RESEARCH PAPER

Angiotensin II causes imbalance between pro- and anti-inflammatory cytokines by modulating GSK-3β in neuronal culture

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BACKGROUND AND PURPOSE

Emerging evidence indicates that the balance between pro-inflammatory cytokines (PICs) and anti-inflammatory cytokines (AICs) within the brain is an important determinant in the outcome of hypertension. However, the mechanism by which this dysregulation occurs is not known. We aimed to investigate whether AngII induces imbalance between PIC and AIC by modulating downstream transcription factors, NFκB and cyclic AMP response element-binding protein (CREB), and whether AngII-induced effects are mediated by glycogen synthase kinase-3β (GSK-3β).

EXPERIMENTAL APPROACH

CATH.a neurons were exposed to AngII (10 nM–1 μM) over a preset time course. In another set of experiments, GSK-3β was knock down by using lentivirus containing short hairpin RNA targeting GSK-3β (L-sh-GSK3β) before AngII exposure. Cell extracts were subjected to RT-PCR, immunoblot and immunoprecipitation.

KEY RESULTS

AngII caused time-dependent increase in PICs (TNF-α and IL-1β) and reduction in AIC (IL-10). AngII exposure caused reduced phosphorylated CREB(Ser-133) and increased p-NFκB(Ser-276) levels, leading to reduced CREB-CBP and increased NFκB-CBP binding. These results were accompanied by increased activation of GSK-3β, as indicated by increased p-GSK3(Tyr-216) to p-GSK3(Ser-9) ratio. In a subsequent study, pretreatment with L-sh-GSK3β attenuated AngII-induced alterations in PICs and IL-10 by augmenting CREB-CBP and attenuating NFκB-CBP binding.

CONCLUSIONS AND IMPLICATIONS

Collectively, these findings are the first to provide direct evidence that AngII-induced dysregulation in cytokines is mediated by GSK-3β-mediated alterations in downstream transcription factors in neuronal cells. Our data also reveal that AngII-induced effects could be alleviated by GSK-3β inhibition, suggesting GSK-3β as an important therapeutic target for hypertension that is characterized by increased PICs and NFκB activation.

Abbreviations

AIC, anti-inflammatory cytokines; AngII, angiotensin II; CBP, CREB-binding protein; CREB, cyclic AMP response element-binding protein; CVDs, cardiovascular diseases; GSK-3β, glycogen synthase kinase-3β; L-sh-GSK3β, lentivirus targeting GSK-3β; PIC, pro-inflammatory cytokines; RAS, renin-angiotensin system

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death in the United States, and of all the CVD conditions, hypertension has the highest prevalence. According to the most recent report from the American Heart Association, an estimated 76.4 million adults ≥20 years of age have high BP (Roger *et al.*, 2011). Despite the success of anti-hypertensive medications such as angiotensin converting enzyme inhibitors, angiotensin receptor blockers and β-adrenergic receptor blockers in reducing BP, the incidence and prevalence of hypertension continues to rise. These statistics clearly suggest the need for novel therapeutic strategies for the treatment of hypertension.

Inflammation is a well-known risk factor for various CVDs including hypertension (Agarwal *et al.*, 2011; Guggilam *et al.*, 2011). Pro-inflammatory cytokines (PICs), such as TNF-α (Dorffel *et al.*, 1999), IL-1β (Dorffel *et al.*, 1999; Peeters *et al.*, 2001) and IL-6 (Chae *et al.*, 2001; Peeters *et al.*, 2001), have been reported to increase with the severity of hypertension and to be of prognostic significance. Besides circulating cytokines, brain cytokines have also been implicated in the pathogenesis of the disease (Guggilam *et al.*, 2011; Kang *et al.* 2011). Recent discoveries indicate that in addition to elevated levels of circulating and brain PICs (Peeters *et al.*, 2001; Shi *et al.*, 2010), anti-inflammatory cytokines (AICs) such as IL-10 have a significant impact on arterial pressure and cardiac remodelling in experimental models of hypertension (Shi *et al.*, 2010). Additionally, an overactivation of the renin-angiotensin system (RAS) directly or indirectly through PIC plays a vital role in the pathogenesis of hypertension.

The most important transcription factors, viz., NFκB and cyclic AMP response element-binding protein (CREB), are known to play a central role in modulating the gene expression of inflammatory mediators involved in hypertension. However, unlike NFκB, which positively regulates gene expression of PICs (Kang *et al.* 2011), activation of CREB positively regulates expression of AICs such as IL-10 (Avni *et al.*, 2010). Competition between NFκB and CREB for binding to the co-activator CREB-binding protein (CBP) is important in regulating their transcriptional activity (Grimes and Jope, 2001a; Shenkar *et al.*, 2001). Although angiotensin II (AngII), a major effector molecule of RAS, has been shown to elevate PIC levels in the brain, the effects of overactivation of RAS on AICs are not very well understood. Also, the exact mechanisms underlying AngII-induced effects on inflammatory cytokines are still poorly understood.

Recently, glycogen synthase kinase (GSK)-3 has gained increasing attention from the scientific community due to its role in many biological processes. Research findings in the past several years have now established that GSK-3 acts as a regulatory switch that determines the output of numerous signalling pathways initiated by diverse stimuli (Frame and Cohen, 2001; Grimes and Jope, 2001b; Woodgett, 2001). Of the two isoforms (-α and -β), GSK-3β is particularly abundant in the CNS and is neuron-specific (Leroy and Brion, 1999). Recently, GSK-3β has been reported to modulate the production of inflammatory cytokines in an NFκB-dependent manner (Martin *et al.*, 2005; Steinbrecher *et al.*, 2005; Vines *et al.*, 2006; Beurel and Jope, 2009). However, the role of GSK-3β in AngII-induced dysregulation of inflammatory molecules within the brain has not yet been explored.

Therefore, the present series of studies were undertaken to investigate the novel role of GSK-3β in AngII-induced dysregulation of inflammatory cytokines in neuronal cells. We hypothesized that (i) AngII causes an imbalance between PIC and AIC; (ii) AngII-induced imbalance in PIC and AIC is modulated by downstream transcription factors, NFκB and CREB; and (iii) dysregulation in PIC and AIC are mediated by GSK-3β. To address these, we used CATH.a neurons (a hybridoma derived from mouse locus coeruleus), a catecholaminergic cell line that expresses AT1R and AT2R and has been identified as a reliable cell culture model for investigating AngII intra-neuronal signalling (Sun *et al.*, 2002; 2003; Li *et al.*, 2007). In this study, we constructed highly efficient lentiviral short hairpin RNA (shRNA) targeting GSK-3β to examine the role of GSK-3β in AngII-mediated effects. The results of this study will help us develop newer therapeutics targets for the treatment of hypertension.

Materials and methods

Neuronal cell culture

The CATH.a neurons (stock no. CRL-11179, American Type Culture Collection, Manassas, VA, USA) were grown in RPMI 1640 media containing 4% (v/v) FBS, 8% horse serum and 100 IU·mL^{-1} penicillin, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Unless otherwise stated, cells were plated at a density of 4×10^6 cells per 60 mm dish or 1×10^7 cells per 100 mm plate. All experiments were performed when the cultures were 70–80% confluent. Before treatment, the cells were allowed to differentiate in serum-free media for 48–72 h (h). To investigate the effects of AngII on inflammatory cytokines, CATH.a cells were exposed to AngII or vehicle. We first performed a pilot experiment to validate the best concentration and time point for AngII stimulation of CATH.a cells, using a concentration range from 10 nM to 1 μM of AngII in culture medium over a stipulated time course (0–24 h). At a defined time point, cells were harvested for real-time RT-PCR for mRNA analysis of TNF-α, we chose TNF-α mRNA levels as the end point because of the focus of the present study. As depicted in Figure 1, we observed that 100 nM is the lowest concentration of AngII that exerts maximum effect on TNF-α mRNA expression in CATH.a neurons. AngII at 1 μM did not cause any additional increase in TNF-α expression and the lowest dose (10 nM) did not produce sufficient increase. The optimal AngII concentration of 100 nM has also been utilized in several previous studies investigating the intra-neuronal AngII signalling in CATH.a cells (Sun *et al.*, 2002; Zimmerman *et al.*, 2004; Mitra *et al.*, 2010; Haack *et al.*, 2012). In all subsequent experiments, cells were exposed to AngII (100 nM) for 6 h, a time point that induces maximal changes in the levels of inflammatory cytokines (Figure 3). In another set of experiments, cells were transfected with lentiviral short hairpin RNA targeting GSK-3β (L-sh-GSK3β) for 48 h before AngII exposure for 6 h. Following exposure to agonists, cells were harvested for real-time RT-PCR, Western blot, immunoprecipitation (IP) and immunofluorescence analysis. Results are presented as the
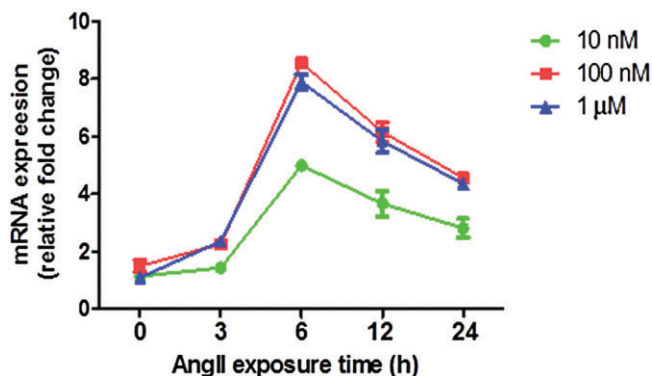
Figure 1

Dose–response relationship between AngII concentration in the culture media and mRNA expression of TNF-α in whole cell lysate in CATH.a cell culture. A pilot experiment was performed to validate the best concentration and time-point for Ang II stimulation of CATH.a cells, using a concentration range from 10 nM to 1 μM of Ang II in culture medium over a stipulated time course (0–24 h). Results showed that 100 nM (at 6 h) is the lowest concentration of Ang II that exerts maximum effect on TNF-α mRNA expression in CATH.a neurons. The results are means ± SD of three independent experiments ($n = 6$ per treatment groups in each experiment).

means ± SD and represent set of three independent experiments in CATH.a cells. In each experiment, $n = 6$ per treatment groups were used.

Lentiviral construction and transduction

We explored the effects of inhibition of GSK-3β by using gene knock-down approach: RNA interference (RNAi) through delivery of a small hairpin RNA (shRNA) against GSK-3β using a lentiviral vector (L-sh-GSK3β) containing the target sequence 5′-CATGAAAGTTAGCAGAGATAA-3′. L-sh-GSK3β was commercially obtained (NitAn Biotech LLC, Columbus, OH, USA) and these vectors were tagged with eGFP. A scrambled sequence of the same length was used as a control (mentioned as L-scrambled in text). Twenty-four hours after plating, CATH.a cells were transduced (in triplicate) separately in 6-well laminin coated plates with 30 MOI (multiplicity of infection, which is equal to ratio of infectious viral particles to cell) of L-sh-GSK3β and scrambled sequence (L-scrambled) viral particles in the presence of 8 μg·mL⁻¹ of polybrene. We use 2 mL of viral supernatant, which contain 2×10^7–10^8 viral particles for each transduction experiment. After 48 h, Western blotting was performed to assess the silencing effects of L-sh-GSK3β. Cells were stimulated with AngII 48 h after transduction. Cells were also transduced with L-scrambled separately in presence of AngII. Densitometric analysis of immunoblot showed that cells transduced with L-sh-GSK3β (MOI 30) had significantly lower (more than 60% reduction) protein expression of GSK-3β when compared to cells transduced with scrambled sequence (Figure 2). These results confirmed efficient suppression of GSK-3β by L-sh-GSK3β in neuronal cells.

RNA extraction and real-time RT-PCR

Semi-quantitative real-time RT-PCR was used to determine the mRNA levels of TNF-α, IL-1β and IL-10 in CATH.a
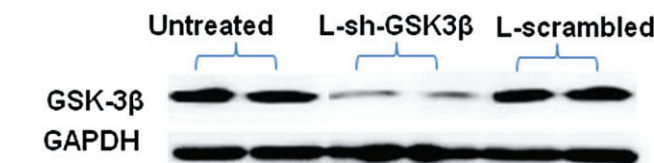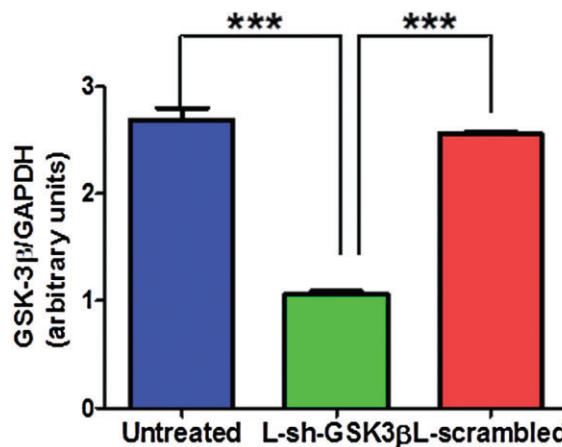
Figure 2

Transduction efficiency of lentiviral shRNA targeting GSK3β (L-sh-GSK3β) in CATH.a cells. Serum-starved CATH.a cells were transduced with L-sh-GSK3β at a multiplicity of infection (MOI) of 30 for 48 h. An immunoblot analysis (upper panel) and a representative blot (lower panel) showing efficient suppression of GSK-3β protein expression by shRNA. The results are means ± SD of three independent experiments ($n = 6$ per treatment groups in each experiment). ***$P < 0.001$.

neurons by using specific primers (Table 1). Total RNA isolation, cDNA synthesis and RT-PCR were performed as previously described (Agarwal et al., 2009). Semi-log amplification curves were evaluated by the comparative quantification method ($2^{-\Delta\Delta Ct}$), and GAPDH was used for normalization of all reported gene expression levels. The data are presented as the fold change of the gene of interest relative to that of control group.

Immunoblot analysis

For whole cell extracts, cells were washed twice with ice-cold PBS and were scraped into in100 μL per dish of cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing protease and phosphatase inhibitors. Samples were incubated on ice for 10 min and then centrifuged (10 000× g, 5 min, 4°C). The supernatants were retained. Protein concentrations were determined by the Bradford method (Bradford, 1976). The lysates were stored at –80°C until used for immunoblotting.

Cell lysates were mixed with Laemmli sample buffer (Bio-Rad Laboratories, Berkeley, CA, USA) and placed in a boiling water bath for 5 min. Proteins (30 μg) were separated by SDS-PAGE using 10–15% (w/v) resolving gels and 6% (w/v) stacking gels, and then transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 1% (w/v) casein

Table 1

Primer sequences used for PCR and amplified product sizes

Gene	Primer	Sequence (5′ to 3′)	Size (bp)
GAPDH	Forward	TGAATGACATCAAGAAGGTGGTGGAG	239
	Reverse	TCCTTGGAGGCCATGTAGGCCAT	
IL-1β	Forward	CTGTGTCTTTCCCGTGGACC	200
	Reverse	CAGCTCATATGGGTCCGACA	
IL-10	Forward	CCAGTTTTACCTGGTAGAAGTGATG	324
	Reverse	TGTCTAGGTCCTGGAGTCCAGCAGACTC	
TNF-α	Forward	CCTCCCTCTCATCAGTTCTA	501
	Reverse	GCAATGACTCTAAAGTAGACCTG	

(for non-phosphorylated antibodies) in PBS or 1% (w/v) BSA (for phosphorylated antibodies) in TBST [20 mM Tris–HCl (pH 7.5), 137 mM NaCl, 0.1% (v/v) Tween 20]. Blots were probed (overnight, 4°C) with the primary antibodies. Specific antibodies used included TNF-α, IL-1β, IL-10, GSK-3β, p-GSK3β(Ser-9), p-GSK3β(Tyr-216), p-CREB(Ser-133) and CBP at 1:1000 dilution. Antibodies were commercially obtained: TNF-α (Abcam Inc., Cambridge, MA, USA); IL-1β (AbD Serotec, Oxon, UK), IL-10 (Abbiotec, San Diego, CA, USA); GSK-3β (BD Transduction Laboratories, San Jose, CA, USA), p-GSK3β(Ser-9), p-GSK3β(Tyr-216), p-CREB(Ser-133) and p-p65(Ser-276) (Cell Signaling Technology, Inc.); and CBP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed in TBST, incubated (60 min, room temperature) with HRP-conjugated secondary antibodies (1:10 000) in blocking solution. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL Plus, Amersham, Piscataway, NJ, USA);, band intensities were quantified using Versa-Doc MP 5000 imaging system (Bio-Rad Laboratories) and were normalized with GAPDH.

Immunoprecipitation

To determine the role of GSK-3β in regulating downstream transcription factors, we used the catch and release IP system (Upstate Biotechnology, Billerica, MA, USA) as described previously (Martin *et al.*, 2005). For these experiments, protein CBP was immunoprecipitated by incubating cell lysates with 2 μg of CBP monoclonal antibody (Pharmingen, San Diego, CA, USA) overnight at 4°C. Samples were incubated with 60 μL of protein G sepharose beads (Amersham) for 1 h at 4°C with gentle agitation. The immune complexes were washed three times with lysis buffer. Samples in Laemmli buffer were placed in a boiling water bath, proteins were separated by SDS-PAGE, and samples were immunoblotted with anti-p-CREB(Ser-133) or anti-p-p65(Ser-276). The membranes were re-probed with an anti-CBP antibody to confirm the efficiency and specificity of IP.

Statistical analysis

Statistical analysis was completed by either unpaired *t*-test or one-way ANOVA with Bonferroni post hoc test using Graph Pad Prism software (version 5.0) (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the fold change of each gene of interest relative to controls. Results were considered significant when $P < 0.05$.

Results

AngII causes an imbalance between pro- and anti-inflammatory cytokines in neuronal cells

To investigate the influence of AngII on PICs and AIC in the neuronal cells, CATH.a cells were exposed to AngII (100 nM) for indicated time and then we examined the mRNA (Figure 3A) and protein (Figure 3B) levels of TNF-α, IL-1β, and IL-10 in whole cell extracts. We observed that AngII-treated cells exhibited time-dependent increase in TNF-α and IL-1β levels with maximal effects at 6 h of exposure. At mRNA level, AngII exposure (6 h) resulted in eightfold increase in TNF-α and about fourfold increase in IL-1β expression in CATH.a neurons (Figure 3A). TNF-α and IL-1β levels in AngII-exposed cells were reduced after 12 and 24 h of exposure when compared with 6 h; however, it remains elevated in comparison with vehicle-treated cells. On the contrary, IL-10 levels in cells treated with AngII for 6 h were significantly lower when compared to vehicle-treated cells. At mRNA level, AngII exposure (6 h) resulted in more than twofold decrease in IL-10 expression (Figure 3A). At 12 and 24 h, IL-10 levels remained lower in comparison with vehicle groups, although the differences were not significant. Noteworthy, AngII exposure for 1 h significantly up-regulated IL-10 levels, whereas TNF-α level was slightly higher at this time point.

To further confirm that AngII causes an imbalance between PIC and AIC in neuronal cells, we determined the alterations in ratio of TNF-α to IL-10 protein levels in cells treated with AngII or vehicle. A significant increase of more than sixfold in TNF-α /IL-10 protein ratio was observed upon 6 h of AngII exposure when compared to all other groups (Figure 3B). These data provide evidence that AngII exposure results in an imbalance between PIC and AIC in favour of PICs in CATH.a neurons.

AngII induces activation of GSK-3β in neuronal cells

To investigate whether AngII exposure for 6 h (maximal effective exposure time) activates GSK-3β, we determined the
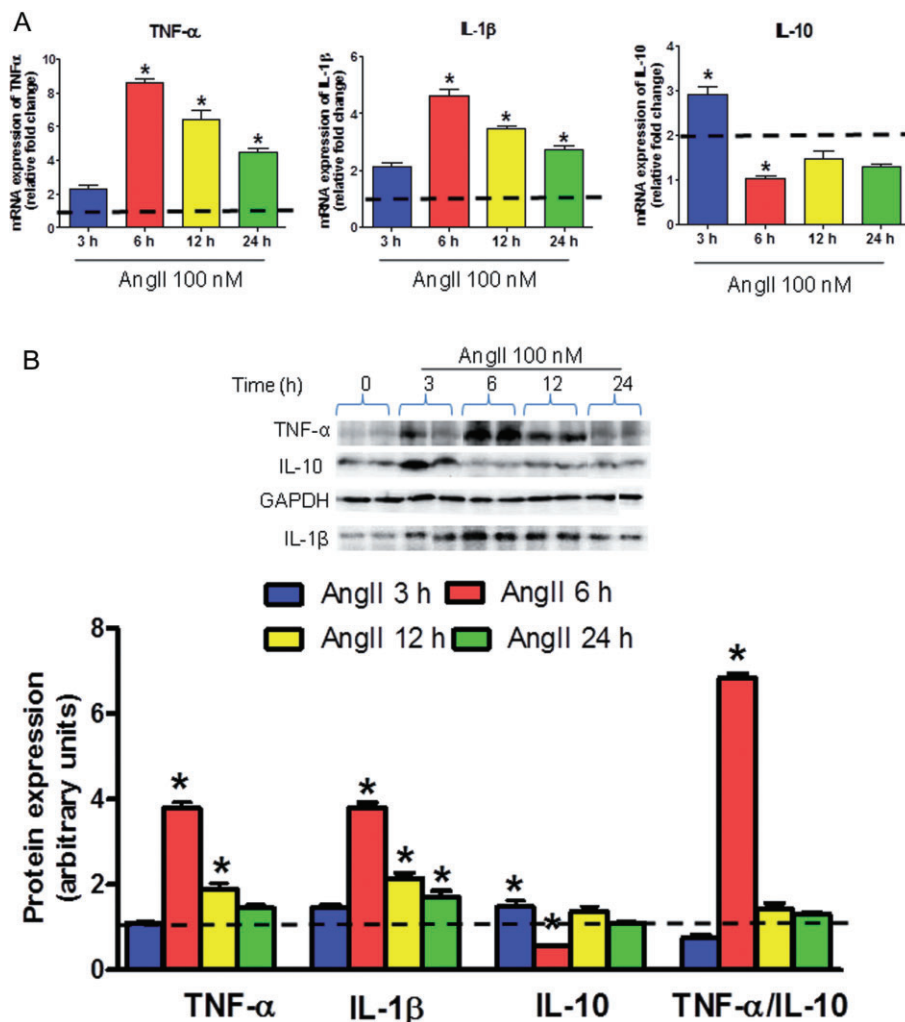
Figure 3

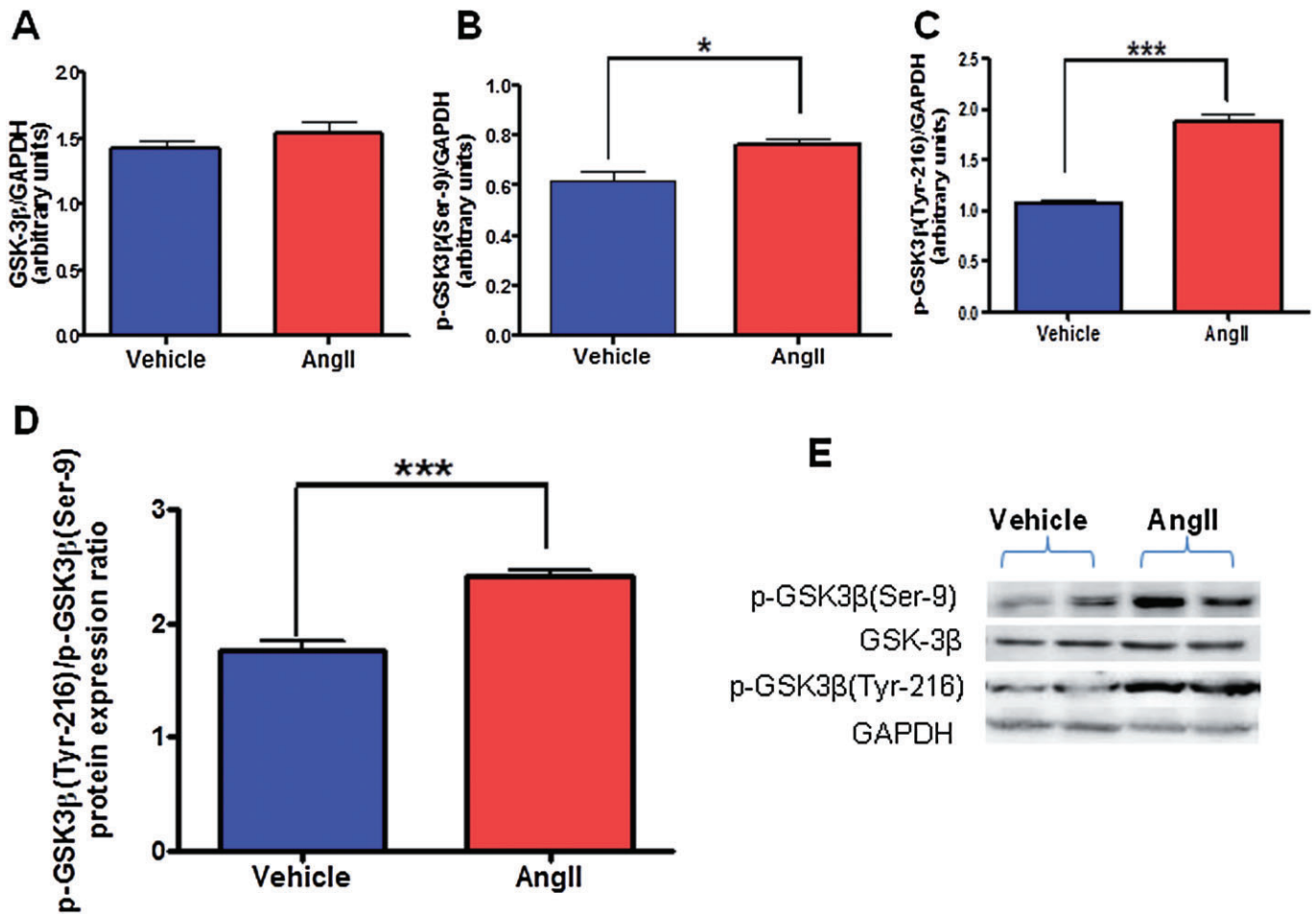Effects of AngII treatment on TNF-α, IL-1β and IL-10 expression levels in neuronal cells. Serum-starved CATH.a cells were stimulated with 100 nM AngII for the indicated time. (A) mRNA expression of TNF-α, IL-1β and IL-10. (B) A representative Western blot and densitometric analysis of protein expression of TNF-α, IL-1β and IL-10. Quantitative Western blot analysis is shown as the ratio of intensities of the protein of interest and GAPDH, relative to unstimulated control cells (represented by the dashed line). AngII resulted in increased TNF-α and IL-1β, and reduced IL-10 levels indicating an imbalance between PIC and AIC in the CATH.a cells in time-dependent manner with maximum alterations at 6 h of AngII treatment. The results are means ± SD of three independent experiments ($n = 6$ per treatment groups in each experiment). *$P < 0.05$ compared to their respective vehicle-treated groups.

protein expression levels of p-GSK3β(Ser-9) and p-GSK3β(Tyr-216) by immunoblot analysis in neuronal cells exposed with AngII or vehicle. The activity of GSK-3β is mainly regulated by post-translational phosphorylation with N-terminal phosphorylation of GSK-3β at Ser-9 has an inhibitory effect, whereas phosphorylation of Tyr-216 activates it (Forde and Dale, 2007). Our immunoblot analysis demonstrated that GSK-3β are expressed in CATH.a neurons and there was a slight but significant increase in the phosphorylation of GSK3β(Ser-9) (Figure 4B; quantitation in Figure 4E). Simultaneously, however, AngII dramatically up-regulated phosphorylation of GSK3β(Tyr-216), in CATH.a cells (Figure 4C; quantitation in Figure 4E). Densitometric analysis further revealed that the ratio of protein expression of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) is significantly up-

regulated on AngII exposure (Figure 4D). As phosphorylation of GSK3β(Tyr-216) is essential for their catalytic activity, these results indicate overall activation of GSK-3β upon AngII (100 nM) exposure. The same blots were stripped and re-probed for native GSK-3β showing no significant difference on native GSK-3β expression between the vehicle and AngII-treated groups (Figure 4A). These findings suggest that AngII-induced effects in neuronal cells could be mediated by activation of GSK-3β.

AngII exposure resulted in altered binding of CBP with CREB and NFκB in neuronal cells

To investigate whether AngII induced imbalance in PIC and AIC is mediated by alterations in downstream transcription factors NFκB subunit p65 and CREB, we assessed the binding
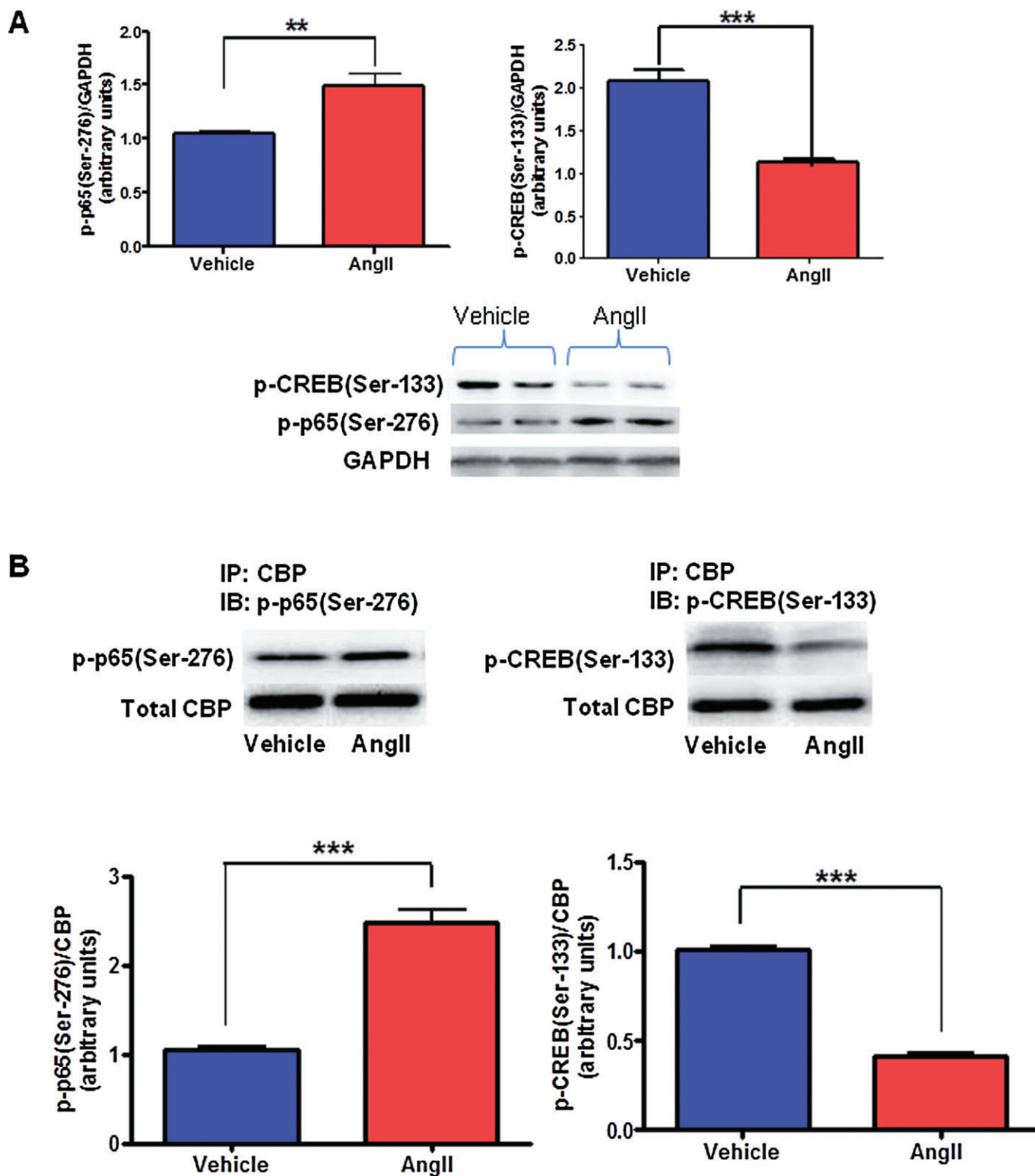
Figure 4

Effects of AngII treatment on total and phosphorylated GSK-3β expression in neuronal cells. Serum-starved CATH.a cells were stimulated with 100 nM AngII for 6 h and cell extracts were then subjected to protein analysis by Western blot. Densitometric analysis of Western blot results showing protein expression of (A) total GSK-3β, (B) p-GSK3β(Ser-9), (C) p-GSK3β(Tyr-216), (D) p-GSK3β(Tyr-216)/p-GSK3β(Ser-9) ratio, and (E) a representative Western blot. AngII caused significant activation of GSK-3β as indicated by reduced p-GSK3β(Ser-9), increased p-GSK3β(Tyr-216), and increased ratio of p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) protein expression in CATH.a neurons. AngII exposure did not alter total GSK-3β protein levels. The results are means ± SD of three independent experiments. *$P < 0.05$; ***$P < 0.001$ compared with cells treated with vehicle.

of CBP (co-activator protein) with p65 and CREB by IP analysis of vehicle- and AngII-treated groups. As phosphorylation of CREB at Ser-133 and p65 at Ser-276 have been shown to be essential for their binding with CBP, we also determined the protein levels of p-CREB(Ser-133) and p-p65(Ser-276). AngII exposure resulted in significant reduction in p-CREB(Ser-133) expression and increased p-p65(Ser-276) (Figure 5A), leading to decreased CREB-CBP binding and increased NFκB-CBP binding as confirmed by IP analysis. As demonstrated in Figure 5B, in rat neuronal cells, binding between CBP and p65 has been increased and binding between CBP and CREB has been decreased, as reflected by increased presence of p-p65(Ser-276) and decreased presence of p-CREB(Ser-133) in CBP immunoprecipitates of AngII-exposed cells when compared to cells treated with vehicle. These data suggest that AngII causes an imbalance in cytokine levels by modulating the downstream transcription factors.

Inhibition of GSK-3β by lentivirus reversed AngII-mediated imbalance in PIC and AIC in neuronal cells

As shown in Figure 6, AngII-exposed cells had significantly increased levels of TNF-α and IL-1β, and decreased levels of IL-10 when compared to vehicle-treated cells. Interestingly, pretreatment of cells with L-sh-GSK3β resulted in significant reduction in mRNA and protein levels of TNF-α and IL-1β when compared to AngII-treated cells. In addition, IL-10 mRNA and protein levels were significantly higher in AngII + L-sh-GSK3β compared with AngII groups. There were no significant differences between vehicle-treated and L-sh-GSK3β + AngII-treated groups. Furthermore, densitometric analysis showed that TNF-α /IL-10 protein ratio was significantly higher in AngII groups in comparison with vehicle, whereas significant reduction in TNF-α /IL-10 ratio was

A

B

Figure 5

Effects of AngII treatment on CBP to NFκB and CREB binding in neuronal cells. The serum-starved (24 h) CATH.a cells were treated without or with AngII (100 nM; 6 h). (A) Densitometric analysis and a representative immunoblot showing increased expression levels of p-p65(Ser-276) and decreased p-CREB(Ser-133) in AngII-treated cells when compared to vehicle. (B) Densitometric analysis and a representative immunoblot showing increased CBP to p-p65(Ser-276) binding and decreased CBP to p-CREB(Ser-133) binding in AngII stimulated cells as measured by immunoprecipitation analysis. The results are means $\pm$ SD of three independent experiments. $**P < 0.01$; $**P < 0.01$, compared with cells treated with vehicle.
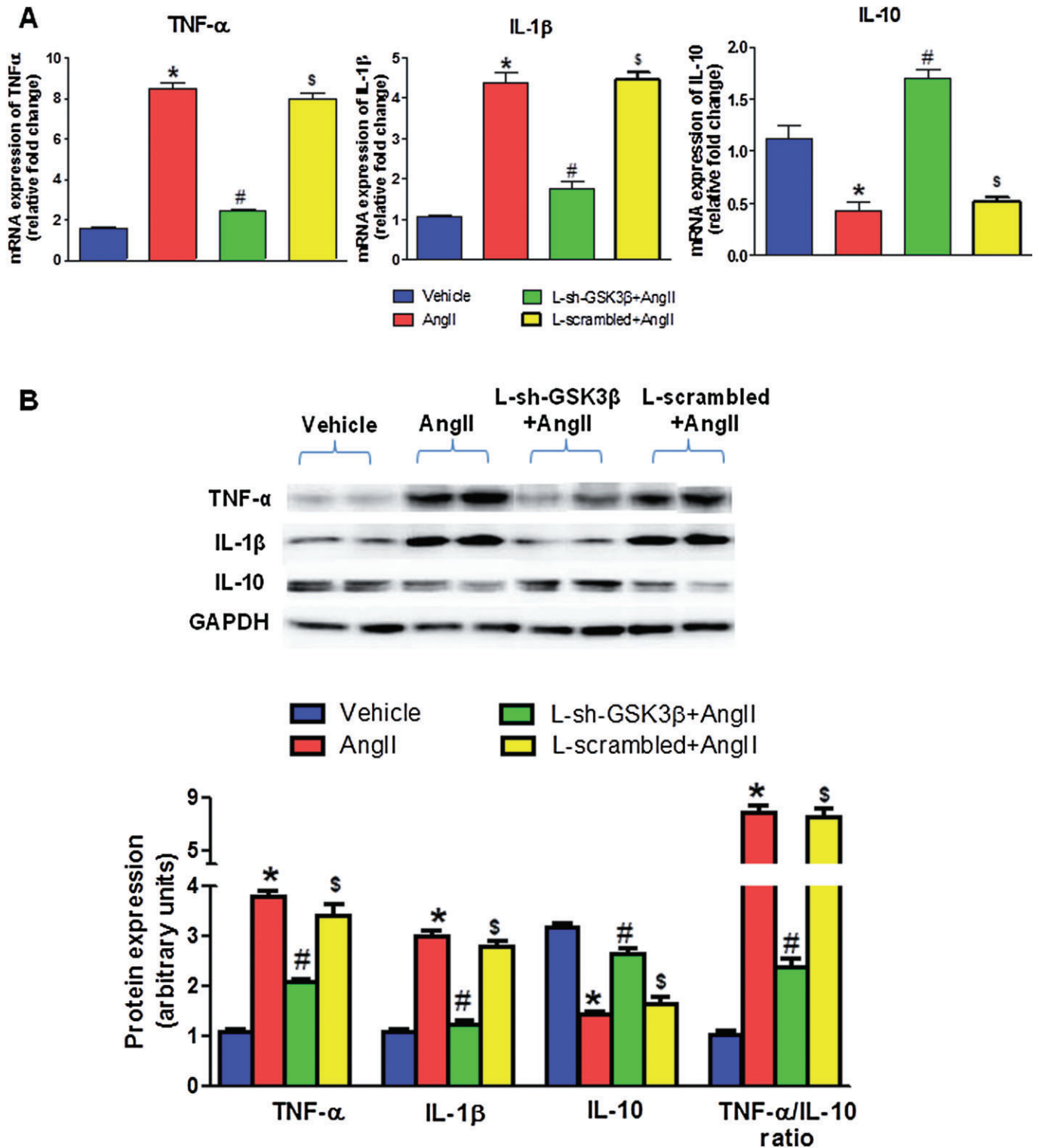
Figure 6

Inhibitory effects of L-sh-GSK3β on AngII-induced imbalance between PIC and AIC in neuronal cells. Serum-starved CATH.a cells were transduced with L-sh-GSK3β at a multiplicity of infection (MOI) of 30 for 48 h and were stimulated with 100 nM AngII for 6 h. (A) mRNA expression of TNF-α, IL-1β and IL-10. (B) Densitometric analysis and a representative immunoblot showing protein expression of TNF-α, IL-1β and IL-10. Pretreatment with L-sh-GSK3β resulted in reversal of AngII-induced increase in TNF-α and IL-1β, and attenuation in IL-10 levels indicating improved balance between PIC and AIC by GSK-3β inhibition. Cells transduced with scrambled sequences separately in presence of AngII did not show any effect. The results are means ± SD of three independent experiments. *$P < 0.05$ AngII versus vehicle-treated cells; #$P < 0.05$ AngII versus L-sh-GSK3β + AngII-treated cells; $P < 0.05$ L-scrambled + AngII versus vehicle-treated cells.
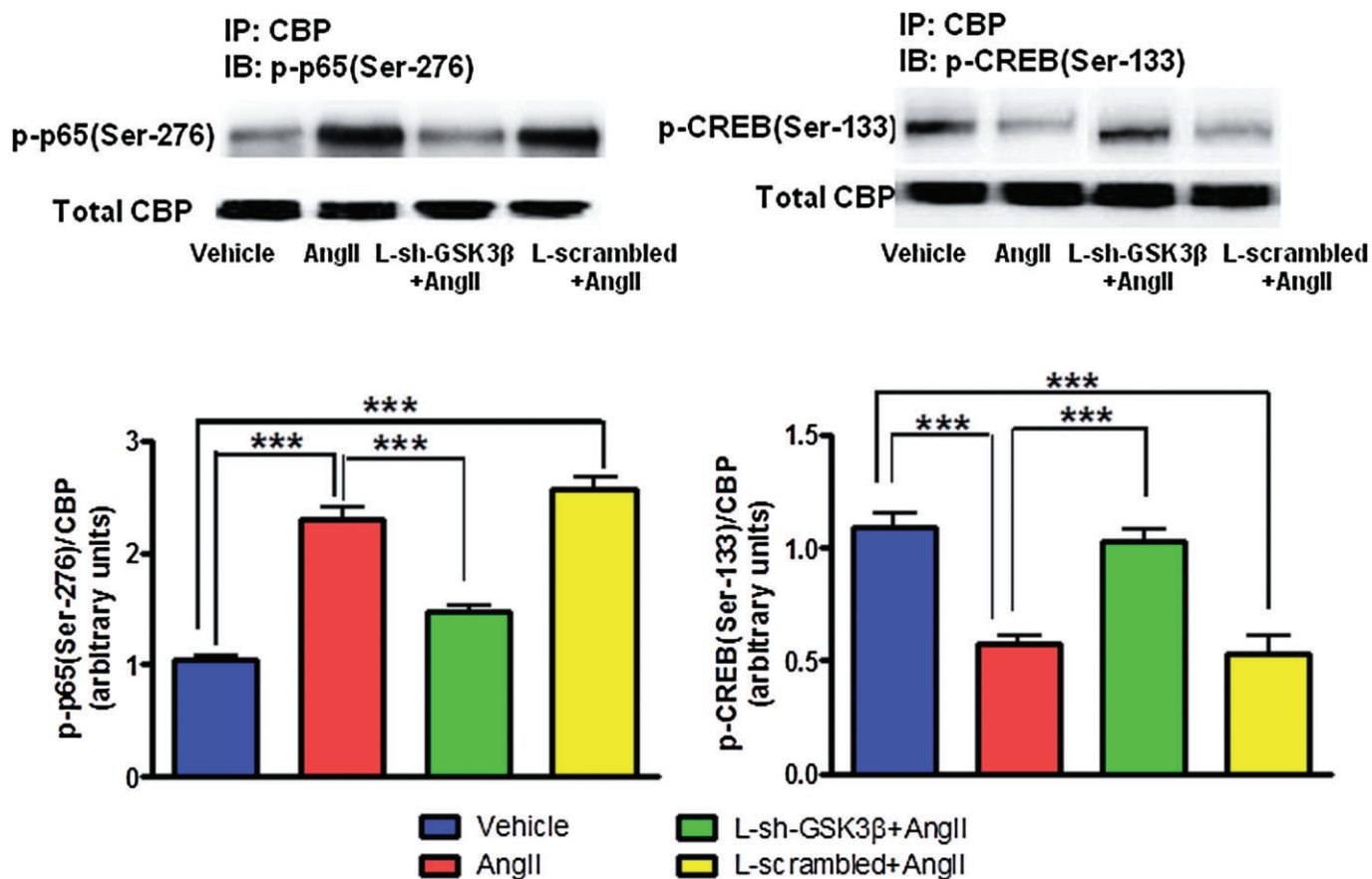
Figure 7
Effects of GSK-3β knock down by lentiviral shRNA (L-sh-GSK3β) on AngII induced alterations in transcription factors in neuronal cells. Serum starved CATH.a cells were transduced with L-sh-GSK3β at a multiplicity of infection (MOI) of 30 for 48 h and were stimulated with 100 nM AngII for 6 h. Cell extracts were then immunoprecipitated with CBP and immunoblotted either with p-p65(Ser-276) or p-CREB(Ser-133). An immunoblot (upper panel) and densitometric analysis (lower panel) showing reversal of AngII-induced increased CBP to p-p65(Ser-276) and reduced CBP to p-CREB(Ser-133) binding in neuronal cells. Cells were also transduced with scrambled sequences in presence of AngII did not show any effect. The results are means ± SD of three independent experiments. ***$P < 0.001$.

observed in L-sh-GSK3β +AngII when compared to AngII-treated cells. Cells transduced with scrambled sequences (L-scrambled) in presence of AngII did not show any effect and there was no difference in these levels between AngII and L-scrambled + AngII-exposed cells. These results demonstrate that pretreatment of cells of lentiviral silencing GSK-3β causes reversal of AngII-induced imbalance between PIC and AIC in neuronal cells.

Inhibition of GSK-3β by lentivirus reversed AngII-mediated altered binding of CBP to NFκB or CREB in neuronal cells

As shown in Figure 7, AngII exposure resulted in increased CBP to p65 binding and decreased CBP to CREB binding in CATH.a cells. Interestingly, pretreatment of cells with L-sh-GSK3β caused significant reduction in AngII-induced elevation in CBP to p65 binding. In addition, CBP-CREB binding was found to be significantly higher in L-sh-GSK3β + AngII group when compared to AngII-exposed cells. Furthermore, L-sh-GSK3β + AngII-treated cells exhibited significantly

elevated levels of p-CREB(Ser-133) and reduced levels of p-p65(Ser-276), in comparison with AngII group. There were no significant differences between vehicle-treated and L-sh-GSK3β + AngII-treated groups. Moreover, cells transduced with L-scrambled did not affect AngII-induced changes in CBP to NFκB or CREB binding. These results indicate that AngII-induced imbalance in cytokine levels and transcription factors are mediated by GSK-3β in neuronal cells.

Discussion

The aim of the present study was to investigate the underlying molecular mechanisms by which AngII causes an imbalance between PIC and AIC, and to elucidate the role of GSK-3β in mediating this dysregulation. Three novel findings emerge from this study. First, AngII causes an imbalance between PIC and AIC in neuronal culture by up-regulating binding of CBP to NFκB and down-regulating binding of CBP to CREB. These data explain the increased NFκB-mediated
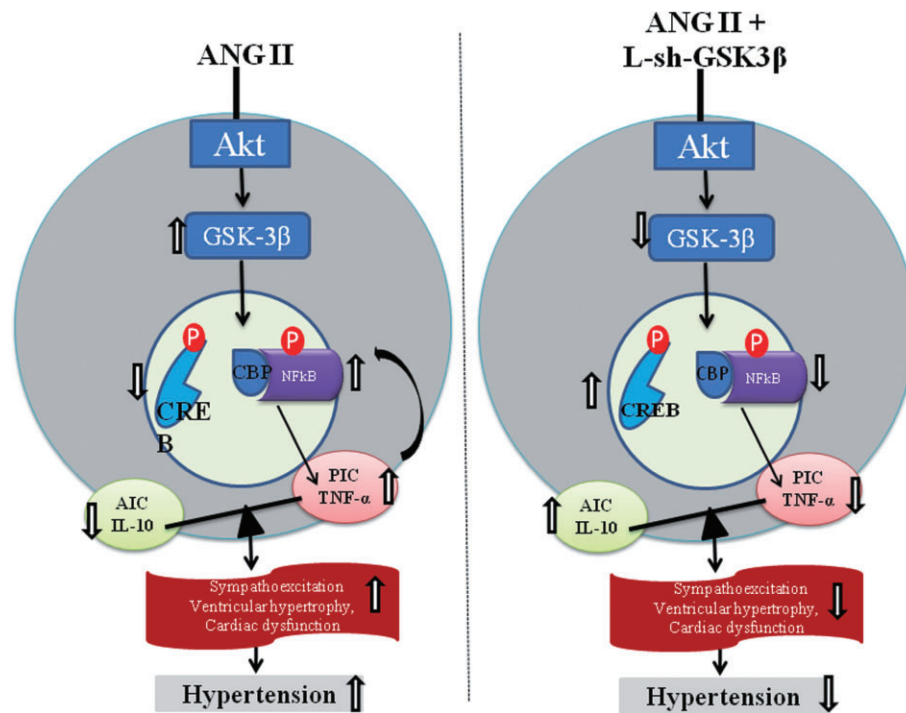
Figure 8

A schematic depicting the proposed pathways (right panel) of AngII-induced dysregulation in inflammatory cytokines in neuronal cells and showing the mechanisms (left panel) by which neuronal cytokines contributes to the pathogenesis of hypertension. It has become clear from the past several years of research that an increased production of PICs in response to overactivated RAS within the cardiovascular regulatory centres of the brain (such as paraventricular nucleus and rostral ventrolateral medulla) causes increased sympathetic outflow leading to increased arterial pressure and cardiac remodelling in experimental models of hypertension. At the cellular level, PICs activate reactive oxygen species which in turn can activate various intracellular signalling pathways, including that of NFκB. Activation of NFκB induces gene transcription of PICs fostering a positive feedback mechanism, and eventually leading to the progression of hypertension. A step further, the results of the present study revealed a novel molecular mechanism that AngII-induced increased phosphorylation of GSK-3β(Tyr-216) and increased p-GSK3β(Tyr-216) to p-GSK-3β(Ser-9) ratio leads to altered activity of downstream transcription factors, NFκB and CREB, in favour of NFκB-mediated gene transcription, thereby, causing an imbalance between PIC and AIC in rat neuronal cells.

transcription of PIC and decreased CREB-mediated transcription of IL-10 on AngII stimulation. Second, AngII causes significantly increased phosphorylation of GSK-3β at Tyr-216 and increased p-GSK3β(Tyr-216) to p-GSK3β(Ser-9) ratio, indicating increased activation of GSK-3β in neuronal cells. Finally, AngII-induced effects in neuronal cells were reversed by lentiviral-mediated silencing of GSK-3β, suggesting that AngII-induced effects are indeed mediated by GSK-3β in neurons. The results of this study reveal a novel molecular mechanism that AngII-induced increased activation of GSK-3β leads to altered activity of downstream transcription factors, NFκB and CREB, in favour of NFκB-mediated gene transcription, thereby causing an imbalance between PIC and AIC in CATH.a neurons (Figure 8). Our data also suggest that AngII-induced effects could be alleviated by GSK-3β inhibition indicating GSK-3β as potential therapeutic target in various CVDs, particularly hypertension as it is characterized by increased PICs and NFκB activation.

Chronic low-grade inflammation is one of the hallmarks of hypertension. PICs, such as TNF-α (Dorffel *et al.*, 1999), IL-1β (Dorffel *et al.*, 1999; Peeters *et al.*, 2001) and IL-6 (Chae *et al.*, 2001; Peeters *et al.*, 2001), have been reported to increase with the severity of hypertension and to be of prog-

nostic significance. Circulating and brain cytokines have both been implicated in the pathogenesis of hypertension. However, emerging evidence indicates that it is not only the PIC (Peeters *et al.*, 2001; Shi *et al.*, 2010) but the balance between pro- and anti-inflammatory cytokines that determines the outcome of the disease, and that these PICs can cross-talk with components of the RAS during the hypertensive response. In the present study, we observed that AngII exposure resulted in upregulation of TNF-α and IL-1β expression in a time-dependent manner in CATH.a neurons with maximal effects at 6 h after AngII exposure. Our results are consistent with those of previous studies from our laboratory which have shown that infusion of AngII in the paraventricular nucleus (PVN), an important cardiovascular regulatory centre in the brain, increases production of TNF-α and IL-1β in rats (Cardinale *et al.*, 2012). Although most of these previous studies have examined the effects of AngII on brain PICs, the effects of overactivation of RAS on anti-inflammatory cytokines are not well understood. In the present study, we observed a significant reduction in IL-10 levels by AngII exposure. Furthermore, the ratio of TNF-α/IL-10 protein expression was found to be dramatically up-regulated in AngII-treated cells. Also, we observed an initial increase in

IL-10 mRNA level at 1 h of AngII exposure which could be due to compensatory and protective response to initial increase in TNF-α. Taken together, these results suggest that, at the cellular level, alterations in RAS components not only increases PIC but also causes an imbalance between PIC and AIC in favour of PIC. These results raise another question: what are the exact mechanisms by which alterations in RAS components cause this dysregulation?

Accumulating evidence has suggested that the NFκB-signalling pathway is activated by AngII via the G-protein coupled angiotensin type I receptor (Wolf and Wenzel, 2004). In the unstimulated cells, NFκB is sequestered in the cytoplasm as an inactive complex with inhibitors of NFκB (IκB) (Ghosh and Karin, 2002). Upon stimulation by some inducers such as AngII, IκB is phosphorylated and degraded, leading to translocation of the p65 subunit of NFκB into the nucleus where it activates gene transcription of TNF-α and IL-1β. In addition to the nuclear translocation of NFκB, its transcriptional activity is regulated by a co-activator CREB-binding protein (CBP) that associates with the C-terminal transactivation domain of p65 (Takahashi et al., 2002). Phosphorylation of p65 at Ser-276 has been shown to be required for recruitment of the CBP and transcriptional activity. Besides NFκB, another transcription factor, CREB, has been shown to be involved in the pathogenesis of hypertension. CREB is a 43 kDa phosphoprotein that positively regulates expression of anti-inflammatory cytokines such as IL-10 (Avni et al., 2010). Although activity of CREB is regulated by complex phosphorylation mechanisms that are not yet completely understood, phosphorylation of CREB at Ser-133 has been shown to be required for recruitment of the CBP and transcriptional activity (Chrivia et al., 1993). Due to limited availability of CBP in the nucleus, competition for CBP by diverse transcription factors is inevitable (Yang et al., 2010). As NFκB and CREB are the key transcription factors in the production of cytokines, it is plausible to investigate whether AngII-induced dysregulation in PIC and AIC is mediated by them. In the present study, we observed that AngII exposure resulted in increased phosphorylation of p65 at Ser-276 and reduced phosphorylation of CREB at Ser-133. Moreover, our IP analysis showed that CBP to NFκB binding was increased in AngII (6 h) exposed neuronal cells, whereas CBP to CREB binding was reduced. These results were also associated with elevated levels of TNF-α and IL-1β, and reduced IL-10 levels in AngII (6 h) exposed cells. Collectively, these results suggest that alterations in phosphorylation status of NFκB and CREB by AngII lead to their altered binding with co-activator CBP which in turn leads to an imbalance between PIC and AIC production.

Recently, glycogen synthase kinase-3 (GSK-3), an enzyme that was originally discovered for its role in insulin-mediated glycogen metabolism (Embi et al., 1980; Rylatt et al., 1980; Woodgett and Cohen, 1984; Hughes et al., 1993; Ali et al., 2001; Woodgett, 2001; Doble and Woodgett, 2003), has now been shown to regulate the activity of several metabolic, signalling and structural proteins (Frame and Cohen, 2001; Woodgett, 2001; MacAulay and Woodgett, 2008). Not only the activity of GSK3 is regulated by its post-translational phosphorylation, it itself phosphorylates a broad range of substrates and thereby regulates their function (Frame and Cohen, 2001; Woodgett, 2001). Among the signalling pro-

teins regulated by GSK-3β are many transcription factors including CREB and NFκB (Plyte et al., 1992; Grimes and Jope, 2001b). Therefore, we postulated that AngII-induced alterations in phosphorylation status of NFκB and CREB as observed in the present study could be mediated by GSK-3β. To investigate this hypothesis, we first examined whether AngII perhaps had any effect on GSK-3β expression. Interestingly, our immunoblot analysis showed that AngII exposure resulted in a slight increase in p-GSK3β(Ser-9) levels in neuronal cells, at first sight suggesting inactivation of GSK-3β. Surprisingly, however, p-GSK3β(Tyr-216) levels were found to be significantly elevated in AngII-treated cells. Moreover, the ratio of p-GSK3β(Tyr-216) to p-GSK3β (Ser-9) was higher in AngII-exposed cells when compared to vehicle-treated cells. As N-terminal phosphorylation of GSK-3β at Ser-9 has an inhibitory effect, whereas phosphorylation of Tyr-216 activates it (Forde and Dale, 2007), these results clearly suggested activation of GSK-3β upon AngII exposure. Although the role of GSK-3β in CVDs is now becoming a major focus of the scientific community, to the best of our knowledge none of the previous studies have explored the effects of AngII, a key mediator of most of the CVDs, on neuronal GSK-3β. Additionally, most of these previous studies have reported the phosphorylation status of GSK-3β at Ser-9 suggesting inhibition of its activity (Javadov et al., 2009; Tateishi et al., 2010). However, these studies have not investigated the phosphorylation level of GSK-3β(Tyr-216) leaving us with insufficient data to conclude whether those stimuli cause inhibition or activation of GSK-3β. In the present study, we observed that AngII exposure caused up-regulation of p-GSK3β(Ser-9) with concomitant and much higher increase in p-GSK3β(Tyr-216), indicating activation of GSK-3β.

Various upstream kinases, such as PI3K, PKB, MAPK, p70 ribosomal S6 kinase, PKA and PKC, have been reported to be responsible for phosphorylation of GSK-3β at Ser-9 upon stimulation with insulin and other growth factors (Doble and Woodgett, 2003). PKB (also termed Akt), a serine/threonine kinase located downstream of PI3K, has been shown to phosphorylate GSK-3β at Ser-9 in vitro and in vivo (Cross et al., 1994; 1995). AngII is known to exert its cellular effects via activation of several downstream kinases such as PI3K, Akt and MAPK (Wei et al., 2009; Zhang et al., 2012). Therefore, the observed increase in p-GSK3β(Ser-9) levels in AngII-exposed cells in this study could be due to activation of one or more of these kinases. Although which of these kinases is primarily responsible for AngII-induced phosphorylation of GSK-3β(Ser-9) is not clear at this time. However, we found that AngII-exposed neuronal cells had significantly higher levels of p-Akt(Ser-473) (Figure 9), indicating its activation as phosphorylation of Akt at Ser-473 is known to be crucial for its activation (Alessi et al., 1996). However, the upstream kinase or kinases responsible for AngII-induced increased phosphoylation of Tyr-216 is not known at this time and could be a focus of future studies.

As GSK-3β acts as a key regulator of transcription factors NFκB and CREB, it is plausible to speculate that GSK-3β could be the missing link in AngII-induced alterations in inflammatory cytokines. In this study, we observed that suppression of GSK-3β by highly efficient lentiviral shRNA prevented an AngII-induced increase in TNF-α and IL-1β, and a decrease in IL-10 levels in neuronal cells. Furthermore, GSK-3β suppres-
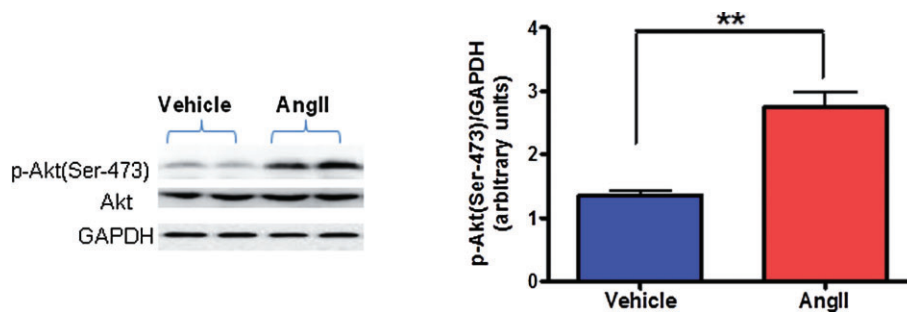
Figure 9

Effects of AngII treatment on phosphorylated Akt expression in neuronal cells. Serum-starved CATH.a cells were stimulated with 100 nM AngII for 6 h and cell extracts were then subjected to protein analysis by Western blot. AngII caused significant activation of Akt as indicated by increased p-Akt(Ser-473) protein expression in CATH.a neurons. AngII exposure did not alter total Akt protein levels. The results are means $\pm$ SD of three independent experiments. $**P < 0.01$ compared with cells treated with vehicle.

sion in AngII-exposed cells led to increased CBP to CREB binding and attenuated CBP to NFκB binding. The altered binding capability of NFκB and CREB to CBP was observed to be due to altered phosphorylation status of both of these transcription factors. Our results showed that GSK-3β silencing caused reduced phosphorylation of NFκB at Ser-276, whereas it increased phosphorylation of CREB at Ser-133. It has been shown previously that phosphorylation of NFκB and CREB at Ser-276 (Reber *et al.*, 2009) and Ser-133 (Chrivia *et al.*, 1993), respectively, is essential for their binding with the CBP and subsequent transactivation. These results suggest that AngII-induced alterations in NFκB and CREB activity are mediated by GSK-3β in neuronal cells. In line with our results, Grimes and Jope, (2001a) showed that inhibition of GSK-3β by lithium facilitates CREB activity in human neuroblastoma SH-SY5Y cells. However, the activity of NFκB is known to be regulated by phosphorylation of IκB and its subsequent nuclear transport. Therefore, the possibility that suppression of GSK-3β affects NFκB regulation at levels other than CBP binding cannot be ignored. Although we have not studied the effects of GSK-3β suppression on phosphorylation of IκB, it has been suggested that GSK-3β does not disrupt NFκB nuclear import in embryonic fibroblasts isolated from GSK3β-null mice (Doble and Woodgett, 2003). Nonetheless, our current results showed that inhibition of GSK-3β in AngII-stimulated neuronal cells altered activity of NFκB and CREB in favour of CREB by modulating their phosphorylation status (Figure 7) and eventually altering their ability to recruit the co-activator CBP. Importantly, these results provide strong evidence that AngII-induced alterations in phosphorylation of NFκB and CREB (Figure 4) in favour of NFκB-mediated transcription of PIC was mediated, at least in part, by GSK-3β.

Past few years of research showed that PICs act as neuromodulators and play a pivotal role in sympathetic regulation of BP (Shi *et al.*, 2010; Agarwal *et al.*, 2011). It is also clear from these studies that increased production of PICs in response to overactivated RAS within the cardiovascular regulatory centres of the brain (such as PVN and rostral ventrolateral medulla) causes increased sympathetic outflow leading to increased arterial pressure and cardiac remodelling in experimental models of hypertension. At the cellular level,

PICs activate reactive oxygen species (Zimmerman *et al.*, 2004; Xia *et al.*, 2011), which, in turn, can activate various intracellular signalling pathways, including that of NFκB. Activation of NFκB induces gene transcription of PICs, which leads to further increase in ROS production, fostering a positive feedback mechanism, and eventually leading to the progression of hypertension. A step further, the results of the present study provide evidence of GSK-3β as an important link between RAS, transcription factors and inflammatory cytokines. A summary of the pathway that our data support is depicted in Figure 8.

Limitations

In the present study, we used CATH.a neurons as our neuronal cell model. Clearly, the CATH.a cell line may not exhibit the exact phenotype of neurons located in areas of the brain that regulate BP (e.g. PVN and rostral ventrolateral medulla). However, CATH.a neurons are commonly used to examine AngII-dependent signalling mechanisms, as these cells express the AngII type 1 (AT1R) and type 2 (AT2R) receptors (Sun *et al.*, 2003). For instance, this cell line has been found to be a suitable model for studies involving AngII-induced oxidative stress in hypertension, NFκB (Okada *et al.*, 2006; Mitra *et al.*, 2010), neuronal activation (Sun *et al.*, 2002), expression of AT1R and JNK pathway (Liu *et al.*, 2006), Elk-1(Stefano *et al.*, 2006) and AP-1(Swanson *et al.*, 1998), as well as the effects of AngII on voltage-gated potassium channels (Gao *et al.*, 2010). Therefore, we believe that this cell line is an adequate and widely validated neuronal cell model to determine the AngII signalling pathways. However, future studies involving primary neuronal cell cultures from the cardiovascular regulatory centres of the brain as well as *in vivo* experiments using animal models of hypertension are still warranted and could be an important perspective of this study.

Another limitation of the present study relates to the involvement of AngII receptors in AngII-induced changes observed in this study. Growing body of evidence suggest that the pro-inflammatory effects of AngII are mediated by angiotensin II type 1 receptor (AT1R). In the context of CNS, previous reports from our laboratory have demonstrated that AngII induces up-regulation of several PICs (e.g. TNF-α, IL-1β

and IL-6) in the PVN of Sprague Dawley rats (Kang *et al.*, 2009). More importantly, central (i.c.v.) blockade of AT1R by losartan caused significant down-regulation of PICs in AngII-infused rats (Kang *et al.*, 2009). These findings clearly suggest AT1R as the primary receptor inducing pro-inflammatory effects of AngII in the brain. Therefore, in our opinion, AngII-induced inflammatory response as observed in the present study could be mediated via AT1R activation as well. Nevertheless, future studies could be directed towards dissecting out the role of various AngII receptors *in vitro* as well as *in vivo*.

In summary, the results of the present study suggest that AngII exposure causes up-regulation of TNF-α and IL-1β, and down-regulation of IL-10 in neuronal cells by increasing CBP to NFκB binding and attenuating CBP to CREB binding, and AngII-induced dysregulation in inflammatory cytokines is indeed mediated by GSK-3β. The results of this study explain a novel molecular mechanism by which an overactivation of the RAS in the neuronal cells modulates activity of the transcription factors leading to inflammatory alterations. The identification of GSK-3β as a downstream target of AngII in mammalian cells suggests an effector role for GSK-3β in cellular responses to AngII. The results of this study suggest the therapeutic potential of inhibiting GSK-3β in the treatment of CVDs characterized by chronic inflammation. However, *in vivo* validation of the data presented here could certainly be an important perspective of this study.

Acknowledgements

Conflict of interest

None declared.

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