlier time points, or if secreted factors are not allowed to accumulate (Fig. 6). Because this effect persists with medium changes, we believe that it is a direct effect of Ang II.

IL-6 was an attractive candidate for the secreted factor, since Ang II has been shown to stimulate its secretion from other cell types (Sano et al., 2000; Skurk et al. 2004). We observed that 20 ng/ml of IL-6 evokes a robust activation of STAT3 in podocytes (Fig. 7A, *left*). This effect was not seen in the presence of a commercially-available IL-6-neutralizing antibody (1 µg/ml) to the culture medium (Fig. 7A, *right*). However, adding this IL-6–neutralizing antibody to culture media had no effect on STAT3 activation evoked by 5-24 hr of exposure to Ang II (Fig. 7B). This indicates that Ang II is stimulating secretion or shedding of some other factor capable of activating the STAT3 pathway. Nevertheless, this secreted factor acts on receptors similar to those used by IL-6, as responses require gp130, an essential co-receptor in signaling by IL-6 and several other cytokines (Taga et al., 1997; Fakuda et al., 1996). Specifically, the effects of 24-hr exposure to Ang II were inhibited by a function-blocking antibody against gp130 (2 µg/ml) (Fig. 7C). In addition, we observed that IL-6 activation of STAT3 in podocytes persists in the presence of inhibitors of calcineurin, CaMKII, and TRPC6 (data not shown). This suggests that TRPC6 is required for processes upstream of the gp130 activation, e.g. synthesis, secretion, and/or shedding of this unknown factor.

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Discussion

The main result of this study is that activation of TRPC6 channels by Ang II or OAG can stimulate secretion of an unknown STAT3-activating factor from mouse podocytes. The stimulatory effects of this factor overcome a small but direct effect of Ang II to suppress tyrosine phosphorylation of STAT3. Secretion of cytokines or growth factors is a novel output of TRPC6 signaling in podocytes, and one that may be of considerable interest in the context of pathophysiology.

Previous work in podocytes has identified three biochemically distinct classes of stimuli that cause TRPC6 channels to become active: canonical lipid signals such as DAG generated during G protein signaling, for example AT_1 receptors for Ang II (Anderson et al., 2014); mechanical stimuli, which appear to act directly on the channel complex and which proceed independent of any G protein signaling in these cells (Anderson et al., 2013); and oxidative effects caused by agents or treatments that increase the local concentration of reactive oxygen species (ROS) (Kim et al., 2012, 2013; Liu et al., 2013) and which increase steady-state surface expression of TRPC6 channels in podocytes. These stimuli can produce additive effects on total TRPC6 activation, and we have presented evidence that ROS generated by NADPH oxidases play an important role in mobilization of TRPC6 channels in response to circulating and locally produced factors such as Ang II (Anderson et al., 2014).

All three classes of stimuli are likely to be engaged in podocytes during inflammatory glomerular diseases. For example, there is evidence of marked oxidative stress in nephrotoxic serum models of mouse glomerulonephritis, and in particular marked up-regulation of NADPH oxidase (NOX2) and increased generation of superoxide free radicals (Kinoshita et al, 2011).

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Interestingly, these effects were reduced by AT_1 antagonists which have long been known to reduce histopathological changes and proteinuria in this model (Suzuki et al., 1998; Mii et al., 2009; Aki et al., 2010). In addition there is an increase in glomerular intracapillary pressure in these diseases (Maddox et al., 1975; Brenner, 1978) which may lead to increased mechanical stimulation of podocytes (Endlich and Endlich, 2006). The synergistic effects of these stimuli on TRPC6, if sustained, could provide a mechanism for secretion of pro-inflammatory cytokines.

In other cell types, the secretion of cytokines proceeds by variety of classical and non-classical pathways (Stow et al., 2009). For instance IL-6 and tumor necrosis factor-α (TNFα), are ultimately released by a Ca^{2+}-dependent exocytotic process (Bost et al., 1995; Kuhns et al., 1998; Rao et al., 2013). Indeed, the degranulation of mast cells evoked by crosslinking of the high affinity IgE receptor appears to be mediated by channels of the TRPC3/6/7 family (Sanchez-Miranda et al., 2010). It is possible that a similar process occurs in podocytes. Previous work has shown that all of the apparatus required for Ca^{2+}-dependent exocytosis is present in podocytes, which express secretory vesicles, Rab3A, synaptotagmins, synaptobrevins, and synaptophysins (Rastaldi et al., 2003, 2006). It bears noting, however, that voltage-activated Ca^{2+} channels are not present in podocytes, and transient receptor potential family channels such as TRPC6 must therefore carry most or all of the Ca^{2+} influx required to activate this mode of secretion. It is also possible that Ca^{2+} influx stimulates ectodomain shedding of molecules that are in some way able to drive or modulate pathways that feed into STAT3. For example, TNFα and macrophage colony-stimulating factor-1 (M-CSF) are secreted by Ca^{2+}-dependent cleavage of membrane-spanning precursor proteins (Massague and Pandiella, 1993; Le Gall et al., 2009). In this regard, monocyte chemoattractant protein-1 (MCP-1), M-CSF, TNFα, and

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certain EGF receptor ligands are among the cytokines capable of causing activation of STAT3 (Darnell et al., 1994; Yu et al., 2009; Fitzgerald et al., 2008). Moreover MCP-1 and TNFα are known to cause changes in podocyte function, leading to increases in albumin permeability of the glomerular filtration barrier (Matsui and Meldrum, 2012; Fornoni et al., 2008).

These data therefore suggest a model in which sustained TRPC6 activation in podocytes contributes to glomerular inflammation by inducing the secretion of a cytokine (or even multiple cytokines). These cytokines could act in an autocrine and paracrine manner to regulate gene expression or other aspects of podocyte cell biology, and they could conceivably cause changes in the behavior of other neighboring cell types, for example parietal cells (Migliorini et al., 2013). The resulting changes may be adaptive in the short run, but over time it is possible that they compromise glomerular function and lead to dysregulation. The secreted factor activates STAT3 signaling by means of a receptor that contains gp130. The role of gp130 signaling in podocytes is not well understood, and has only been studied in the role of IL-6 signaling in this cell type (Kim and Park, 2013). In rat podocytes, IL-6 evokes an increase in expression of apoptotic markers that coincides with stimulation of p53 expression (Kim and Park, 2013), along with expression of inflammatory markers (lee et al., 2012).

The consequences of STAT3 activation have not been comprehensively studied in podocytes, but it is known that proliferative podocyte diseases are reduced by podocyte-specific deletion of STAT3 (Dai et al., 2013; Gu et al., 2013; Feng et al., 2009) and that inhibition of STAT3 can reduce proliferation of cultured podocytes (He et al. 2004). STAT3 may also function in podocytes to regulate expression of molecules such as vascular endothelial growth factor (VEGF) (Korgaonkar et al., 2008). It is possible therefore that STAT3 activation in

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podocytes is responsible for changes in both the differentiation state and in control of cell cycle, and that this can occur in response to stimuli that cause sustained activation of TRPC6.

In summary, we have demonstrated that Ang II and a DAG analog simulate phosphorylation of the transcription factor STAT3 in podocytes. This effect requires TRPC6 channels, and is mediated by secretion of an unidentified gp130 ligand into the medium.

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Itemized author contributions

Participated in research design: Abkhezr, Dryer.

Conducted experiments: Abkhezr.

Performed data analysis: Abkhezr, Dryer

Wrote or contributed to the writing of the manuscript: Abkhezr, Dryer

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Footnotes

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Figure legends

Figure 1. Angiotensin II (Ang II) evokes tyrosine phosphorylation of STAT3. A, Representative immunoblot showing biphasic changes in phosphorylation of STAT3 on tyrosine 705 (Y705) but not on serine 727 (S727). These blots were obtained using antibodies that selectively recognize the phosphorylated forms of STAT3. The antibody against STAT3 Y705 often shows two bands, one of which is non-specific. The lower molecular weight band (arrow) is Y705. There is a discernible decrease in tyrosine phosphorylation at early time points (20-60 min of continuous exposure) followed by an increase in tyrosine phosphorylation at 6-24 h. Quantitative densitometric analysis of3 repetitions of this experiment are shown in B (for Y705) and C (for S272). In this and subsequent figures, bar graphs denote mean ± s.e.m.

Figure 2. Ang II evokes a rapid increase in the phosphorylation of mTOR but has no effect on expression of nephrin in cultured podocytes. A, Representative immunoblot. Quantitative analyses of 3 repetitions of this experiment are shown in B and C.

Figure 3. OAG, a membrane-permeable analog of diacylglycerol that activates TRPC6 channels, causes biphasic change in tyrosine phosphorylation of STAT3. A, Representative immunoblot. B, Densitometric analysis of 3 repetitions of this experiment. Note decrease in phosphorylation of Y705 at 40 and 60 min, and sustained increase after 6-24 hours of continuous exposure to OAG.

Figure 4. Knockdown of TRPC6 channels eliminates Ang II-evoked increases in STAT3 phosphorylation. A, Immunoblot analysis of proteins extracted from podocytes exposed to

control siRNA or siRNAs that target TRPC6. Ang II was applied for 24 hr. B, Densitometric analysis of 3 repetitions of this experiment.

Figure 5. Effects of various signaling inhibitors on STAT3 signaling in podocytes. Inhibitors of CaMK II (KN-93), JAK (AG490) and calcineurin (cyclosporine) eliminate increases in STAT3 phorphorylation evoked by 24-hr exposure to Ang II (A, B). Inhibitors of protein kinase C (chelerythrine) and mTOR (rapamycin) have no effect (C, D).

Figure 6. Ang II-evoked stimulation of STAT3 signaling in podocytes requires secretion of an unknown factor into the medium. In one group of cells (control or Ang II-treated, as indicated), media was changed every 2 hr (*left*). In another group of cells (control or Ang II-treated) the medium was undisturbed for the entire 8 hr duration of the experiment, so that anything secreted into the medium had time to accumulate (*right*). Ang II treatment evoked a large activation when medium was undisturbed but actually caused a decrease in STAT3 phospyhorylation when medium was changed every 2 hr. A, shows a representative immunoblot and B shows densitometric analysis of three repetitions of this experiment. The suppression seen when medium is changed frequently corresponds to the suppression seen at early time points in Fig. 1.

Figure 7. Ang II-evoked signaling is not mediated by secretion of IL-6, but requires activation of receptors containing gp130. A, An IL-6– neutralizing antibody is able to suppress STAT3 phosphorylation evoked by IL-6. B, Effects of Ang II persist in the presence of a neutralizing

antibody against IL-6. The neutralizing antibody has no effect by itself. C, An antibody that blocks function of gp130 inhibits STAT3 signaling evoked by 24-hr exposure to 100 nM Ang II.

Figure 8. A model of Ang II and STAT3 signaling in podocytes. Ang II and mechanical stimuli causes activation of TRPC6 channels in podocytes through mechanisms described elsewhere (Anderson et al., 2013, 2014). The resulting Ca^{2+} influx causes activation of a variety of signaling pathways including calcineurin/NFAT and CaMKII, which results in secretion of an unknown cytokine or signaling molecule, possibly by exocytosis. The autocrine and paracrine actions of this factor cause activation of a receptor complex that includes gp130, resulting in tyrosine phosphorylation of STAT3. This transcription factor can then translocate into the nucleus and regulate gene expression. TRPC6 channels can stimulate their own expression (Nijenhuis et al. 2011) and thereby become self-sustaining. This could cause cytokine secretion to become chronic, resulting in global changes in glomerular function. Ang II also appears to produce a cell-autonomous inhibitory effect on STAT3 signaling that is seen at early time points or when gp130 signaling is inhibited.

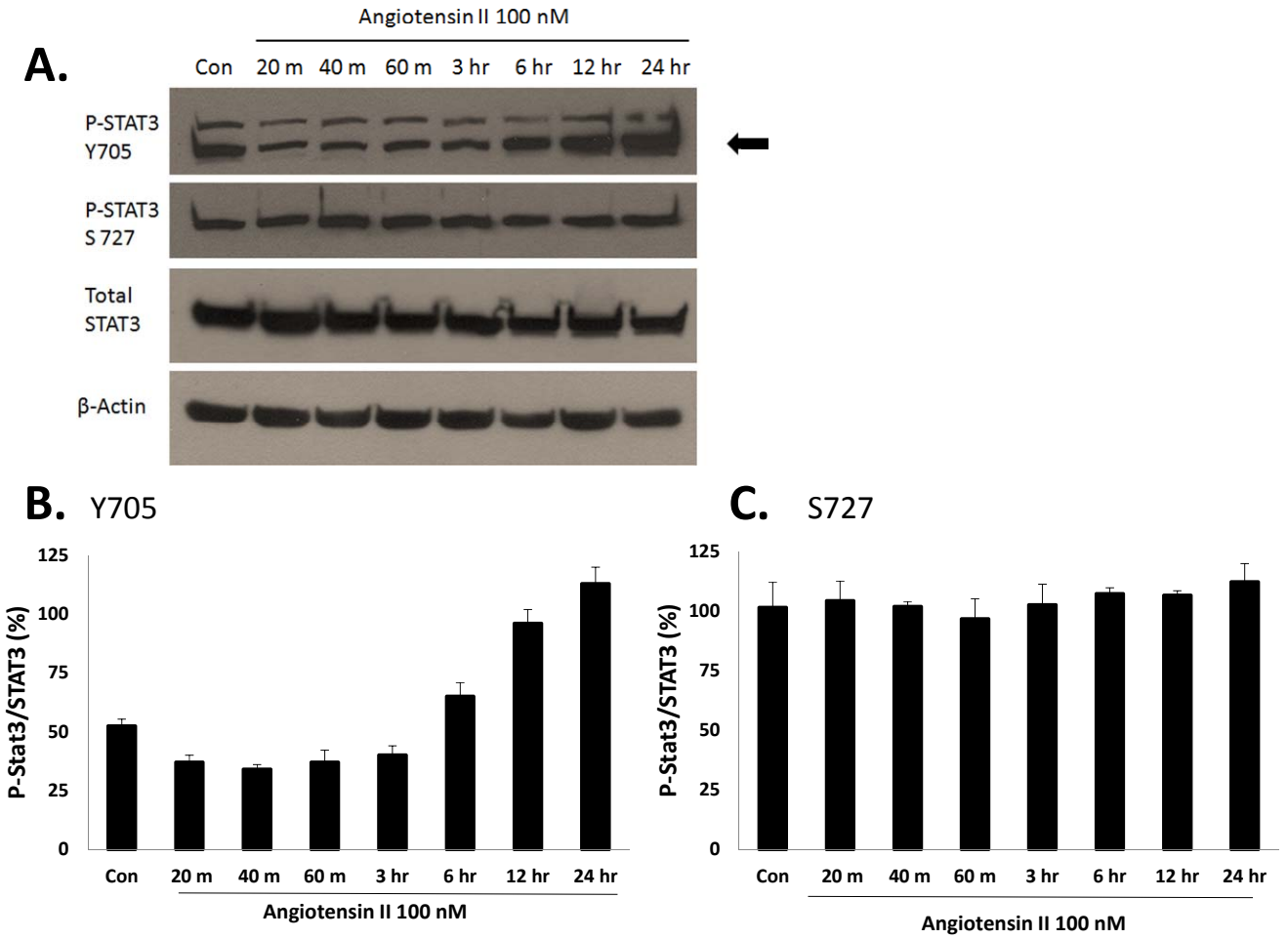

Figure 1

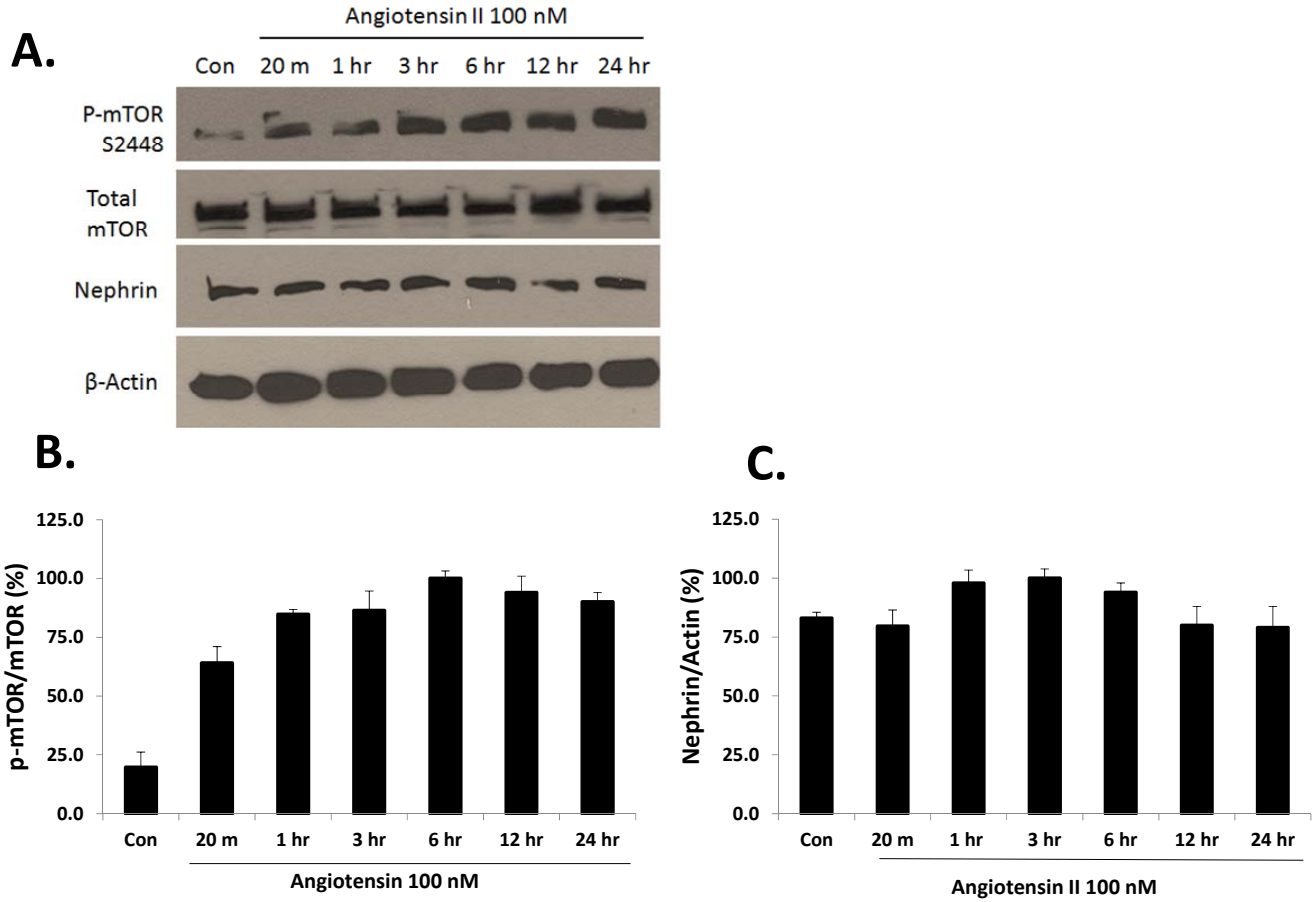

Figure 2

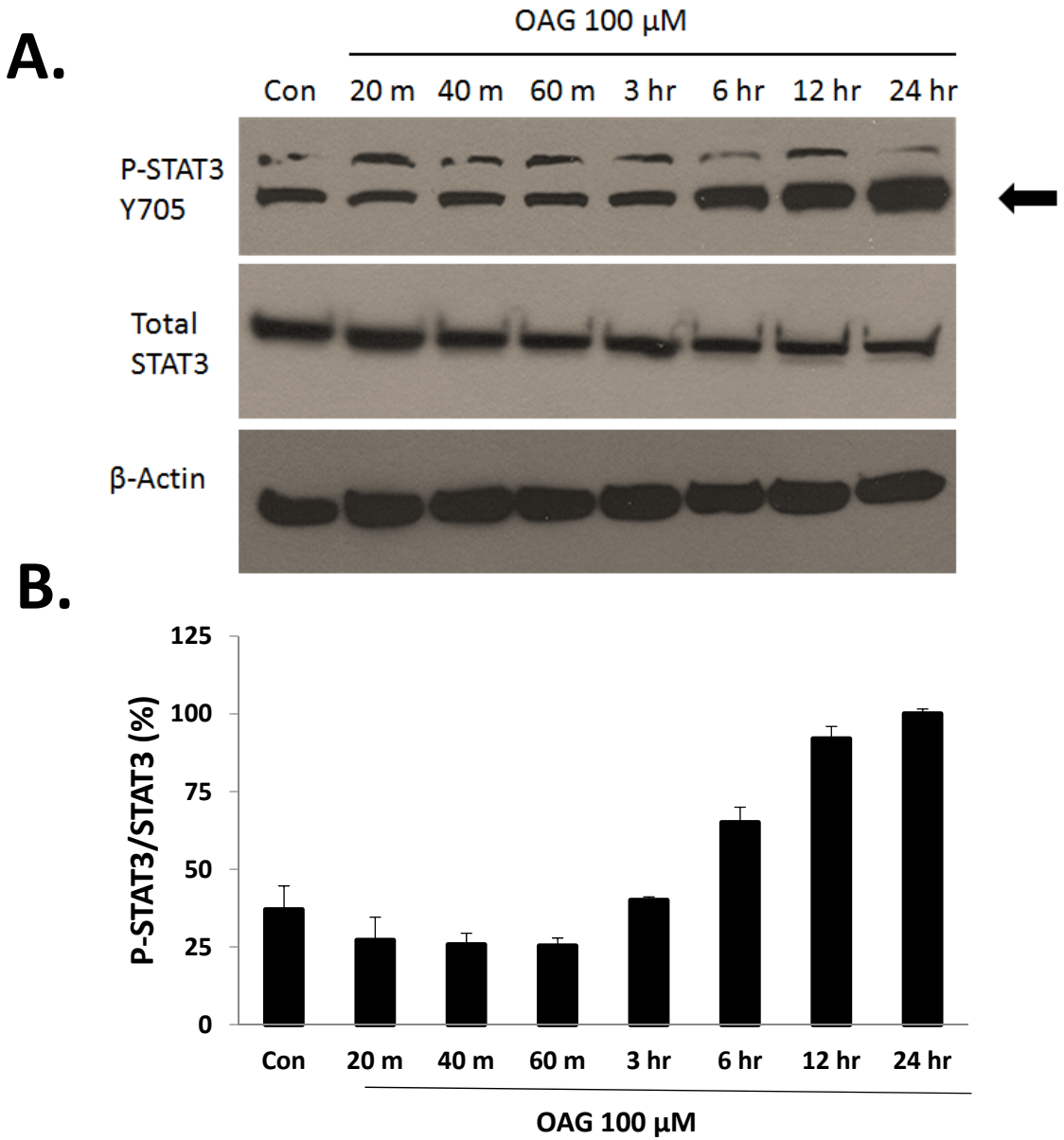

Figure 3

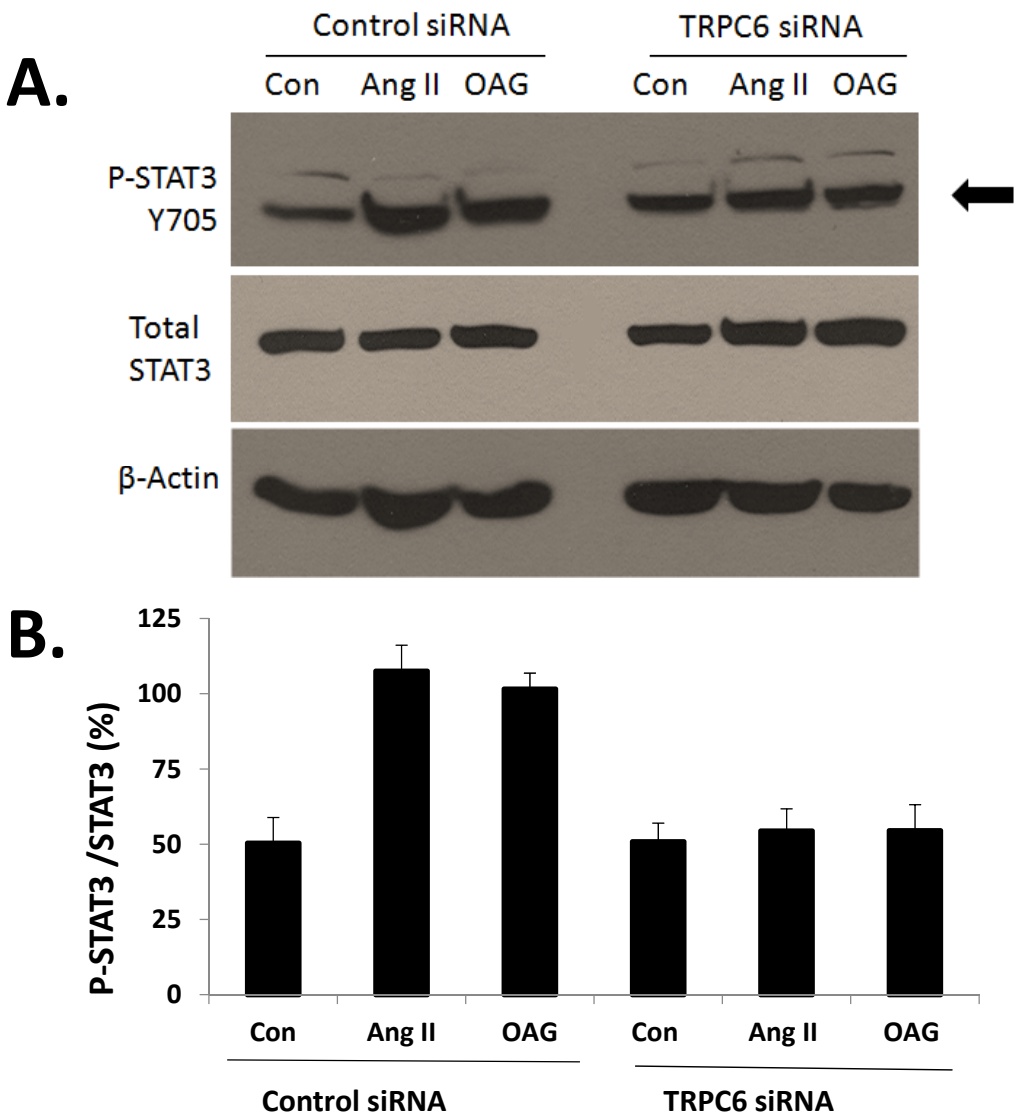

Figure 4

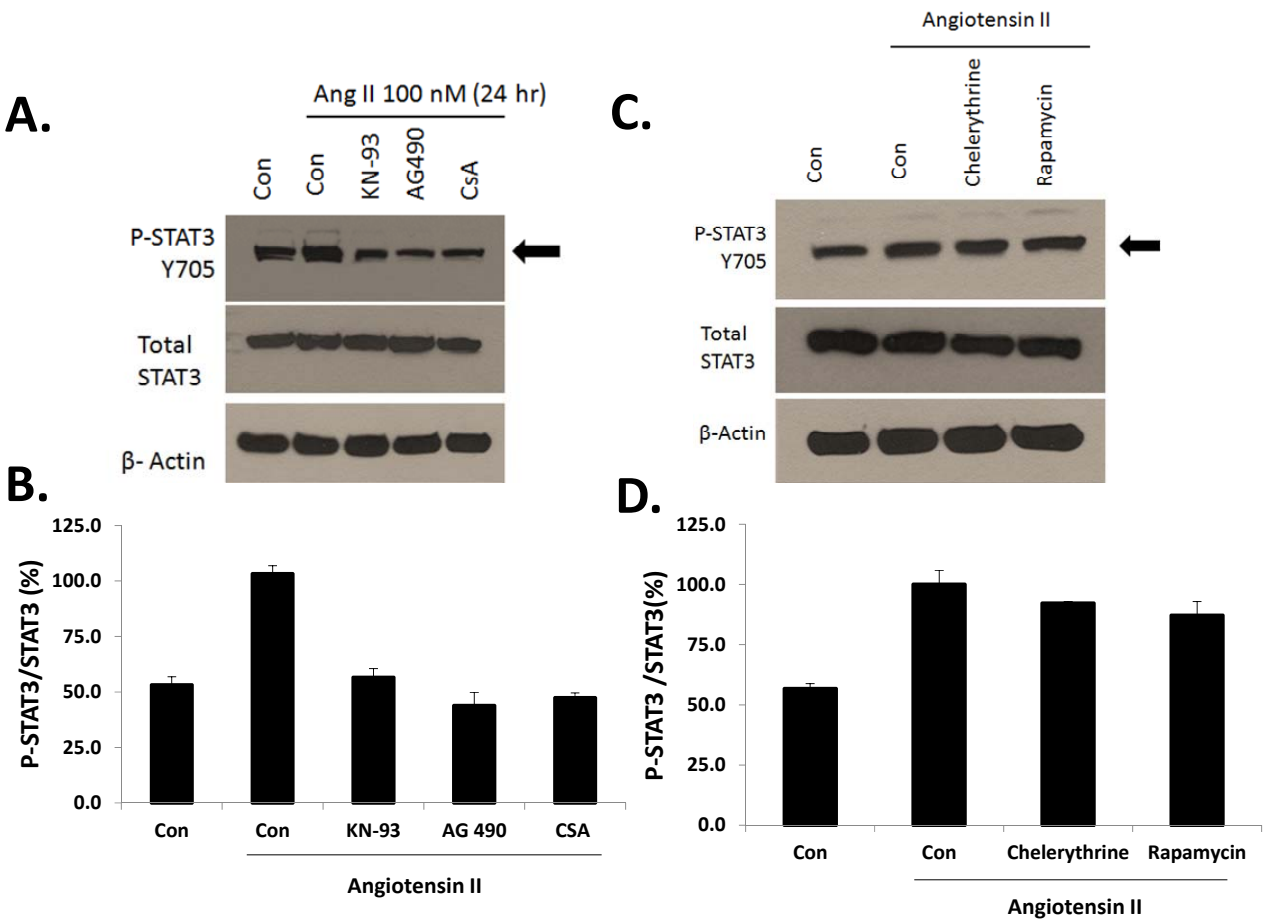

Figure 5

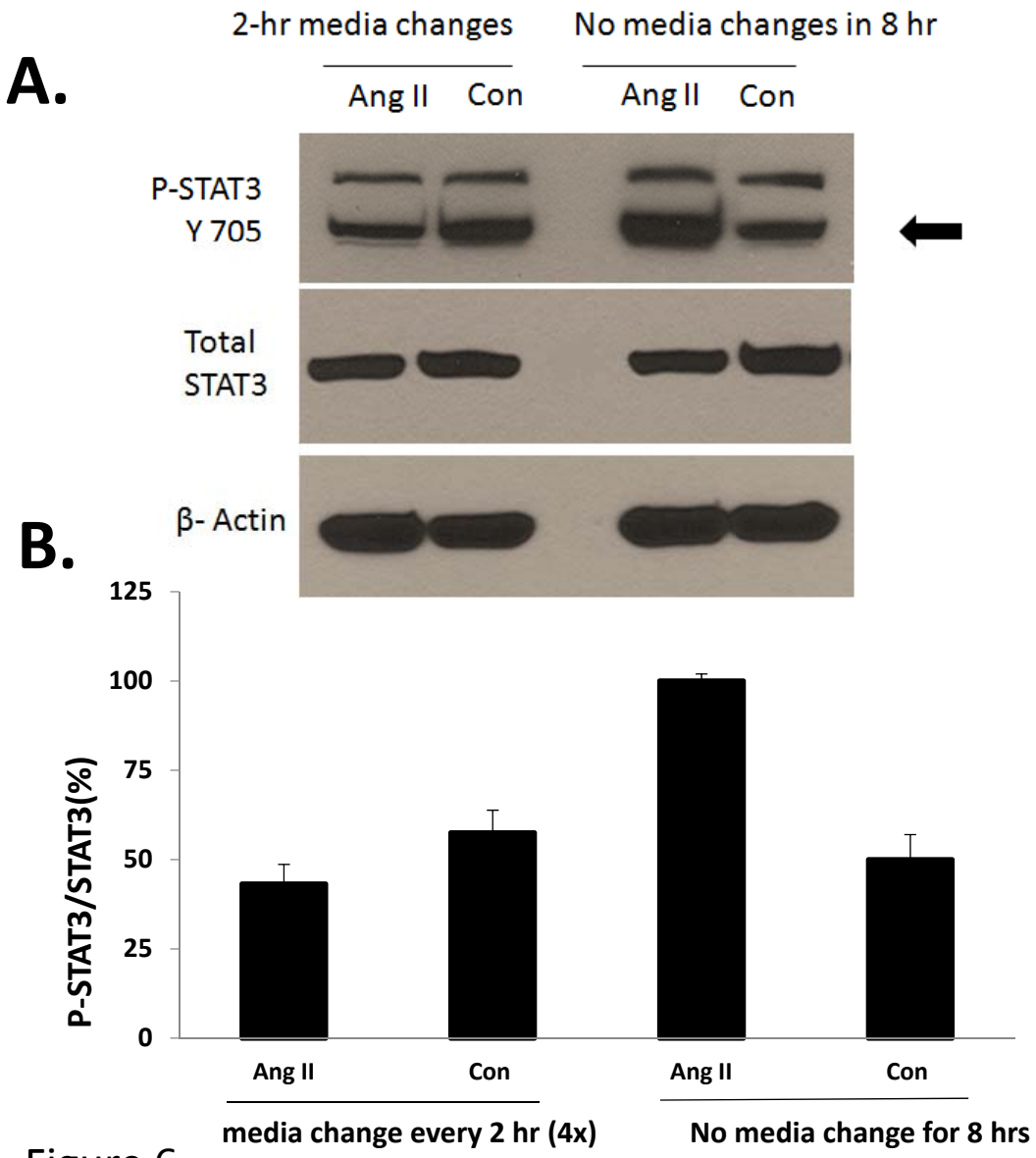

Figure 6

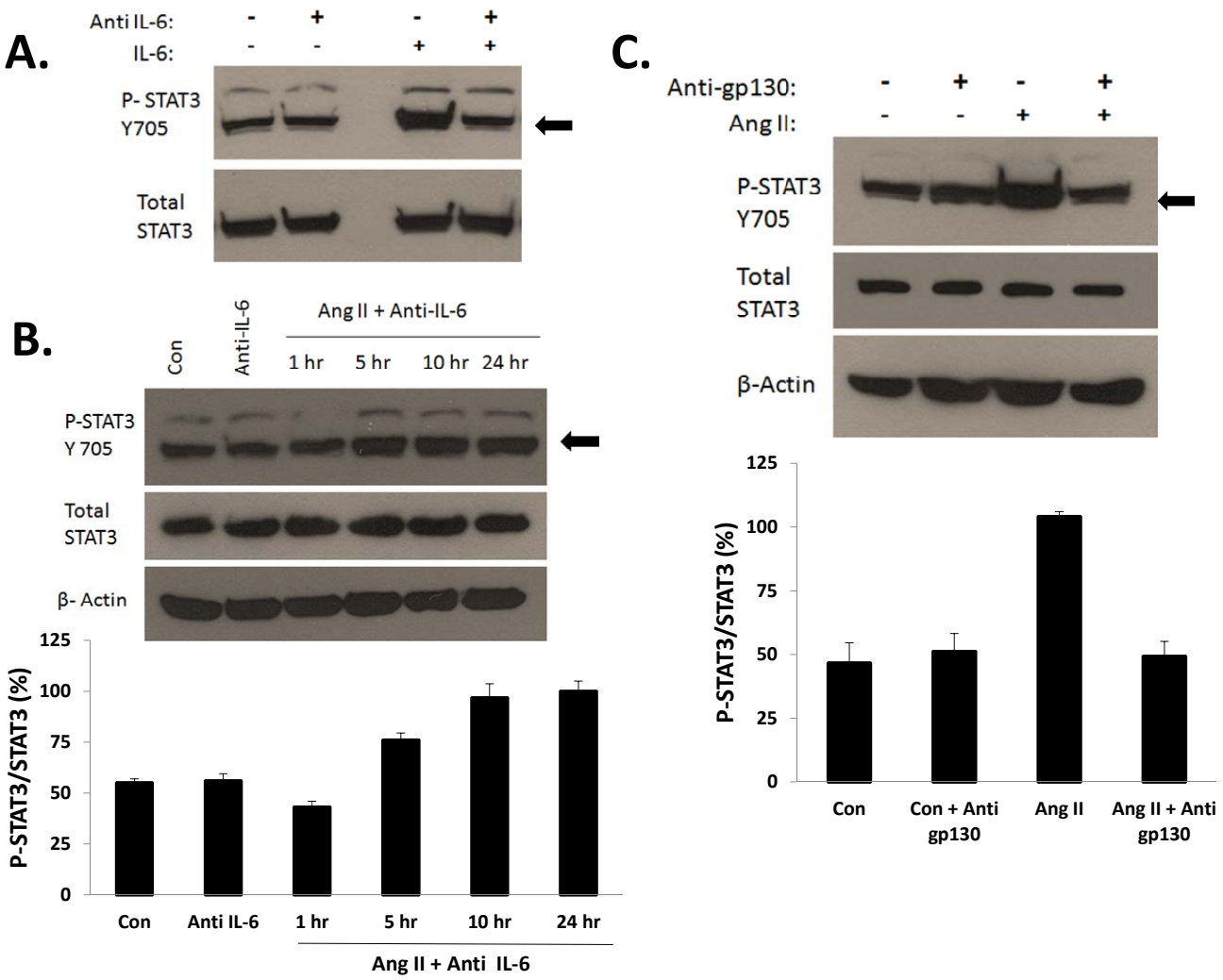

Figure 7

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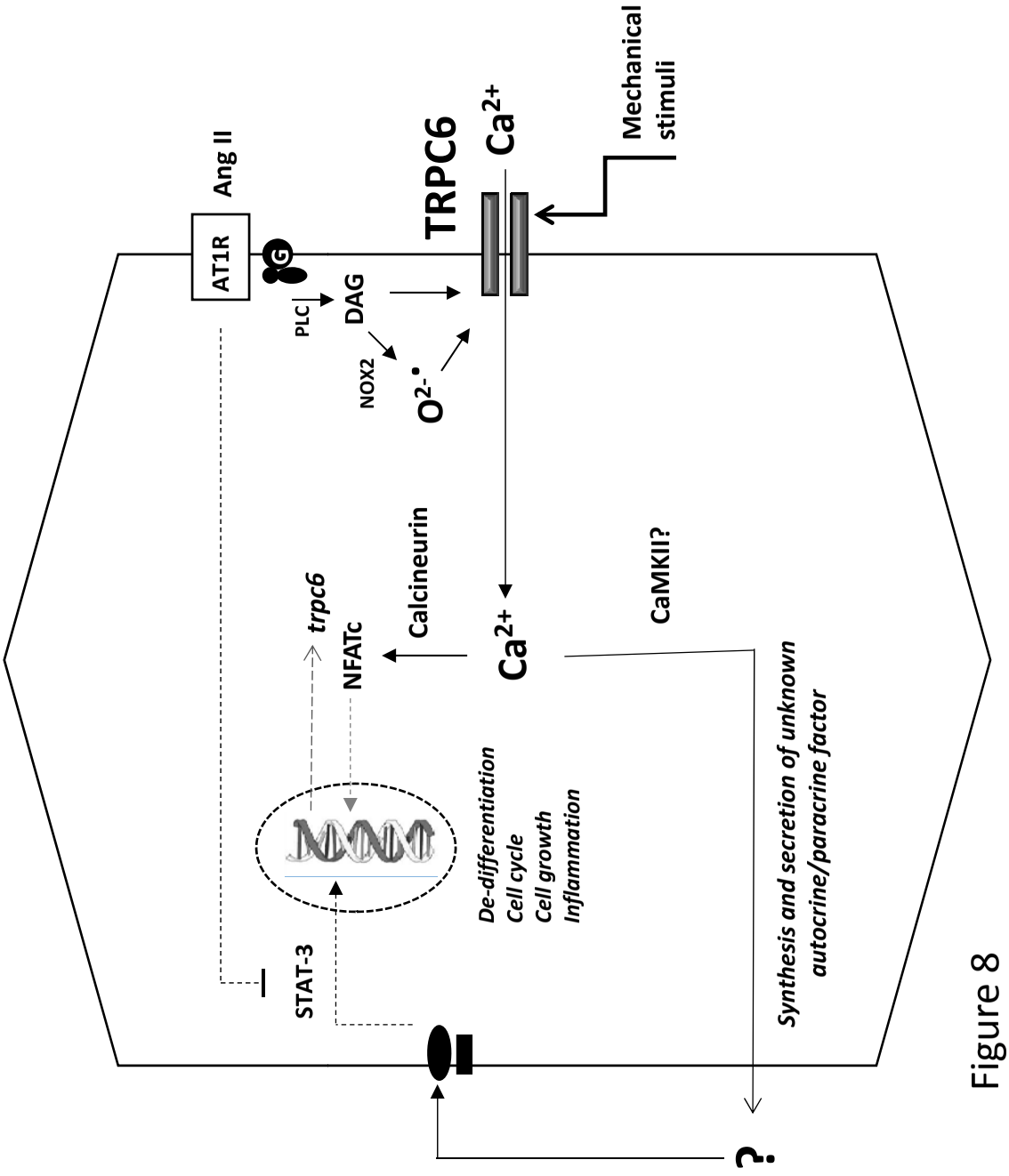

Figure 8