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Angiotensin II Type-2 Receptors Modulate Inflammation Through Signal Transducer and Activator of Transcription Proteins 3 Phosphorylation and TNFα Production

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Angiotensin subtype-1 receptor (AT_1R) influences inflammatory processes through enhancing signal transducer and activator of transcription proteins 3 (STAT3) signal transduction, resulting in increased tumor necrosis factor-α (TNF-α) production. Although angiotensin subtype-2 receptor (AT_2R), in general, antagonizes AT_1R-stimulated activity, it is not known if AT_2R has any anti-inflammatory effects. In this study, we tested the hypothesis that AT_2R activation plays an anti-inflammatory role by reducing STAT3 phosphorylation and TNF-α production. Changes in AT_2R expression, TNF-α production, and STAT3 phosphorylation were quantified by Western blotting, Bio-Plex cytokine, and phosphoprotein cellular signaling assays in PC12W cells that express AT_2R but not AT_1R, in response to the AT_2R agonist, CGP-42112 (CGP, 100 nm), or AT_2R antagonist PD-123319 (PD, 1 μm). A 100% increase in AT_2R expression in response to stimulation with its agonist CGP was observed. Further, AT_2R activation reduced TNF-α production by 39% and STAT3 phosphorylation by 83%. In contrast, PD decreased AT_2R expression by 76%, increased TNF-α production by 84%, and increased STAT3 phosphorylation by 67%. These findings suggest that increased AT_2R expression may play a role in the observed decrease in inflammatory pathway activation through decreased TNF-α production and STAT3 signaling. Restoration of AT_2R expression and/or its activation constitute a potentially novel therapeutic target for the management of inflammatory processes.

Chronic activation of inflammatory pathways likely influences the pathogenesis of many common and disabling diseases in older adults, including cardiovascular diseases, inflammatory and autoimmune disorders, neurodegenerative conditions, infection, and cancer (Fujita and others 1996; Ershler and Keller 2000; Maggio and others 2006). The role of the renin–angiotensin system in the development of chronic inflammation observed in older adults is still being elucidated. Angiotensin II (Ang II) acts through 2 G-protein-coupled receptor subtypes: angiotensin subtype-1 receptor (AT_1R) and angiotensin subtype-2 receptor (AT_2R). These receptors have substantial differences in tissue distribution and intracellular signaling pathways (Carey and Siragy 2003). The activation of AT_1R leads to a powerful pro-inflammatory effect (Suzuki and others 2003), induction of reactive oxygen species (Nickenig and Harrison 2002), hypertrophy, and apoptosis (Bascands and others 2001) and stimulation of fibroblast proliferation and collagen synthesis (Cipollone and others 2004). By contrast, AT_2R exerts effects that are the opposite of AT_1R, including anti-inflammatory (Matsubara 1998), anti-proliferative (Matsubara 1998), and anti-apoptotic actions (Bascands and others 2001).

Although blocking AT_1R results in decreased production of the tumor necrosis factor-α (TNF-α) *in vitro* (Hahn and others 1994; Beasley 1997; Han and others 1999; Tsutamoto and others 2000; Siragy and others 2003), it is unclear whether this results from direct inhibition of AT_1R or indirectly via unopposed AT_2R activation.

Ang II activates signal transducer and activator of transcription proteins 3 (STAT3) via AT_1R (Omura and others 2000). STAT3 is a key signal transduction protein that mediates cell differentiation, proliferation, apoptosis, inflammation, and tumor cell evasion of the immune system (Costantino and Barlocco 2008). Binding sites have been identified for STAT3 within the promoter region of TNF-α (Chappell and others 2000). Mutation of the 3 base pairs of the STAT3 binding site had considerable effects on TNF-α promoter activity, demonstrating that STAT3 upregulates TNF-α expression (Chappell and others 2000). Given this and the lack of knowledge as to how AT_2R influences inflammatory processes, we set out to determine the role of AT_2R activation in inflammation. We hypothesized that stimulation of AT_2R decreases TNF-α production through altered STAT3 signaling. We tested this hypothesis using PC12W

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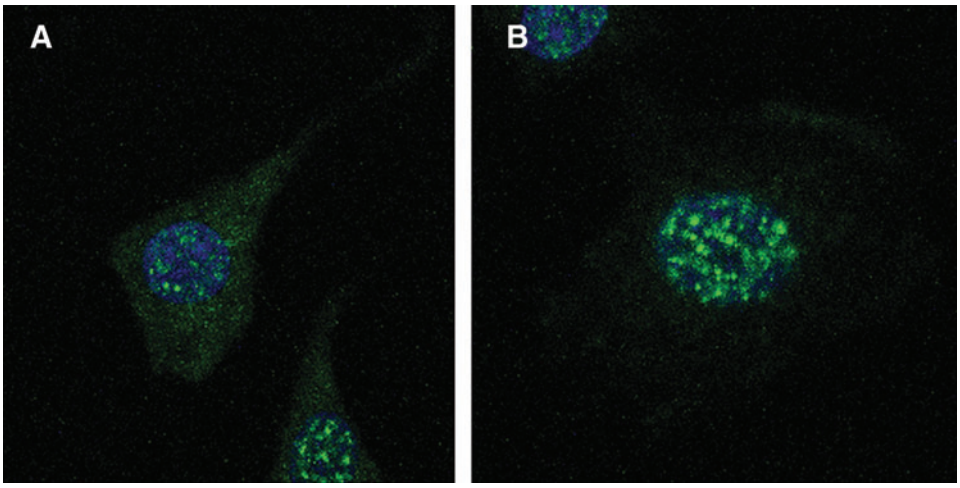

FIG. 1. Laser scanning confocal images of AT_2R in PC12W cells, **(A)** and **(B)** 100×magnification. Green fluorescent labeling for the AT_2R was detected on the cell membrane and in the nuclear and perinuclear region of the PC12W cells. AT_2R, angiotensin subtype-2 receptor.

cells that exclusively express AT_2R in the absence of AT_1R, to exclude the effects of any crosstalk between AT_1R and AT_2R on STAT3 signaling and TNF-α production (Omura and others 2000).

Northern and Western blot analyses were used to validate that AT_1R are not expressed in PC12W cells (data not shown). The Biorad Radiance 2100 laser scanning confocal/multiphoton microscopy system (Mills and others 2003) was utilized to identify AT_2R on the cell membrane and in the perinuclear and nuclear region of serum-starved PC12W cells (Fig. 1). The system consists of a Nikon TE300 epifluorescence microscope, a Plan Fluor 100×NA 1.4 oil immersion objective lens, argon ion laser (457,488,514), HeNe 543, and 633 nm (www.cellscience.bio-rad.com). The anti-receptor antibodies for AT_2R reacted specifically with AT_2R (Ozono and others 1997, 2000; Wang and others 1998). AT_2R antibodies were labeled with Alexa fluorophores 488 to demonstrate localization of AT_2R using confocal microscopy with higher spatial resolution beyond the limits of conventional microscopy. Images are representative of 3 experiments.

To determine the percentage change in AT_2R expression after administration of AT_2R agonist CGP-42112A or antagonist PD-123319, PC12W cells were treated with AT_2R agonist CGP42112 (CGP) 100 nm or antagonist PD123319 (PD) 1 μm for 24 h. Western immunoblot detection with anti-AT_2R in PC12W cells was used to quantify protein expression. Differences between groups were quantified using densitometry (Bio Rad GS-670 Imaging Densitometer). Stimulation of AT_2R led to an ∼100% increase in the expression of AT_2R, whereas inhibition led to a 76% decrease in expression of AT_2R (Fig. 2A, B). Previous studies demonstrated an increase in AT_2R expression during this receptor stimulation with Ang II (Shibata and others 1997; Zahradka and others 1998). AT_2R stimulation seems to increase the AT_2R gene promoter activity, an effect that was prevented by AT_2R blockade with PD (De Paolis and others 1999). Our current study is consistent with these previous studies and confirms that AT_2R stimulation enhances its gene activity. Thus, AT_2R stimulation may have a positive feedback on its expression.

To determine whether direct AT_2R stimulation influences STAT3 phosphorylation, PC12W cells were treated with AT_2R agonist CCGP42112A (CGP, 100 nmol), or AT_2R antagonist PD123319 (PD, 1 μmol) for 24 h. STAT3 phorphorylation status was determined using bead-based multiplex Luminex xMAP technology assays (Bio-Rad Laboratories, Hercules, CA) to directly detect phosphorylated proteins STAT3 in lysates derived from cell culture using highly specific antibodies (Cell Signaling Technology, Inc., Danvers, MA). Data from the reaction were acquired using the Bio-Plex suspension array system. Activation of AT_2R was associated with 83% decrease in phosphorylation of STAT3 (288 ± 24 U at base line to 49 ± 4 U), whereas inhibition was associated with a 68% increase in STAT3 phosphorylation (288 ± 24 U baseline to 484 ± 28 U [$P < 0.0001$]) (Fig. 3A).

To demonstrate the effects of AT_2R agonist or antagonist on the production of TNF-α. The production of TNF-α was

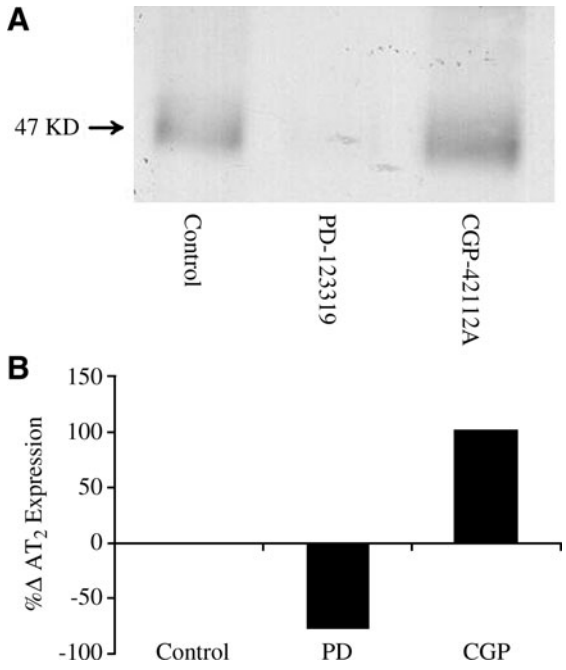

FIG. 2. Immunoblot quantification **(A)** of AT_2R expression in response to AT_2R agonist CGP-42112A (CGP, 100 nmol, □) or AT_2R antagonist PD-123319 (PD, 1 μmol, ■) for 24 h using immunoblot detection with anti-AT_2R in PC12W cells. Bar graphs **(B)** represent the means of the band densities expressed as percentage change from control. Blots are representative of 3 experiments.

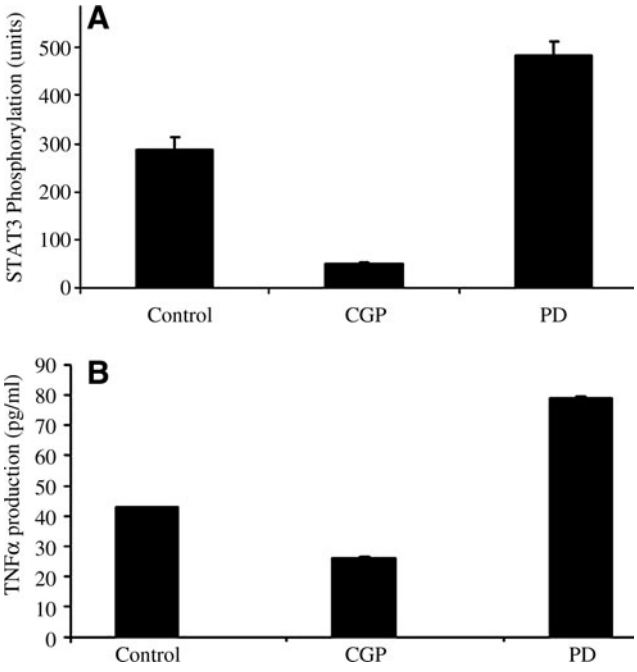

FIG. 3. **(A)** Change in STAT3 phosphorylation in response to treatment with the AT_2R agonist CGP42112A (CGP, 100 nmol) or AT_2R antagonist PD123319 (PD, 1 μmol) for 24 h. Data acquired using the Bio-Plex suspension array system. **(B)** Change in the production of TNF-α in PC12W cells in response to AT_2R agonist CGP-42112A (CGP, 100 nmol) or AT_2R antagonist PD-123319 (PD, 1 μmol) for 24 h. Data shown are average of 3 experiments. STAT3, signal transducer and activator of transcription proteins 3; TNF-α, tumor necrosis factor-α.

measured using Bio-Plex cytokine bead-based assays (xMAP technology) involving diverse matrices that are designed to quantify multiple cytokines. PC12W cells were incubated with AT_2R agonists or antagonists for 24 h, lysed, and centrifuged as per the manufacturer's protocol. Using these 96-well-plate-format assays, we profiled the production of TNF-α. Data from the reaction were acquired using the Bio-Plex suspension array system described above. CGP-42112A stimulation of AT_2R led to a 39% reduction of TNF-α production as compared to the control (42.79 ± 0.179 pg/mL to 26 ± 0.56 pg/mL; $P < 0.0001$). Inhibition of AT_2R caused an 84% increase in TNF-α production (42.79 ± 0.179 pg/mL to 78.82 ± 0.71 pg/mL; $P < 0.0001$) (Fig. 3B).

In this study we demonstrated that stimulation of AT_2R in AT_1R-deficient PC12W cells with an AT_2R agonist upregulates receptor expression level, reduces STAT3 phosphorylation, and reduces TNF-α production. Further, specific antagonism of these same receptors results in decreased AT_2R expression, increased STAT3 phosphorylation, and increased TNF-α production. This result suggests that AT_2R, like AT_1R, may be important in modulating inflammatory pathway activation, and that alterations in ratio of AT_2R to AT_1R may play an important role in chronic inflammatory pathway activation such as those observed in some older adults (Warnholtz and others 1999; Siragy and others 2003; Suzuki and others 2003).

The findings related to STAT3 phosphorylation and TNF-α production are supported by previously published reports demonstrating that mutations of the STAT3 binding sites have considerable effects on the promoter region of TNF-α (Chappell and others 2000). Also, TNF-α induces the tyrosine phosphorylation and DNA binding activity of STAT3 (Guo and others 1998; Miscia and others 2002), suggesting a positive feedback mechanism. Our data that AT_2R stimulation decreases TNF-α expression fit well with AT_2R's presumed vasodilator and cardiovascular protective effects (Siragy and Carey 1999; Siragy and others 2000). Given that the PC12W cells are devoid of AT_1R, the observed effects of stimulation or blockade of AT_2R are receptor-specific and cannot be a result of a receptor cross-talk with the AT_1R.

Previous studies confirmed that AT_1R blockade increases AT_2R activity *in vivo* (Weber 1992; Guan and others 1996), leading to decreased remodeling and cardiac fibrosis, which were completely abolished by simultaneous AT_2R blockade. This suggests that such effects are the result of AT_2R activation rather than AT_1R blockade (Barber and others 1999; Siragy and others 2000; Carey and others 2001; Oishi and others 2006). This also supports a potential role for AT_1R blockers to modulate inflammatory pathways through upregulating AT_2R.

In conclusion, we provide first evidence that AT_2R modulates STAT3 signaling and TNF-α production in a cell line devoid of AT_1R. Further studies in cell lines with AT_1R or in animal models are necessary to further understand the interactions between these receptor subtypes and inflammation.

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Author Disclosure Statement

No competing financial interests exist.

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