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Angiotensin AT_2 Receptor Stimulation is Anti-inflammatory in Lipopolysaccharide-activated THP-1 Macrophages via Increased Interleukin-10 Production

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Abstract

Macrophages, via activation of the Toll-like receptors (TLR4), play an important role in the pathogenesis of hypertension and associated end-organ damage. There is accumulating evidence to suggest a protective role of the angiotensin AT_2 receptor (AT_2R) in pathological conditions involving inflammation and tissue injury. We have recently shown that AT_2R stimulation is renoprotective, in part, via increased anti-inflammatory interleukin-10 (IL-10) production in renal epithelial cells, however the role of AT_2R in macrophage inflammatory behavior is not known. The present study was designed to investigate whether AT_2R activation exerts an anti-inflammatory response in TLR4-induced inflammation. The anti-inflammatory mechanisms of AT_2R agonist C21 (1 μmol/ml) pre-treatment on the cytokine profile of THP-1 macrophages after activation by LPS (1 μg/ml) was studied. The AT_2R agonist dose-dependently attenuated LPS-induced TNF-α and IL-6 production but increased IL-10 production. IL-10 was critical for the anti-inflammatory effect of AT_2R stimulation, since IL-10 neutralizing antibody dose-dependently abolished the AT_2R–mediated decrease in TNF-α level. Further, the enhanced IL-10 levels were associated with a sustained, selective increase in phosphorylation of extracellular signal-regulated kinase (ERK1/2), but not p38 MAPK. Blocking the activation of ERK1/2 prior to C21 pre-treatment completely abrogated this increased IL-10 production in response to AT_2R agonist C21, while there was a partial reduction in IL-10 levels on inhibition of p38. We conclude that AT_2R stimulation exerts a novel anti-inflammatory response in THP-1 macrophages via enhanced IL-10 production as a result of sustained, selective ERK1/2 phosphorylation, and thus may have protective role in hypertension and associated tissue injury.

Keywords

AT_2 receptor; inflammation; macrophage; Interleukin-10

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CONFLICT OF INTREST/ DISCLOSURE STATEMENT

None

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INTRODUCTION

Chronic inflammation, characterized by elevated cytokine and chemokine expression, has been shown to play a central role in the pathophysiology of hypertension and associated endorgan damage, including renal injury[1–4]. At the cellular level, chronic inflammation is mediated largely by macrophages. Also, macrophage infiltration invariably accompanies hypertensive organ damage, such as that to the blood vessels[5], the heart[6] and the kidneys[7]. Moreover, circulating monocytes are activated in hypertensive patients and produce increased amounts of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF-α), interleukin-1 (IL-1) and transforming growth factor- β (TGF-β)[8]. In fact, recent evidence indicates that, rather than being a mere consequence of elevated blood pressure, macrophage activation actually serves as a causative factor in the development of hypertension[9, 10].

The renin-angiotensin system (RAS) is a critical hormonal system that regulates blood pressure and is abnormally activated in hypertensive patients. Macrophages also express all major components of the RAS[11]. Angiotensin II (Ang II), via the AT_1 receptor has been demonstrated to participate in the activation and pro-inflammatory polarization of leukocytes[12–14]. However, the precise cellular mechanisms are not well defined. One potential inflammatory pathway that has been implicated in hypertension is activation of innate immune receptors, specifically Toll-like receptor-4 (TLR4) signaling[15], which leads to the production of an array of pro-inflammatory mediators. In fact, peripheral monocyte TLR4 expression is markedly increased in hypertensive patients compared to normotensive controls[16]. Further, Ang II via the AT_1 receptor has been shown to up-regulate TLR4 expression[17] and exacerbate pro-inflammatory cytokine production in response to TLR4 activation by lipopolysaccharide (LPS) in macrophages[18]. Accumulating evidence suggests that the AT_2 receptor, which is generally considered to be a functional antagonist of the AT_1 receptor, exerts an anti-inflammatory response[19–21]. We have previously shown that stimulation of AT_2 receptor by the selective agonist Compound 21 (C21) attenuates pro-inflammatory signaling in response to LPS-activation of proximal tubule epithelial cells via increased IL-10 production[22]. However, whether AT_2 receptor can induce IL-10 production and exert an anti-inflammatory response in LPS-activated macrophages has not been investigated.

In macrophages, multiple pathways exist to promote IL-10 production[23]. Activation of mitogen-activated protein kinases (MAPKs), specifically via p38 and extracellular signal-regulated kinase (ERK-1/2), has been shown to be required to increase IL-10 in macrophages and inhibition of either MAPK blunts IL-10 production[24–28]. Moreover, high ERK1/2 activation correlates well with the extent of IL-10 production in this cell-type[23]. Since the AT_2 receptor has been linked to a sustained increase in ERK1/2 phosphorylation, it is possible that this might be a potential molecular mechanism by which the AT_2 receptor agonist can enhance IL-10 production in macrophages.

The present study was designed to test the hypothesis that stimulation of the AT_2 receptor attenuates TLR4-mediated pro-inflammatory cytokine production in macrophages via increased IL-10 production. We evaluated the effect of C21 on the production of TNF-α,

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IL-6 and IL-10 in LPS-activated THP-1 macrophages. We demonstrate that pre-treatment with C21 significantly attenuated the levels of pro-inflammatory cytokines. This was found to be dependent on increased IL-10 production in response to AT_2 receptor activation. Moreover, this up-regulation of IL-10 is mediated via a sustained, selective increase in ERK1/2 phosphorylation.

MATERIALS AND METHODS

Cell culture and treatments

The human THP-1 monocytic cell-line (ATCC) was cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic/antimycotic cocktail (penicillin 100U/ml, streptomycin 100μg/ml and Amphotericin B 250 ng/ml) at 37°C in a humidified atmosphere with 5% CO_2. Cell culture media and supplements were all purchased from (HyClone, ThermoFisher Scientific, Inc.). To differentiate monocytes to macrophages, 5×10^5 cells/well were treated with 40 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) for 48 hours in RPMI-1640 containing 5% FBS. At the end of the 48 hour incubation period, the medium was aspirated and cells were washed with RPMI-1640 without FBS and antibiotic/antimycotic and were incubated in this medium for 6–8 hours. To eliminate pre-existing cytokine production, the medium was replaced by fresh serum free medium before treatments were initiated. LPS (1μg/ml) (E.coli O55:B5, Sigma) was used to induce the production of pro-inflammatory cytokines. Cells were pre-treated with C21 (Custom synthesized) 60 minutes prior to the addition of LPS and the drug remained in the medium for the entire duration of treatment. Treatments with specific inhibitors including Candesartan (1μmol/l) (AstraZeneca) PD123319 (10μmol/l) (Pfizer), SB203580 (10μmol/l) (Cell Signaling Technology) and PD98059 (10μmol/l) (Cell Signaling Technology) were carried out as indicated in the text and figure legends.

Immunoblotting

Macrophages were washed twice with PBS and lysed on the plate using ice-cold cell lysis buffer (Cell Signaling Technology) containing protease (Roche) and phosphatase (Sigma) inhibitor cocktails. Equal amounts of protein in Laemmli buffer were loaded per well (15 μg/lane for AT_1R, 45 μg/lane for AT_2R and 30 μg/lane for ERK1/2 and p38 MAPK) and separated by SDSPAGE using a Tris-Glycine system. Proteins were then transferred to a PVDF membrane using the wet transfer protocol. The membrane was incubated in 5% non-fat dry milk in PBST for 1 hour, following overnight incubation with primary antibodies for AT_1R (Biomolecular Integrations), AT_2R (EZ Biolabs, Inc), p-ERK1/2 (Cell Signaling Technology) and p-p38 MAPK (Cell Signaling Technology) at a 1:1000 dilution. This was followed by washing with PBST and 1 hour incubation with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc). Chemiluminesence was detected by the addition of Luminol HRP substrate (Santa Cruz Biotechnology, Inc.) and quantified by software-assisted densitometric analysis (Alpha Innotech Corp.). To ensure equal loading, blots were stripped and re-probed for β-Actin (BioVision) for AT_1R and AT_2R, total-p38 and total-ERK1/2 for p-p38 and p-ERK1/2, respectively (Cell Signaling Technology).

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ELISA

Cytokines in the medium were assessed by kit-based ELISA using the manufacturers protocols (R&D Systems).

mRNA Expression by RT-PCR

Total RNA from the cells was extracted using the RNEasy kits (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by RT-PCR from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System (Life Technologies). This cDNA was used as a template for the quantitative RT-PCR analysis of gene expressions of *TNFA*, *IL6* and *IL10* using TaqMan gene expression assays (Applied Biosystems). Relative quantification was determined using the delta-delta Ct method with GAPDH as a control.

Statistical Analysis

Data are presented as means ±SEM. Student's *t*-test was used to compare means of two groups. One-way ANOVA with post-hoc test (Tukey) for multiple comparisons was used to compare variations between more than 2 groups. A value of $p<0.05$ was considered statistically significant, with n=5–8 experiments per group.

RESULTS

Expression of angiotensin AT_1 and AT_2 receptors in THP-1 macrophages

THP-1 macrophages express both, AT_1R and AT_2R. The expression of both receptor subtypes was not altered by C21 or LPS treatment (Figure 1A, B); however C21 pre-treatment lowered AT_1R expression by ~50% in response to LPS (Figure 1A), which is in agreement with a number of reports demonstrating the AT_2R-mediated down-regulation of AT_1R in pathophysiological conditions[29–31].

Effect of AT_2R agonist (C21) and AT_1R antagonist (candesartan) on cytokine production by LPS-activated THP-1 macrophages

THP-1 Macrophages were treated with LPS (1 μg/ml) for 24 hours to induce the expression of pro-inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokine, IL-10. Pre-treatment with AT_2R agonist C21 (1 μmol/L) attenuated the LPS-induced TNF-α and IL-6 production by ~33% and ~50%, respectively, with a concurrent 75% increase in IL-10 production. This anti-inflammatory response was blocked by AT_2R antagonist PD123319 (10 μmol/L), suggesting an AT_2 receptor-mediated effect. However, AT_1R antagonist candesartan (1 μmol/L) pre-treatment did not alter the cytokine levels in response to LPS (Figure 2 A–C).

Anti-inflammatory effect of AT_2R agonist (C21) on cytokine production by LPS-activated THP-1 macrophages is mediated via increased IL-10 production

AT_2R agonist C21 pre-treatment dose dependently (0.1–10 μmol/L) attenuated the production of TNF-α (Figure 3A) while IL-10 production was concurrently enhanced (Figure 3B) in LPS-activated THP-1 macrophages 24 hours post-LPS. Pre-treatment with C21 before LPS-activation resulted in lower TNF-α levels starting as early as 60 minutes

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post-LPS and this trend continued up to 4 hours (Figure 3C). Cells pre-treated with C21 for 1 hour produced measurable amounts of antiinflammatory IL-10 at the time of addition of LPS and the IL-10 levels continued to be higher up to 4 hours compared to LPS treated control cells (Figure 3D). Thus, AT_2R agonist exerts an antiinflammatory effect on early cytokine production in THP-1 macrophages and this response is dose-dependent.

Cytokine production at the level of mRNA and protein released in the media was assessed at 24 hours post-LPS activation to determine the effect of chronic stimulation of AT_2R. C21 pre-treatment significantly attenuated pro-inflammatory TNF-α and IL-6 mRNA as well as protein expression (Figure 4A, 4B, 4D, 4E). Conversely, IL-10 in the media was significantly higher in C21 pre-treated cells after LPS stimulation (Figure 4F). Interestingly, IL-10 mRNA expression was higher in C21 treated cells, even in the absence of LPS activation (Figure 4C), though this did not translate to higher IL-10 protein expression in this treatment group (Figure 4F). Further, a neutralizing antibody to IL-10 dose-dependently ameliorated the effect of C21 on LPS-induced TNF-α production (Figure 5). Thus, the increase in IL-10 production in response to C21 in the presence of LPS appears to be critical for attenuating the anti-inflammatory response to LPS.

Involvement of p38 and ERK1/2 MAPKs in AT_2R-mediated increase in IL-10 production

The activation of MAPKs, specifically p38 and ERK1/2, has been shown to be a key requirement for the regulation of IL-10 production in macrophages[25, 28, 32, 33]. LPS treatment led to a rapid phosphorylation of p38 and ERK1/2 which peaked at 30 minutes post-LPS and returned to basal levels by 2 hours post-LPS (Figure 6A,B 7A,B). C21 pre-treatment delayed the peak of p38 phosphorylation to 1 hour post-LPS (Figure 6A,B). Incubation with a p38 inhibitor (SB203580; 10μmol/L) prior to C21 pre-treatment partially abolished the C21-mediated increase in IL-10 at 24 hours post-LPS activation (Figure 6C).

On the other hand, C21 pre-treatment resulted in a delayed, sustained increase in ERK1/2 phosphorylation (Figure 7A,B), which persisted up to 24 hours post-LPS treatment (Figure 7C). Moreover, pre-incubation of cells with a MEK inhibitor (PD98059; 10 μmol/L), which prevents ERK1/2 activation, prior to C21 pre-treatment completely prevented the C21-mediated increase in IL-10 at 24 hours post-LPS activation (Figure 7D), suggesting that the sustained, selective ERK1/2 phosphorylation associated with AT_2R agonist is essential for the increase in IL-10 production. C21 treatment alone did not induce ERK1/2 phosphorylation at 24 hours (Figure 7C) or earlier time points (data not shown), suggesting that LPS-mediated signaling is required for C21 to exert its anti-inflammatory effect.

DISCUSSION

Macrophages, via activation of the TLR4-mediated pro-inflammatory signaling, play an important role in the initiation and progression of hypertension and associated end-organ damage. Macrophages express AT_2 receptors[11], which have been demonstrated to attenuate TLR-mediated pro-inflammatory cytokine production in proximal tubule epithelial cells[22]. In the present study, we have demonstrated that pre-treatment of THP-1 macrophages with AT_2R agonist C21 attenuates TNF-α and IL-6 production in response to activation of TLR4

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by LPS. This effect is mainly a result of increased IL-10 production by macrophages via a sustained, selective increase in ERK1/2 phosphorylation.

Ang II via the AT_1 receptor has been documented to promote pro-inflammatory cytokine and chemokine production in a manner similar to TLR4-mediated signaling in a number of tissues including endothelial cells[34, 35], renal tubular epithelial cells[36], dendritic cells[37–39], and T lymphocytes[40–42]. However, its precise role in macrophages/monocytes is controversial. Incubation of monocytes with Ang II has been shown to induce the expression of the chemokine monocyte chemoattractant protein-1 (MCP-1)[43], while no effect was observed on the production of cytokines[44]. The RAS is up-regulated during monocyte differentiation and macrophages express a relatively higher level of AT_1 and AT_2 receptors compared to monocytes[11]. Incubation of macrophages with Ang II via AT_1R activation resulted in increased IL-6 production, without affecting TNF-α[18, 45]. Further, the interaction between Ang II and enhanced TLR4 signaling has been reported[17, 46–48]. However, interpretation of these findings has been made complicated by the fact that the commonly used AT_1R blockers, including losartan and candesartan, have been found to exert anti-inflammatory actions independent of the AT_1R[18, 44].

Conversely, the anti-inflammatory role of the AT_2R, which is generally considered to act as a functional antagonist of the AT_1R, has been demonstrated in a number of *in vitro* and *in vivo* models[19–22, 49]. Here, we report that at higher concentration of LPS (1 μg/ml compared to 50 ng/ml used by Larrayoz et al.[44]), candesartan was ineffective in lowering pro-inflammatory cytokine production while AT_2R agonist C21 significantly lowered both, TNF-α and IL-6 which was associated with an increase in the anti-inflammatory cytokine IL-10 production. Since this alteration in cytokine profile could be blocked by AT_2R antagonist PD123319, we conclude this anti-inflammatory effect was a specific AT_2 receptor mediated response. We found that pre-treatment with C21 in the presence of LPS also attenuated AT_1R expression. The down-regulation of AT_1R in response to AT_2R stimulation under pathophysiological conditions has been reported in a number of experimental models[29–31]. In the present study, however, this observation may be unrelated to the anti-inflammatory response to AT_2R agonist since the increase in pro-inflammatory cytokine levels did not appear to be mediated via AT_1R activation.

We have previously shown that AT_2R stimulation resulted in enhanced IL-10 secretion by proximal tubule epithelial cells[22]. A similar observation was reported in a specific subset of splenic $CD8^+AT2R^+$ T cells which produced uncharacteristically high amounts of IL-10 and AT_2R stimulation by Ang II as well as by C21 further augmented the IL-10 production[50]. Here we report that C21 alone increased the IL-10 gene expression, however, this did not translate to increased IL-10 protein secretion, except in the presence of TLR4 activation by LPS. This could be a result of post-transcriptional modifications to IL-10 mRNA that have been shown to occur in immune cells as a means of regulation of IL-10 production in the absence of an inflammatory stimulus[51].

Though there is considerable evidence to suggest an anti-inflammatory effect of AT_2 receptor stimulation, the signaling pathways involved in mediating this response lack clear definition and are still a subject of debate. Moreover, the cell-types and experimental

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conditions greatly influence the downstream signaling cascades activated by the AT_2R. Typically, AT_2R stimulation results in the activation of phosphatases, including MAP kinase phosphatase-1 (MKP-1)[52–54] and SH-2 domain containing phosphatase-1 (SHP-1)[55–57], which ultimately leads to AT_2R-mediated apoptosis. On the other hand, AT_2R stimulation has also shown to promote cellular differentiation via a sustained increase in ERK1/2 phosphorylation[58–61] which is independent of cAMP-mediated signaling[62]. In the present study, AT_2R agonist pre-treatment resulted in a delayed increase in ERK1/2 phosphorylation which was sustained up to 24 hours post-LPS activation, however, AT_2R agonist alone did not promote ERK1/2 phosphorylation at any of the time points studied, nor was IL-10 detectable in the medium. Thus, it appears that LPS-mediated signaling pathways are required for the augmented IL-10 production by AT_2R agonist. It may be speculated that C21 pre-treatment 'primes' macrophages such that in the presence of an activating signal such as LPS, their polarization to the 'alternatively activated', anti-inflammatory M2 phenotype is favored over the pro-inflammatory, 'classically activated' M1 phenotype.

In macrophages, multiple pathways exist that can regulate the production of IL-10 depending upon the activating stimulus[28, 63–66]. Of these, activation of p38 and ERK1/2 MAPKs has been shown to be critical for induction of IL-10 synthesis[23–28]. We report that inhibition of p38 activation partially abrogated the AT_2R-mediated increase in IL-10 while inhibition of ERK1/2 activation resulted in a complete lack of IL-10 production in response to AT_2R stimulation, suggesting that p38 MAPK may contribute to, but is not essential for AT_2R-mediated IL-10 expression. This observation could also be linked to the altered kinetics of p38 MAPK phosphorylation in response to LPS in the presence and absence of AT_2R agonist pretreatment. However, the precise mechanisms that orchestrate these changes in time course of MAPK phosphorylation require further investigation.

Over the past decade, AT_2R stimulation has emerged as a potential therapeutic target for the treatment of hypertension and end-organ damage[21, 22, 29, 67, 68], particularly with concomitant AT_1R blockade[69, 70]. Further, AT_2R activation at the level of the kidney has been shown to promote vasodilation and natriuresis, thus affording renoprotection in the setting of hypertension[29, 71, 72]. Though administration of C21 has been shown to have a modest, if any, effect on lowering blood pressure[21, 68, 73–75], its protective effects on inflammation, oxidative stress, fibrosis and vascular remodeling underscore the potential benefit of the addition of an AT_2R agonist to the currently used classical anti-hypertensive drugs to retard the progression of hypertension and end-organ damage. Here, we identify macrophages, which play a central role in the initiation and progression of hypertension-associated target organ damage, as an additional target of AT_2R stimulation.

In conclusion, the present study demonstrates a novel anti-inflammatory role for AT_2R stimulation in macrophages involving the attenuation of TLR4-mediated pro-inflammatory cytokine production. We further demonstrate that ERK1/2-dependent increased IL-10 production is a key event involved in mediating this anti-inflammatory response. Thus, it may be speculated that stimulation of the AT_2 receptor may be beneficial in hypertension owing to its protective effects not only on hemodynamic factors, such as vasodilation and Na^+ excretion, but also as a consequence of attenuation of inflammation and associated end-organ injury.

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REFERENCES

1. Bautista LE. Inflammation, endothelial dysfunction, and the risk of high blood pressure: Epidemiologic and biological evidence. J Hum Hypertens. 2003; 17:223–230. [PubMed: 12692566]
2. Chrysohoou C, Pitsavos C, Panagiotakos DB, Skoumas J, Stefanadis C. Association between prehypertension status and inflammatory markers related to atherosclerotic disease: The ATTICA Study. Am J Hypertens. 2004; 17(7):568–573. [PubMed: 15233975]
3. Heijnen BFJ, Essen H, Schalkwijk CG, Janssen BJA, Struijker-Boudie HAJ. Renal inflammatory markers during the onset of hypertension in spontaneously hypertensive rats. Hypertens Res. 2014; 37:100–109. [PubMed: 23985702]
4. Stumpf C, John S, Jukic J, Yilmaz A, Raaz D, et al. Enhanced levels of platelet P-selectin and circulating cytokines in young patients with mild arterial hypertension. J Hypertens. 2005; 23:995–1000. [PubMed: 15834285]
5. Clozel M, Kuhn H, Hefti F, Baumgartner HR. Endothelial dysfunction and subendothelial monocyte macrophages in hypertension. Effect of angiotensin converting enzyme inhibition. Hypertension. 1991; 18:132–141. [PubMed: 1653185]
6. Haller H, Behrend M, Park JK, Schaberg T, Luft FC, Distler A. Monocyte infiltration and c-fms expression in hearts of spontaneously hypertensive rats. Hypertension. 1995; 25:132–138. [PubMed: 7843744]
7. Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, et al. Renal injury from angiotensin II-mediated hypertension. Hypertension. 1992; 19:464–474. [PubMed: 1568765]
8. Chen NG, Abbasi F, Lamendola C, McLaughlin T, Cooke JP, Tsao PS, et al. Mononuclear cell adherence to cultured endothelium is enhanced by hypertension and insulin resistance in healthy nondiabetic volunteers. Circulation. 1999; 100:940–943. [PubMed: 10468524]
9. Machnik A, Neuhofer W, Jantsch J, Dahlmann A, Tammela T, Machura K, Park JK, Beck FX, Müller DN, Derer W, Goss J, Ziomber A, Dietsch P, Wagner H, van Rooijen N, Kurtz A, Hilgers KF, Alitalo K, Eckardt KU, Luft FC, Kerjaschki D, Titze J. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. Nat Med. 2009; 15(5):545–552. [PubMed: 19412173]
10. Kriska T, Cepura C, Gauthier KM, Campbell WB. Role of macrophage PPARγ in experimental hypertension. Am J Physiol –Heart C. 2014; 306:H26–H32.
11. Okamura A, Rakugi H, Ohishi M, Yanagitani Y, Takiuchi S, Moriguchi K, Fennessy PA, Higaki J, Ogihara T. Upregulation of renin-angiotensin system during differentiation of monocytes to macrophages. J Hypertens. 1999; 17:537–545. [PubMed: 10404956]
12. Ma LJ, Corsa BA, Zhou J, Yang H, Li H, Tang YW, Babaev VR, Major AS, Linton MF, Fazio S, Hunley TE, Kon V, Fogo AB. Angiotensin type 1 receptor modulates macrophage polarization and renal injury in obesity. Am J Physiol Renal Physiol. 2011; 300(5):F1203–F1213. [PubMed: 21367915]
13. Rafatian N, Milne RW, Leenen FH, Whitman SC. Role of renin-angiotensin system in activation of macrophages by modified lipoproteins. Am J Physiol Heart Circ Physiol. 2013; 305(9):H1309–H1320. [PubMed: 23997101]
14. Yamamoto S, Yancey PG, Zuo Y, Ma LJ, Kaseda R, Fogo AB, Ichikawa I, Linton MF, Fazio S, Kon V. Macrophage polarization by angiotensin II-type 1 receptor aggravates renal injury-acceleration of atherosclerosis. Arterioscler Thromb Vasc Biol. 2011; 31(12):2856–2864. [PubMed: 21979434]
15. Bomfim GF, Dos Santos RA, Oliveira MA, Giachini FR, Akamine EH, Tostes RC, Fortes ZB, Webb RC, Carvalho MHC. Toll-like receptor 4 contributes to blood pressure regulation and vascular contraction in spontaneously hypertensive rats. Clin Science. 2012; 122:535–543.

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16. Marketou ME, Kontaraki JE, Zacharis EA, Kochiadakis GE, Giaouzaki A, Chlouverakis G, Vardas PE. TLR2 and TLR4 gene expression in peripheral monocytes in nondiabetic hypertensive patients: the effect of intensive blood pressure-lowering. J Clin Hypertens (Greenwich). 2012; 14(5):330–335. [PubMed: 22533660]
17. Wolf G, Bohlender J, Bondeva T, Roger T, Thaiss F, Wenzel UO. Angiotensin II upregulates toll-like receptor 4 on mesangial cells. J Am Soc Nephrol. 2006; 17(6):1585–1593. [PubMed: 16675600]
18. An J, Nakajima T, Kuba K, Kimura A. Losartan inhibits LPS-induced inflammatory signaling through a PPARgamma-dependent mechanism in human THP-1 macrophages. Hypertens Res. 2010; 33(8):831–835. [PubMed: 20505677]
19. Rompe F, Artuc M, Hallberg A, Alterman M, Stroder K, Thone-Reineke C, Reichenback A, Schacherl J, Dahlof B, Bader M, Alenina N, Schwaninger M, Zuberbier T, Funke-Kaiser H, Schmidt C, Schunck W, Unger T, Steckelings UM. Direct angiotensin II type 2 receptor stimulation acts as anti-inflammatory through epoxyeicosatrienoic acid and inhibition of nuclear factor κB. Hypertension. 2010; 55:924–931. [PubMed: 20157051]
20. Abadir PM, Walton JD, Carey RM, Siragy HM. Angiotensin II type 2 receptors modulate inflammation through signal transducer and activator transcription proteins 3 phosphorylation and TNF-α production. J Interferon Cytokine Res. 2011; 31:471–474. [PubMed: 21288138]
21. Matavelli LC, Jiang H, Siragy HM. Angiotensin AT_2 receptor stimulation inhibits early renal inflammation in renovascular hypertension. Hypertension. 2011; 57:308–313. [PubMed: 21189405]
22. Dhande IS, Ali Q, Hussain T. Proximal tubule Angiotensin AT_2 receptors mediate an anti-inflammatory response via Interleukin-10: Role in renoprotection in obese rats. Hypertension. 2013; 61:1218–1226. [PubMed: 23547236]
23. Saraiva M, O'Garra A. The regulation of Interleukin-10 production by immune cells. Nat Rev Immunol. 2010; 10(3):170–181. [PubMed: 20154735]
24. Zhang P, Martin M, Michalek SM, Katz J. Role of mitogen-activated protein kinases and NF-κB in the regulation of proinflammatory and anti-inflammatory cytokines by Porphyromonas gingivalis hemagglutinin B. Infect Immun. 2005; 73(7):3990–3998. [PubMed: 15972486]
25. Ma W, Lim W, Gee K, Aucoin S, Nandan D, Kozlowski M, Diaz-Mitoma F, Kumar A. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. J Biol Chem. 2001; 276(17):13664–13674. [PubMed: 11278848]
26. Yi AK, Yoon JG, Yeo SJ, Hong SC, English BK, Krieg AM. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. J Immunol. 2002; 168(9):4711–4720. [PubMed: 11971021]
27. Koscsó B, Csóka B, Selmeczy Z, Himer L, Pacher P, Virág L, Haskó G. Adenosine augments IL-10 production by microglial cells through an A_2B adenosine receptor-mediated process. J Immunol. 2012; 188(1):445–453. [PubMed: 22116830]
28. Chanteux H, Guisset AC, Pilette C, Sibille Y. LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms. Respir Res. 2007; 8:71. [PubMed: 17916230]
29. Ali Q, Wu Y, Hussain T. Chronic AT_2 receptor activation increases renal ACE2 activity, attenuates AT1 receptor function and blood pressure in obese Zucker rats. Kidney Int. 2013; 84(5):931–939. [PubMed: 23823602]
30. Shum M, Pinard S, Guimond MO, Labbé SM, Roberge C, Baillargeon JP, Langlois MF, Alterman M, Wallinder C, Hallberg A, Carpentier AC, Gallo-Payet N. Angiotensin II type 2 receptor promotes adipocyte differentiation and restores adipocyte size in high-fat/high-fructose diet-induced insulin resistance in rats. Am J Physiol Endocrinol Metab. 2013; 304(2):E197–E210. [PubMed: 23149621]
31. Yang J, Chen C, Ren H, Han Y, He D, Zhou L, Hopfer U, Jose PA, Zeng C. Angiotensin II AT(2) receptor decreases AT(1) receptor expression and function via nitric oxide/cGMP/Sp1 in renal proximal tubule cells from Wistar-Kyoto rats. J Hypertens. 2012; 30(6):1176–1184. [PubMed: 22504846]

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32. Lucas M1, Zhang X, Prasanna V, Mosser DM. ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. J Immunol. 2005 Jul 1; 175(1):469–477. [PubMed: 15972681]
33. Kaiser F, Cook D, Papoutsopoulou S, Rajsbaum R, Wu X, Yang HT, Grant S, Ricciardi-Castagnoli P, Tsichlis PN, Ley SC, O'Garra A. TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. J Exp Med. 2009; 206(9):1863–1871. [PubMed: 19667062]
34. Pastore L, Tessitore A, Martinotti S, Toniato E, Alesse E, Bravi MC, et al. Angiotensin II stimulates intercellular adhesion molecule-1 (ICAM-1) expression by human vascular endothelial cells and increases soluble ICAM-1 release *in vivo*. Circulation. 1999; 100:1646–1652. [PubMed: 10517737]
35. Pueyo ME, Gonzalez W, Nicoletti A, Savoie F, Arnal JF, Michel JB. Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-kappaB activation induced by intracellular oxidative stress. Arterioscler Thromb Vasc Biol. 2000; 20:645–651. [PubMed: 10712386]
36. Rice EK, Tesch Gregory H, Cao Zemin, Cooper Mark E, Metz Christine N, Bucala Richard, Atkins Robert C, Nikolic-Paterson David J. Induction of MIF synthesis and secretion by tubular epithelial cells: A novel action of angiotensin II. Kidney Int. 2003; 63:1265–1275. [PubMed: 12631343]
37. Lapteva N, Ide K, Nieda M, Ando Y, Hatta-Ohashi Y, Minami M, Dymshits G, Egawa K, Juji T, Tokunaga K. Activation and suppression of renin-angiotensin system in human dendritic cells. Biochem Biophys Res Commun. 2002; 296:194–200. [PubMed: 12147250]
38. Muller DN, Shagdarsuren E, Park JK, Dechend R, Mervaala E, Hampich F, Fiebeler A, Ju X, Finckenberg P, Theuer J, et al. Immunosuppressive treatment protects against angiotensin II-induced renal damage. Am J Pathol. 2002; 161:1679–1693. [PubMed: 12414515]
39. Nahmod KA, Vermeulen ME, Raiden S, Salamone G, Gamberale R, Fernandez-Calotti P, Alvarez A, Nahmod V, Giordano M, Geffner JR. Control of dendritic cell differentiation by angiotensin II. FASEB J. 2003; 17:491–493. [PubMed: 12514109]
40. Crowley SD, Frey CW, Gould SK, Griffiths R, Ruiz P, Burchette JL, Howell DN, Makhanova N, Yan M, Kim HS, et al. Stimulation of lymphocyte responses by angiotensin II promotes kidney injury in hypertension. Am J Physiol Renal Physiol. 2008; 295:F515–F524. [PubMed: 18495795]
41. Jurewicz M, McDermott DH, Sechler JM, Tinckam K, Takakura A, Carpenter CB, Milford E, Abdi R. Human T and natural killer cells possess a functional renin-angiotensin system: further mechanisms of angiotensin II-induced inflammation. J Am Soc Nephrol. 2007; 18:1093–1102. [PubMed: 17329576]
42. Kvakan H, Kleinewietfeld M, Qadri F, Park JK, Fischer R, Schwarz I, Rahn HP, Plehm R, Wellner M, Elitok S, et al. Regulatory T cells ameliorate angiotensin II-induced cardiac damage. Circulation. 2009; 119:2904–2912. [PubMed: 19470887]
43. Dai Q, Xu M, Yao M, Sun B. Angiotensin AT1 receptor antagonists exert anti-inflammatory effects in spontaneously hypertensive rats. Br J Pharmacol. 2007; 152:1042–1048. [PubMed: 17922026]
44. Larrayoz IM1, Pang T, Benicky J, Pavel J, Sánchez-Lemus E, Saavedra JM. Candesartan reduces the innate immune response to lipopolysaccharide in human monocytes. J Hypertens. 2009; 27(12):2365–2376. [PubMed: 19730394]
45. Nakamura A1, Johns EJ, Imaizumi A, Yanagawa Y, Kohsaka T. Effect of beta(2)-adrenoceptor activation and angiotensin II on tumour necrosis factor and interleukin 6 gene transcription in the rat renal resident macrophage cells. Cytokine. 1999; 11(10):759–765. [PubMed: 10525314]
46. Ji Y, Liu J, Wang Z, Liu N. Angiotensin II Induces Inflammatory Response Partly Via Toll-Like Receptor 4-Dependent Signaling Pathway in Vascular Smooth Muscle Cells. Cell Physiol Biochem. 2009; 23:265–276. [PubMed: 19471094]
47. Bondeva T, Roger T, Wolf G. Differential Regulation of *Toll-Like Receptor 4* Gene Expression in Renal Cells by Angiotensin II: Dependency on AP1 and PU.1 Transcriptional Sites. Am J Nephrol. 2007; 27:308–314. [PubMed: 17495427]
48. Wu J, Yang X, Zhang YF, Zhou SF, Zhang R, Dong XQ, Fan JJ, Liu M, Yu XQ. Angiotensin II up-regulated Toll-like receptor 4 and enhances lipopolysaccharide-induced CD40 expression in rat peritoneal mesothelial cells. Inflamm Res. 2009; 58:473–482. [PubMed: 19271152]

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49. Sabuhi R, Ali Q, Asghar M, Al-Zamily NRH, Hussain T. Role of angiotensin II AT_2 receptor in inflammation and oxidative stress: Opposing effects in lean and obese rats. Am J Renal Physiol. 2011; 300:F700–F706.
50. Curato C, Slavic S, Dong J, Skorska A, Altarche-Xifro W, Miteva K, Kaschina E, Thiel A, Imboden H, Wang I, Steckelings U, Steinhoff G, Unger T, Li J. Identification of non-cytotoxic and IL-10 producing CD8+AT_2R+ T-cell population in response to ischemic heart injury. J Immunol. 2010; 185:6286–6293. [PubMed: 20935205]
51. Powell MJ, Thompson SA, Tone Y, Waldmann H, Tone M. Posttranscriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region. J Immunol. 2000; 165(1):292–296. [PubMed: 10861064]
52. Hayashida W, Horiuchi M, Dzau VJ. Intracellular third loop domain of angiotensin II type-2 receptor. Role in mediating signal transduction and cellular function. J Biol Chem. 1996; 271:21985–21992. [PubMed: 8703004]
53. Yamada T, Horiuchi M, Dzau VJ. Angiotensin II type 2 receptor mediates programmed cell death. Proc Natl Acad Sci. 1996; 93:156–160. [PubMed: 8552595]
54. Horiuchi M, Hayashida W, Kambe T, Yamada T, Dzau VJ. Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. J Biol Chem. 1997; 272:19022–19026. [PubMed: 9228085]
55. Bedecs K, Elbaz N, Sutren M, Masson M, Susini C, Strosberg AD, Nahmias C. Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. Biochem J. 1997; 325:449–454. [PubMed: 9230127]
56. Elbaz N, Bedecs K, Masson M, Sutren M, Strosberg AD, Nahmias C. Functional trans-inactivation of insulin receptor kinase by growth-inhibitory angiotensin II AT_2 receptor. Mol Endocrinol. 2000; 14:795–804. [PubMed: 10847582]
57. Shibasaki Y, Matsubara H, Nozawa Y, Mori Y, Masaki H, Kosaki A, Tsutsumi Y, Uchiyama Y, Fujiyama S, Nose A, et al. Angiotensin II type 2 receptor inhibits epidermal growth factor receptor transactivation by increasing association of SHP-1 tyrosine phosphatase. Hypertension. 2001; 38:367–372. [PubMed: 11566906]
58. Gendron L, Laflamme L, Rivard N, Asselin C, Payet MD, Gallo-Payet N. Signals from the AT_2 (angiotensin type 2) receptor of angiotensin II inhibit p21ras and activate MAPK (mitogen-activated protein kinase) to induce morphological neuronal differentiation in NG108-15 cells. Mol Endocrinol. 1999; 13(9):1615–1626. [PubMed: 10478850]
59. Stroth U, Blume A, Mielke K, Unger T. Angiotensin AT(2) receptor stimulates ERK1 and ERK2 in quiescent but inhibits ERK in NGF-stimulated PC12W cells. Brain Res Mol Brain Res. 2000; 78(1–2):175–180. [PubMed: 10891597]
60. Hansen JL, Servant G, Baranski TJ, Fujita T, Iiri T, Sheikh SP. Functional reconstitution of the angiotensin II type 2 receptor and G(i) activation. Circ Res. 2000; 87(9):753–759. [PubMed: 11055978]
61. De Paolis P, Porcellini A, Savoia C, Lombardi A, Gigante B, Frati G, Rubattu S, Musumeci B, Volpe M. Functional cross-talk between angiotensin II and epidermal growth factor receptors in NIH3T3 fibroblasts. J Hypertens. 2002; 20(4):693–699. [PubMed: 11910305]
62. Gendron L, Oligny JF, Payet MD, Gallo-Payet N. Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II AT_2 receptor signaling pathway in NG108-15 cells. J Biol Chem. 2003; 278(6):3606–3614. [PubMed: 12464615]
63. MacKenzie KF, Clark K, Naqvi S, McGuire VA, Nöehren G, Kristariyanto Y, van den Bosch M, Mudaliar M, McCarthy PC, Pattison MJ, Pedrioli PG, Barton GJ, Toth R, Prescott A, Arthur JS. PGE(2) induces macrophage IL-10 production and a regulatory-like phenotype via a protein kinase A-SIK-CRTC3 pathway. J Immunol. 2013; 190(2):565–577. [PubMed: 23241891]
64. Okenwa C, Kumar A, Rego D, Konarski Y, Nilchi L, Wright K, Kozlowski M. SHP-1-Pyk2-Src protein complex and p38 MAPK pathways independently regulate IL-10 production in lipopolysaccharide-stimulated macrophages. J Immunol. 2013; 191(5):2589–2603. [PubMed: 23904162]
65. Elcombe SE, Naqvi S, Van Den Bosch MW, MacKenzie KF, Cianfanelli F, Brown GD, Arthur JS. Dectin-1 regulates IL-10 production via a MSK1/2 and CREB dependent pathway and promotes

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the induction of regulatory macrophage markers. PLoS One. 2013; 8(3):e60086. [PubMed: 23533666]

66. Iyer SS, Ghaffari AA, Cheng G. Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. J Immunol. 2010; 185(11): 6599–6607. [PubMed: 21041726]
67. Carey RM, Padia SH. Role of angiotensin AT(2) receptors in natriuresis: Intrarenal mechanisms and therapeutic potential. Clin Exp Pharmacol Physiol. 2013; 40(8):527–534. [PubMed: 23336117]
68. Rehman A, Leibowitz A, Yamamoto N, Rautureau Y, Paradis P, Schiffrin EL. Angiotensin type 2 receptor agonist compound 21 reduces vascular injury and myocardial fibrosis in stroke-prone spontaneously hypertensive rats. Hypertension. 2012; 59(2):291–299. [PubMed: 22184324]
69. Carey RM, Howell NL, Jin XH, Siragy HM. Angiotensin type 2 receptor-mediated hypotension in angiotensin type-1 receptor-blocked rats. Hypertension. 2001; 38(6):1272–1277. [PubMed: 11751702]
70. Barber MN, Sampey DB, Widdop RE. AT(2) receptor stimulation enhances antihypertensive effect of AT(1) receptor antagonist in hypertensive rats. Hypertension. 1999; 34(5):1112–1116. [PubMed: 10567191]
71. Ali Q, Hussain T. AT_2 receptor non-peptide agonist C21 promotes natriuresis in obese Zucker rats. Hypertens Res. 2012; 35(6):654–660. [PubMed: 22297475]
72. Hilliard LM, Jones ES, Steckelings UM, Unger T, Widdop RE, Denton KM. Sex-specific influence of angiotensin type 2 receptor stimulation on renal function: a novel therapeutic target for hypertension. Hypertension. 2012; 59(2):409–414. [PubMed: 22158645]
73. Bosnyak S, Welungoda IK, Hallberg A, Alterman M, Widdop RE, Jones ES. Stimulation of angiotensin AT_2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats. Br J Pharmacol. 2010; 159(3):709–716. [PubMed: 20128808]
74. Paulis L, Becker ST, Lucht K, Schwengel K, Slavic S, Kaschina E, Thöne-Reineke C, Dahlof B, Baulmann J, Unger T, Steckelings UM. Direct angiotensin II type 2 receptor stimulation in Nω-nitro-L-arginine-methyl ester-induced hypertension: the effect on pulse wave velocity and aortic remodeling. Hypertension. 2012; 59(2):485–492. [PubMed: 22215717]
75. Gelosa P, Pignieri A, Fändriks L, de Gasparo M, Hallberg A, Banfi C, Castiglioni L, Turolo L, Guerrini U, Tremoli E, Sironi L. Stimulation of AT_2 receptor exerts beneficial effects in stroke-prone rats: focus on renal damage. J Hypertens. 2009; 27(12):2444–2451. [PubMed: 19680135]

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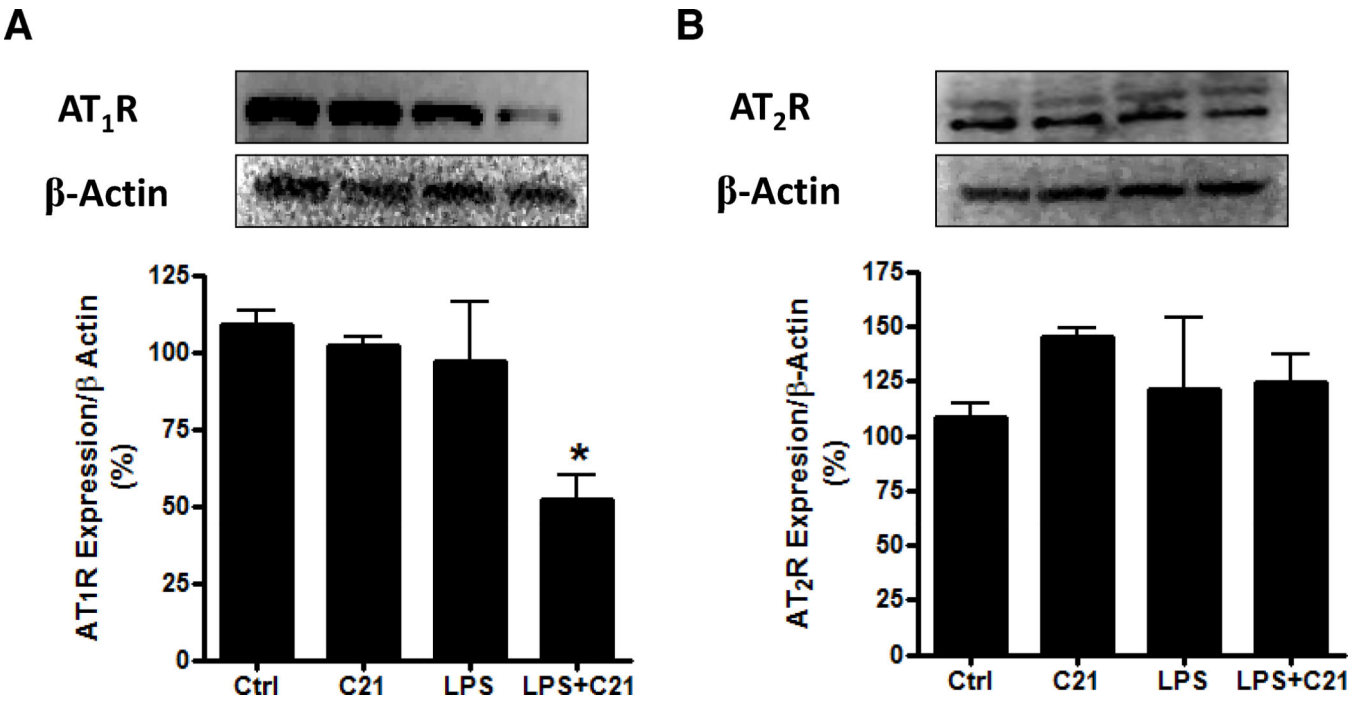

Figure 1. Expression of angiotensin II receptors in THP-1 macrophages

Protein expression by immunoblotting of angiotensin AT_1 (**A**) and AT_2 (**B**) receptors in THP-1 macrophages after 24 hours treatment with vehicle (Ctrl), AT_2R agonist C21 (C21; 1μmol/l), LPS (1μg/ml) or both LPS+C21. Cells were pre-treated with C21 (1μmol/l) for 60 minutes prior to LPS-activation. Data are represented as mean ± SEM. * indicates $p<0.05$ vs Ctrl THP-1 macrophages (n=5).

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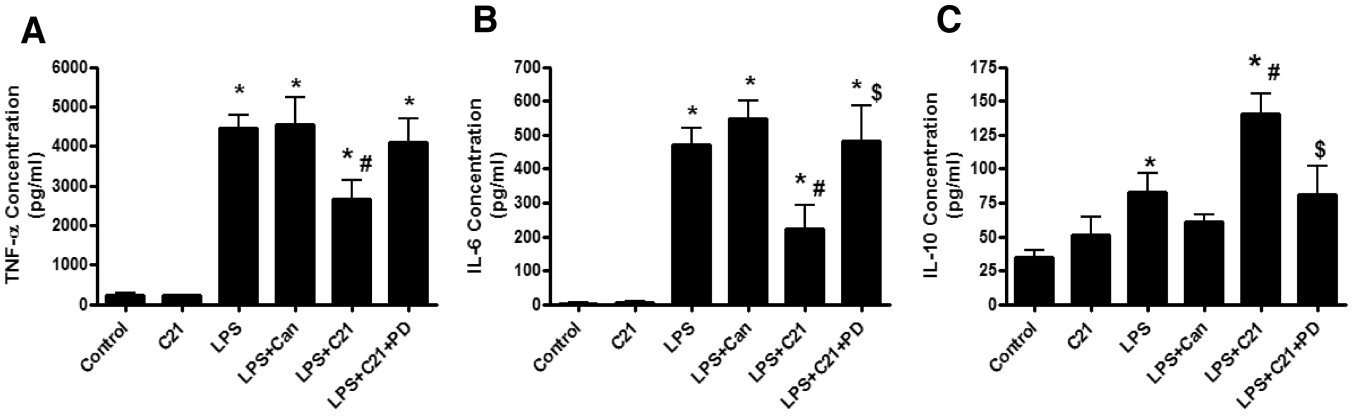

Figure 2. Effect of AT_2R agonist (C21) and AT_1R antagonist (Candesartan) on cytokine production by LPS-activated THP-1 macrophages

THP-1 macrophages were incubated with either AT_2R agonist (C21; 1μmol/l) or AT_1R antagonist Candesartan (Can; 1μmol/l) for 60 minutes prior to activation with LPS (1μg/ml). To demonstrate the specificity of C21, an additional group of cells were incubated with AT_2R antagonist PD123319 (PD; 10μmol/l) for 30 minutes prior to C21 pre-treatment. The cytokines TNF-α (**A**), IL-6 (**B**) and IL-10 (**C**) were assessed in the media 24 hours after LPS-activation by ELISA. Data are represented as mean ± SEM. * indicates $p<0.05$ vs Ctrl, # indicates $p<0.05$ vs LPS treated and **$** indicates $p<0.05$ vs LPS+C21 treated THP-1 macrophages (n=6).

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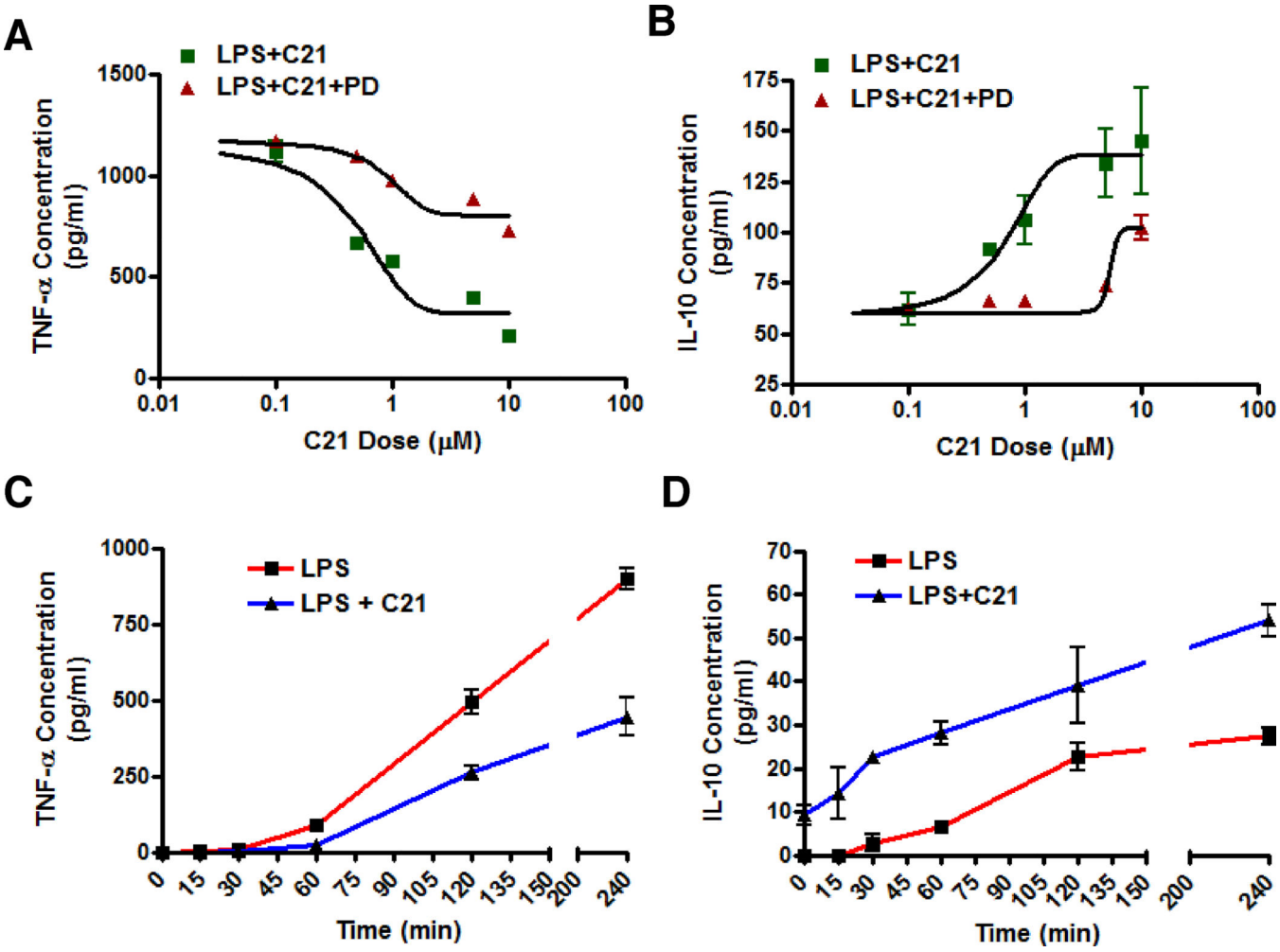

Figure 3. Dose-dependent and time-dependent effects of AT_2R agonist (C21) on cytokine production by LPS-activated THP-1 macrophages

The dose-dependent effect of AT_2R agonist C21 on TNF-α (**A**) and IL-10 (**B**) was assessed at 24 hours following LPS-activation (1μg/ml). C21 pre-treatment (0.1–10μmol/l) was given 60 minutes before LPS and cytokines in the media were assessed by ELISA. An additional set of cells was incubated with AT_2R antagonist PD123319 (PD; 10μmol/l) for 30 minutes prior to C21 pre-treatment to demonstrate the receptor specificity of C21. The TNF-α (**C**) and IL-10 (**D**) production at earlier time points was also determined in the media at the indicated times after LPS-activation following C21 (1μmol/ml) pretreatment. Data are represented as mean ± SEM. * indicates $p<0.05$ vs respective control (n=6).

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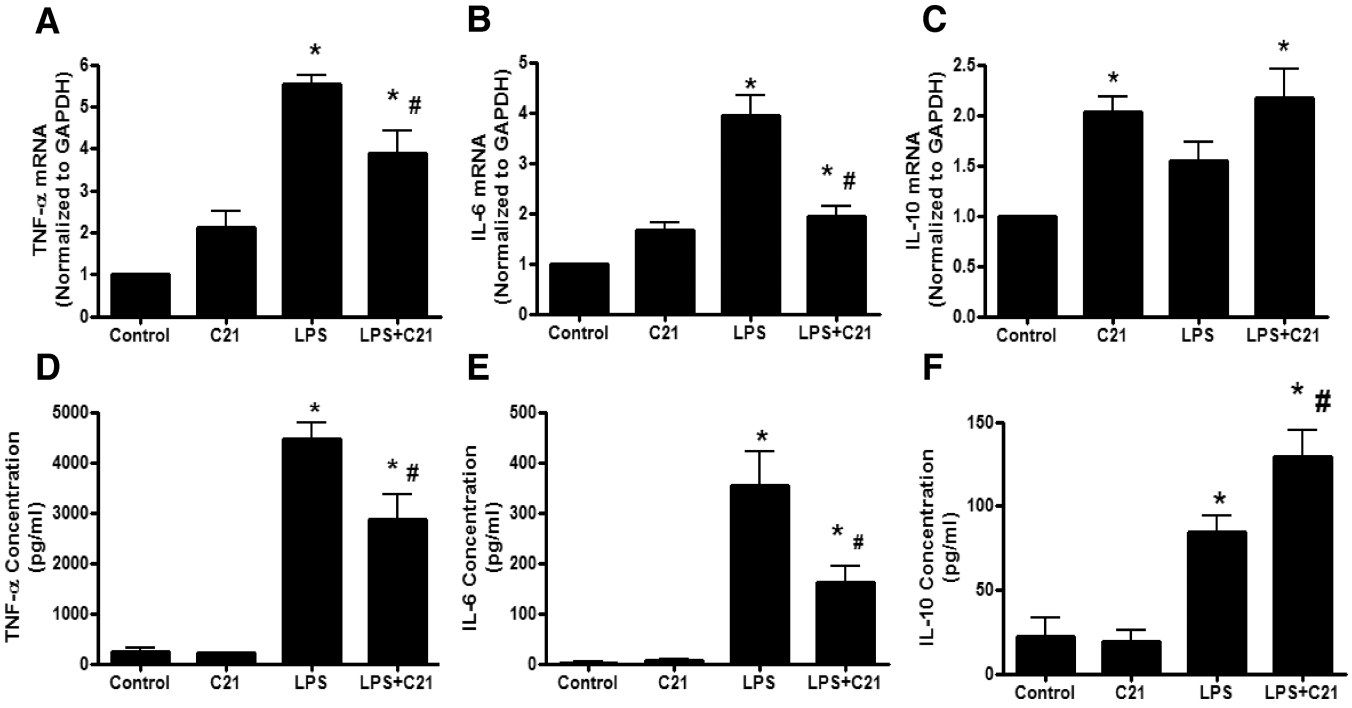

Figure 4. Effect of AT_2R agonist (C21) on mRNA and protein expression of cytokines by LPS-activated THP-1 macrophages

Macrophages were treated with vehicle (Control), C21 (1μmol/ml), LPS (1μg/ml) or both, LPS and C21 (LPS+C21). C21 (1μmol/l) pre-treatment was started 60 minutes prior to LPS-activation. Expression of TNF-α, IL-6 and IL-10 at the level of mRNA (**A, B, C**) was determined by RT-PCR while TNF-α, IL-6 and IL-10 protein was quantified in the media (**D, E, F**) by ELISA 24 hours after LPS-activation. Data are represented as mean ± SEM. * indicates $p<0.05$ vs Control and # indicates $p<0.05$ vs LPS treated THP-1 macrophages (n=5–8).

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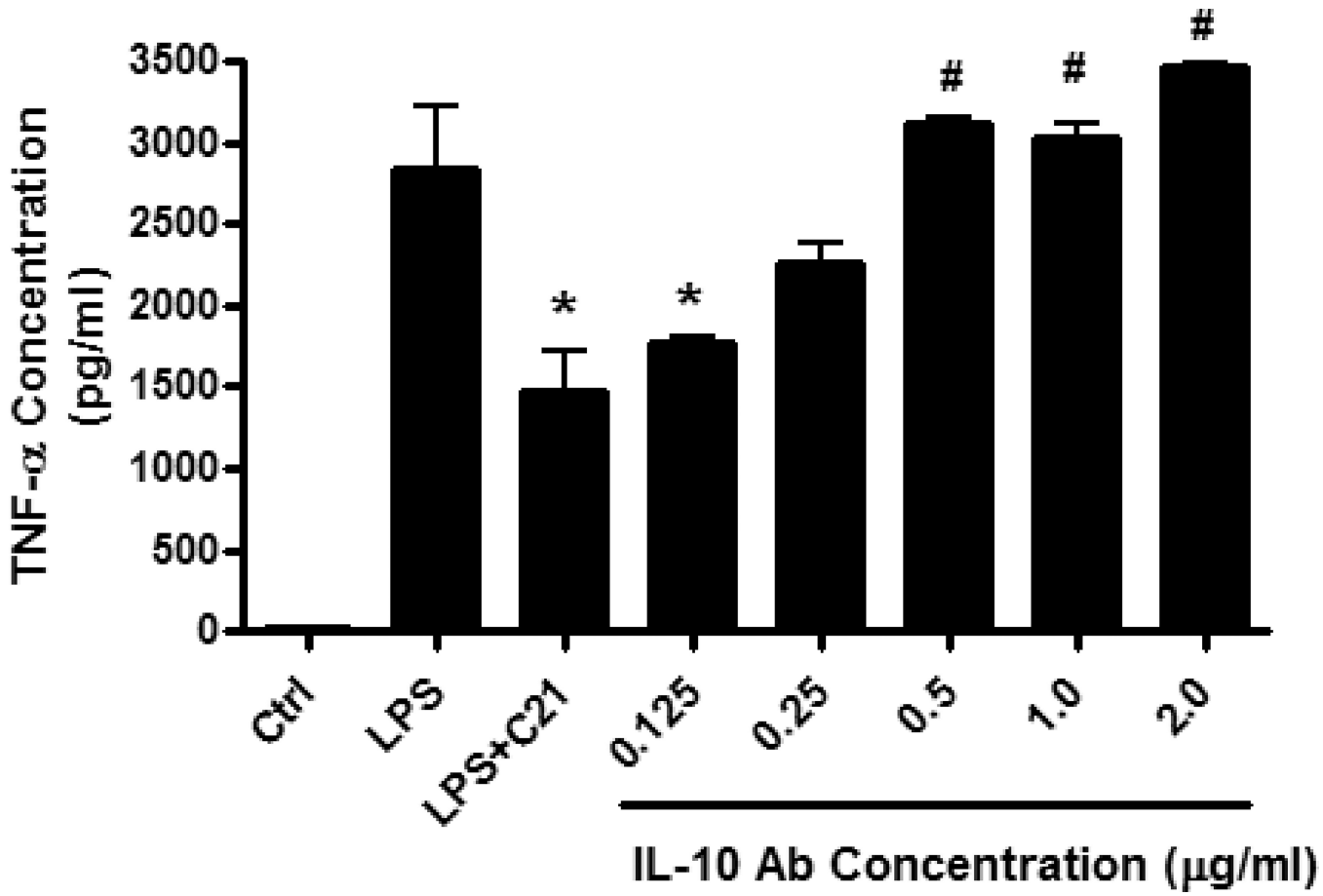

Figure 5. Dose-dependent inhibition of on the anti-inflammatory effect of AT_2R agonist (C21) on TNF- α production by neutralizing interleukin-10 (IL-10) antibody

Cells were incubated with increasing doses of neutralizing IL-10 antibody (0.125–2.5μg/ml) for 30 minutes prior to C21 pre-treatement (1μmol/l). Cells were activated with LPS (1μg/ml) 60 minutes after addition of C21. Cytokine concentrations in media were measured by ELISA. Data are represented as mean ± SEM. * indicates $p<0.05$ vs LPS, # indicates $p<0.05$ vs LPS+C21 treated THP-1 macrophages (n=5).

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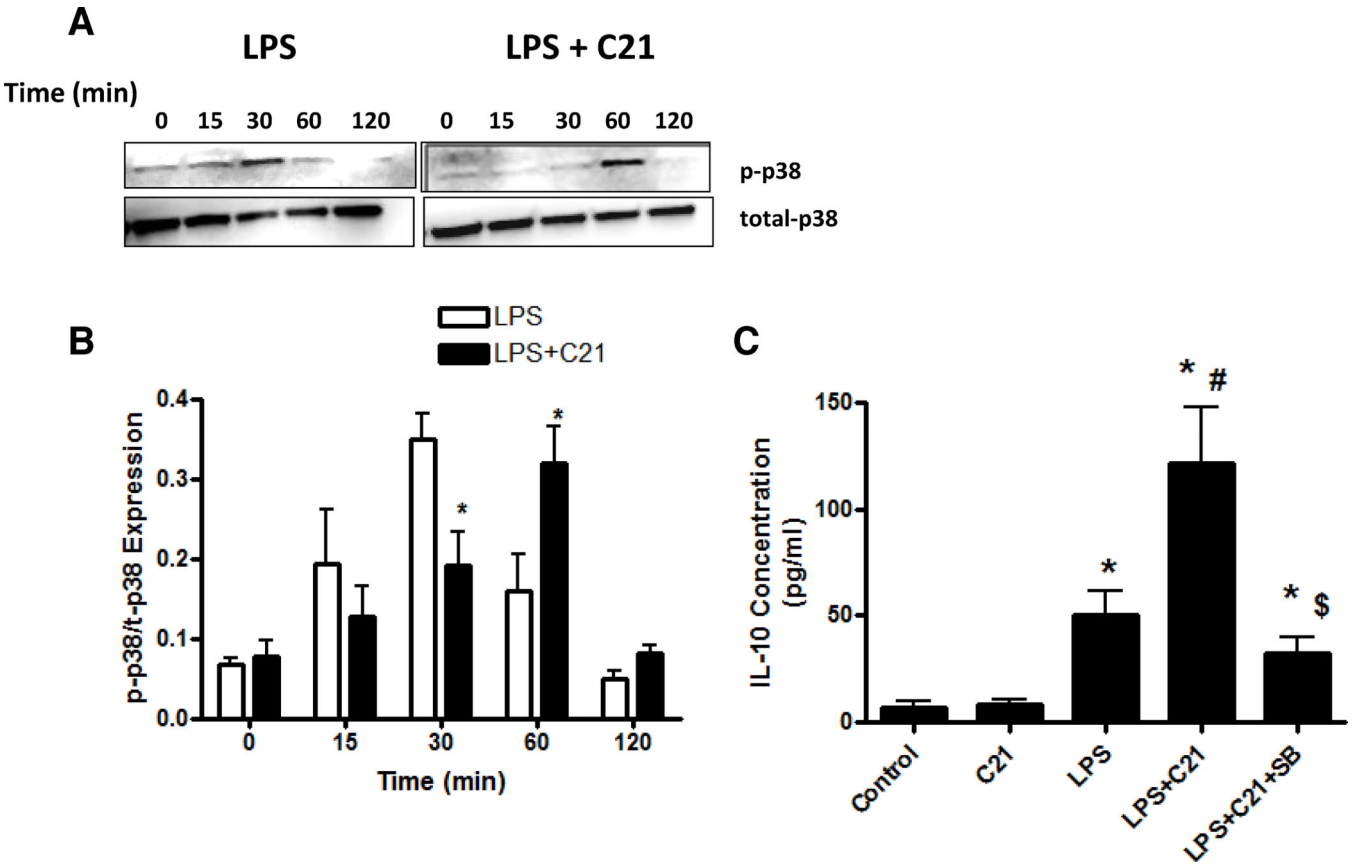

Figure 6. Effect of pre-treatment with AT_2R agonist (C21) on the phosphorylation of p38 MAPK

The time course of p38 phosphorylation in response to LPS-activation with or without C21 was determined by immunoblotting (**A**) and the ratio of phophorylated to toal p38 was quantified (**B**). C21 pre-treatment (1μmol/l) was started 60 minutes prior to the addition of LPS (1μg/ml). Times indicated are after the addition of LPS. The effect of p38 inhibition on IL-10 production was also assessed (C). Macrophages were activated with LPS (1μg/ml) 60 minutes after addition of C21 (1μmol/l). An additional set of cells was pre-treated with p38 inhibitor SB203580 (10μmol/l) for 60 minutes before C21 was added. Cytokine concentrations in media were measured by ELISA 24 hours after LPS treatment. Data are represented as mean ± SEM. * indicates $p<0.05$ vs Ctrl, # indicates $p<0.05$ vs LPS treated and **$** indicates $p<0.05$ vs LPS+C21 treated THP-1 macrophages (n=6).

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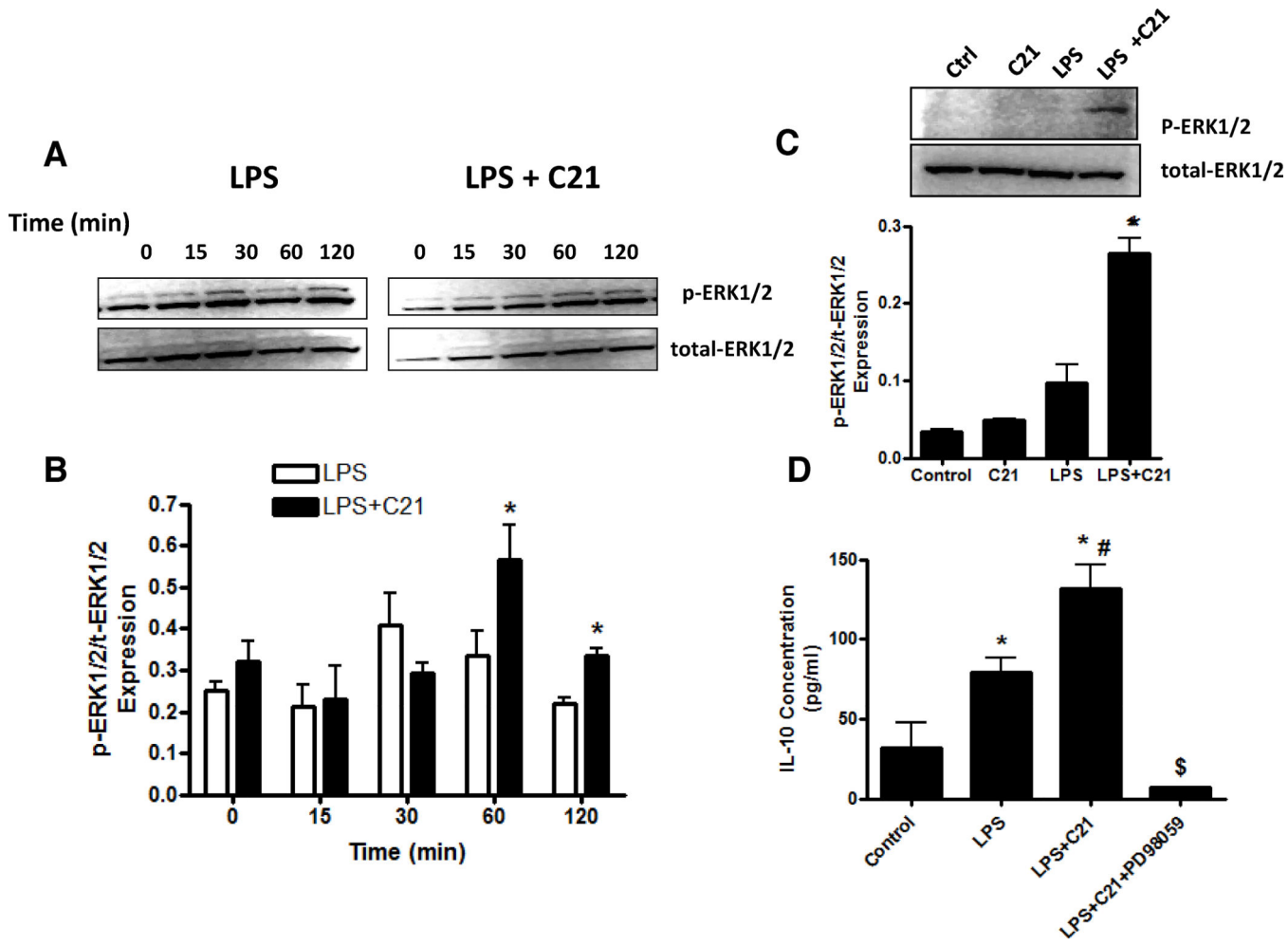

Figure 7. Effect of pre-treatment with AT_2R agonist (C21) on the phosphorylation of ERK1/2

The time course of ERK1/2 phosphorylation in response to LPS-activation with or without C21 was determined by immunoblotting (**A**) and the ratio of phophorylated to total ERK1/2 was quantified (**B**). C21 pre-treatment (1μmol/l) was started 60 minutes prior to the addition of LPS (1μg/ml). Times indicated are after the addition of LPS. To assess whether AT_2R agonist led to a sustained increase in ERK1/2 phosphorylation, macrophages were activated with LPS (1μg/ml) 60 minutes after addition of C21 (1μmol/l) and cells were collected after 24 hours post-LPS treatment. Subsequently, the ratio of phophorylated to total ERK1/2 was quantified by immunoblotting (**C**). The effect of inhibition of ERK1/2 phosphorylation on IL-10 production was also assessed (**D**). Macrophages were activated with LPS (1μg/ml) 60 minutes after addition of C21 (1μmol/l). An additional set of cells was pre-treated with MEK inhibitor PD98059 (10μmol/l) for 60 minutes before C21 was added. Cytokine concentrations in media were measured by ELISA 24 hours after LPS treatment. Data are represented as mean ± SEM. * indicates $p<0.05$ vs Ctrl, # indicates $p<0.05$ vs LPS treated and **$** indicates $p<0.05$ vs LPS+C21 treated THP-1 macrophages (n=6).