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Angiotensin II induces cyclooxygenase 2 expression in rat astrocytes via the angiotensin type 1 receptor

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ABSTRACT

We previously showed that Angiotensin (Ang) II stimulated pro-inflammatory and mitogenic actions in astrocytes suggesting that astrocytes are emerging as key players in neuroinflammation. Evidence suggests that neuroinflammation may contribute to central sympathetic overactivity and elevated blood pressure. Further, cyclooxygenase (Cox)-derived prostanoids were implicated in Ang II-dependent hypertension. Cox2 is one of two Cox isoenzymes that is responsible for the formation of prostanoids from arachidonic acid. Constitutively expressed Cox2 has a protective and homeostatic role in the cardiovascular and renal systems. Inducible Cox2 has been associated with pathogenic stimuli resulting in inflammatory conditions and cancers. In this study, we investigated the effect of Ang II on Cox2 protein and mRNA expression in brainstem and cerebellum astrocytes, and determined whether any differences in Cox2 expression exist in spontaneously hypertensive rat (SHR) astrocytes compared to their normotensive control Wistar rats. We demonstrated that Ang II increased Cox2 protein and mRNA levels relative to untreated controls in a time-dependent manner, in Wistar and SHR brainstem and cerebellum astrocytes. Increases in Cox2 protein expression were evident within 4 h, with subsequent sustained elevation for several hours followed by a decline at 48 h. Ang II-induced Cox2 protein levels were higher in Wistar compared to SHRs in both brainstem and cerebellum astrocytes for the majority of time points examined. The Ang II-induced Cox2 mRNA levels increased within 8 h followed by a rapid decline to almost basal levels at later time points. At the earlier time points, Cox2 mRNA elevation were higher in SHR compared to Wistar rat astrocytes. These Ang II actions were mediated by the Ang type I receptor. Our results corroborate previous reports of Ang II's ability to stimulate neuroinflammatory mediators in astrocytes. Cox2-derived prostaglandins might play a role in brain-renin angiotensin system associated hypertension, and astrocytes could be significant players.

1. Background

Angiotensin (Ang) II, the major biological peptide produced by the renin angiotensin system (RAS), induces the production of various pro-inflammatory cytokines, contributing to impaired neuronal signaling, blood-brain barrier breakdown and sustained neuroinflammation (Haspula and Clark, 2018a, 2018b). Ang II also stimulates proinflammatory actions in astrocytes via multiple signaling cascades suggesting an important role of astrocytes in neuroinflammation (Alanazi et al., 2014, Gowrisankar and Clark, 2016). Neuroinflammation contributes to central sympathetic overactivity and elevated blood pressure (Haspula and Clark, 2018a, 2018b). Thus, an overactive brain RAS may adversely affect central regulation of blood pressure by inducing and/or sustaining neuroinflammation. Astrocytes are now considered significant contributors to neuroinflammation by a complex interplay with neurons that involves autocrine and paracrine signaling. Astrocytes become reactive in response to inflammatory insults, stimulating their proliferation and the production of inflammatory mediators (Sofroniew and Vinters, 2010; Clark et al., 2011). We have previously shown that Ang II via the Ang type I receptor (AT1R) induces

Abbreviations: Ang, angiotensin; AT1R, Angiotensin type I receptor; AT2R, Angiotensin type 2 receptor; BCA, bicinchoninic acid assay; Cox2, Cyclooxygenase 2; DMEM/F12, Dulbecco's modified eagle medium/ F12; EP1, Prostaglandin EP1 receptor; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; NFκb, nuclear factor kappa-light-chain-enhancer of activated B cells; PG, prostaglandin; qPCR, quantitative polymerase chain reaction; RAS, renin angiotensin system; SHR, spontaneously hypertensive rat; TBI, traumatic brain injury; TBST, Tris buffered saline containing 0.1% Tween 20; TGFβ1, transforming growth factor β1; VSMC, vascular smooth muscle cells; WKY, Wistar Kyoto.

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interleukin-6 (IL-6) in rat astrocytes via a nuclear factor kappa b-(NFκb)-mediated mechanism (Gowrisankar and Clark, 2016). NFκb regulates the production of numerous cytokines and inflammatory mediators including cyclooxygenase (Cox) 2 (Poligone and Baldwin, 2001). Cox2 also known as prostaglandin H synthase, is one of two isoenzymes that are responsible for the formation of prostanoids (prostaglandins and thromboxanes) from arachidonic acid (Poligone and Baldwin, 2001; Bos et al., 2004; Ricciotti and FitzGerald, 2011). Cox2 is referred to as the inducible isoform and is also constitutively expressed in a number of tissues. Prostaglandins are bioactive lipids that have divergent homeostatic physiological and pathological functions (Ricciotti and FitzGerald, 2011). There are four main biologically active prostaglandins PGE_2, PGD_2, PGI_2 and $PGF_2\alpha$ (Ricciotti and FitzGerald, 2011). The distribution of prostaglandins depend on the cell type and cellular context. Their levels and expression patterns are markedly altered in inflammatory conditions (Ricciotti and FitzGerald, 2011). The PGE_2 prostaglandin is the major pro-inflammatory mediator; other prostaglandin types may also have pro-inflammatory effects depending on the context and prostaglandin receptor that is activated (Poligone and Baldwin, 2001; Ricciotti and FitzGerald, 2011). Cox2 may therefore exhibit both pro- and anti-inflammatory actions depending on the tissue and the final prostaglandins that are produced (Gilroy et al., 1999; Poligone and Baldwin, 2001).

Previous studies have demonstrated that differences exist in Ang II effects on Cox2 depending on the tissue. In renal tissue, Ang II decreased Cox2 expression (Cheng et al., 1999). In addition, inhibition of the RAS using AT1R blockers resulted in an elevation of Cox2 levels (Wolf et al., 1999). This highlights the complex interplay between the Cox and RAS in the regulation of blood pressure. Ang II induces Cox2 in vascular smooth muscle cells (VSMCs) (Hu et al., 2002) and other cell types (Slice et al., 2005; Beltrán et al., 2009). Tissue differences in Ang II effects are not unusual, similar differences were observed in Ang II effects on iron homeostasis in astrocytes compared to peripheral tissue (Huang et al., 2014). Even though Cox2 is the inducible form of the enzyme, in the brain, stomach and renal tissues it is constitutively expressed (Kirkby et al., 2016). Constitutively expressed Cox2 has protective and homeostatic roles in the cardiovascular and renal systems (Kirkby et al., 2016). Inducible Cox2 is associated with numerous CNS insults including traumatic brain injury (TBI), inflammatory conditions, cancers and pathogenic stimuli (Harris, 2003; Salinas et al., 2007). Therapeutic strategies inhibiting Cox2 production, increases the risk of cardiovascular events (Funk and FitzGerald, 2007). This increased cardiovascular risk has been attributed to the inhibition of not only the inducible, but also the constitutive actions of Cox2, leading to altered expression of prostanoids (Funk and FitzGerald, 2007). Over the past several years, many studies have focused on understanding the physiological and pathophysiological roles of Cox2 and its isoform Cox1. Cox1 is referred to as the constitutive isoform and is expressed in many tissues (Kam and See, 2000). However, there is evidence that Cox1 may also be inducible and may contribute to inflammatory conditions. In addition, Cox1 expression may be upregulated as a compensatory mechanism when Cox2 is inhibited (Kirtikara et al., 1998). Therefore, although some progress has been made in elucidating the roles of the Cox isoforms, some controversies still exist. This may very well be due to the temporal, cell specific or context specific actions of the enzymes. In addition to possible distinct cellular effects, the tissue pattern of Cox2 elevation may also differ depending on the type of stimuli.

The relationship between brain-RAS and Cox2 has the potential to influence central regulation of blood pressure and warrants examination. More specifically, the potent vasoactive and inflammatory hormone Ang II may induce Cox2 overexpression in astrocytes. If this occurs in brain areas responsible for cardiovascular regulation, this may lead to dysregulation of blood pressure by different mechanisms. Furthermore, the effect of Cox2 production is time-dependent; it is argued that initial upregulation may be proinflammatory whereas sustained upregulation may be anti-inflammatory or reparative (Gilroy et al., 1999; Ricciotti and FitzGerald, 2011). Other investigators have hypothesized that in brain injuries, the acute Cox2 response is beneficial whereas chronic elevation is harmful (Strauss et al., 2000; Gopez et al., 2005; Strauss, 2008). In human neonatal tissue, the A2 reactive astrocyte subtype was shown to express Cox2 and elicit harmful effects via PGE_2 and its receptor Prostaglandin E2 receptor 1 (EP1) (Shiow et al., 2017). In addition to reducing neuroinflammatory actions, Cox2 inhibition was shown to have direct pressor effects (Qi et al., 2002). Prostanoids derived from Cox regulate blood pressure, with specific prostanoids having contrasting effects on blood pressure (Bos et al., 2004). Moreover, the two Cox isoforms have been shown to have opposing effects on systemic blood pressure in response to Ang II stimulation (Qi et al., 2002).

This highlights the need for a better understanding of the precise roles of Cox enzymes in individual cell types. Since Cox2 is the isoform that is most commonly implicated in inflammatory conditions (Ricciotti and FitzGerald, 2011), our current study focuses on Cox2. The profile of the Cox-derived prostaglandins in distinct cell types, both under physiological and pathological conditions, would be beneficial in delineating the role of Cox enzymes in disease pathogenesis. Thus, it is essential to determine how Ang II regulates Cox2 in astrocytes, and whether Cox2 regulation differs in brain regions involved in autonomic control compared to other reference regions. To determine this, we used astrocytes isolated from the spontaneously hypertensive rat (SHR). The SHR is a widely used animal model of human essential hypertension (Okamoto and Aoki, 1963). SHRs have a pre-hypertensive phase during which they have normal blood pressure but, hormonal systems such as the RAS are already overactive (Judy et al., 1979; Fisher et al., 2009). We used astrocytes isolated from newborn SHRs to understand the development and progression of essential hypertension. This is important for the development of preventive treatments rather than therapies to treat established hypertension. The newborn SHRs have normal blood pressure however, RAS components are already overactive (Lais et al., 1977; Judy et al., 1979; Fisher et al., 2009).

Constitutively expressed Cox2 gene was previously observed in the brainstem and cerebellum of Cox2 luciferase knock-in reporter mice (Kirkby et al., 2016). Other studies have observed Cox2 upregulation in rat astrocytes treated with basic fibroblast growth factor and/or lipopolysaccharide (LPS) (Lichtenstein et al., 2012). These effects were independent of NFκb activation and mediated via ERK/JNK/FAK (Lichtenstein et al., 2012). Moreover, transforming growth factor β1 (TGFβ1) increased Cox1, Cox2 and PGE_2 in cultured astrocytes (Luo et al., 1998). The TGFβ1-induced Cox1 was absent in neuronal cell types. In addition, the production of PGE_2 was markedly higher in astrocytes compared to neurons (Luo et al., 1998). This further supported the hypothesis that astrocytes are major players in neuroinflammation. Taking into account these findings, upregulation of Cox2 in astrocytes may be a key mechanism in the neuroinflammatory responses of astrocytes to Ang II stimulation. How this mechanism may affect central regulation of blood pressure is not widely studied. Thus, we investigated the pattern of Ang II-induced Cox2 elevation in astrocytes isolated from two brain regions that control distinct functions. The brainstem regulates cardiovascular actions (Gordan et al., 2015) and the cerebellum is responsible for cognitive and motor control (Buckner, 2013). Ang II levels in the brainstem and cerebellum are elevated in SHRs compared to Wistar rats (Phillips and Kimura, 1988). We further determined if the Cox2 elevation pattern is different in SHR astrocytes compared to their normotensive controls (Wistar rats). If Cox2 stimulation by Ang II is dysregulated in astrocytes from the pre-hypertensive rats compared to their normotensive controls, this may provide insight on early pathological changes that may contribute to the development of hypertension.

2. Materials and methods

2.1. Reagents

The reagents were purchased from the sources indicated. Specifically, Ang II was purchased from Bachem (Torrance, CA, USA). AT1R antagonist, Losartan was provided by Du Pont Merck (Wilmington, DE, USA). The AT2R antagonist PD123319, and the anti-actin antibody were purchased from Sigma (St. Louis, MO, USA). The bicinchoninic acid assay (BCA) kit was purchased from Pierce Biotechnology (Rockford, IL, USA). All quantitative PCR (qPCR) supplies including the taqman primers, taqman master mix and cDNA kit were bought from Applied Biosystems (Foster City, CA, USA). Anti-Cox2 antibody, was purchased from Cell Signaling Technology (Danvers, MA, USA). Western blotting supplies were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other general laboratory supplies and reagents were obtained from Sigma (St. Louis, MO, USA), Fisher Scientific (Waltham, MA, USA) or VWR International (Suwanee, GA, USA).

2.2. Astrocyte cultures

Timed pregnant SHRs and Wistar rats, were purchased from Charles River Laboratory (Wilmington, MA, USA). The rats were housed at our Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility with water and food available ad libitum. All animal protocols were approved by the University Institutional Animal Care and Use committee and complied with the ethical treatment of animals as outlined in the NIH Guide for Animal Care and Use. Primary astrocyte cultures were prepared from the brainstem and cerebellum of 2–3 days old rat pups according to methods previously published (Tallant and Higson, 1997). Cultures were prepared from pooled brain regions. The pooled tissues were mechanically dissociated and grown in Dulbecco's modified eagle medium/ F12 (DMEM/F12) supplemented with 10% fetal bovine serum, 10 μg/ml penicillin and 100 units/ml streptomycin. Cells were incubated at 37 °C in a humidified CO_2 incubator with 5% CO_2 and 95% air. Culture media was changed every 3 to 4 days until 85% confluent. Confluent monolayers of astrocytes were shaken overnight to produce an astrocyte enriched system by removing oligodendrocytes and microglia. The cultures contained approximately 95% astrocytes based on qPCR, western blotting, and flow cytometry measurements as previously described (Haspula and Clark, 2016). Expression of the glial fibrillary acidic protein was used as a marker for astrocytes (Haspula and Clark, 2016). The astrocyte enriched cultures were made quiescent by placing in serum free media for 48 h prior to treatments.

2.3. Cell treatments and Western blot

To determine the effect of Ang II on Cox2 protein expression, quiescent cells were treated with 100 nM Ang II for 4 to 48 h. For Ang II receptor inhibition, the cells were treated with the AT1R inhibitor (10 μM Losartan) or the AT2R inhibitor (10 μM PD123319) alone and in combination with Ang II. Following treatments, cells were washed in phosphate buffered saline (PBS) containing sodium orthovanadate (0.01 mM). Cell lysates were then collected in mammalian protein extraction reagent supplemented with 0.01 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonylfluoride, and 0.6 μM leupeptin, pH 7.4. The lysates were centrifuged (12, 000 *g* for 10 min, 4 °C) and the protein concentrations determined using the BCA method according to manufacturer's instructions. Cell lysates without Ang II treatment were also collected and used as the controls.

2.4. Western blot analysis

30 μg of solubilized proteins were ran on 10% polyacrylamide gels then transferred to nitrocellulose membranes. Membranes were blocked in 5% non-fat milk dissolved in Tris buffered saline containing 0.1% Tween 20 (TBST). After blocking, the membranes were washed in TBST then exposed to primary antibody (anti-rat Cox2, 1:1000) overnight at 4 °C. The Cox2 immunoreactive bands were normalize to beta actin; thus, membranes were also exposed to anti-beta actin (1:5000). After incubation with primary antibodies, membranes were incubated with anti-rabbit secondary antibody (1:3000) for 1 h at 25 °C. After washing, the immunoreactive bands were visualized with enhanced chemiluminescence reagent and quantified using Image J software (National Institute of Health (NIH), Bethesda, MD, USA).

2.5. Cell treatment and quantitative polymerase chain reaction analysis

To determine the effect of Ang II on Cox2 mRNA expression, qPCR was used. Quiescent cells were treated with 100 nM Ang II for 4 to 24 h. For Ang II receptor inhibition, the cells were treated with the AT1R inhibitor (10 μM Losartan) or the AT2R inhibitor (10 μM PD123319) alone with or without Ang II. Total RNA was extracted from the cells using the trizol method. Total RNA concentrations were determined using the NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). A high capacity complementary strand DNA kit was used to reverse transcribe 2 μg of total RNA to complementary strand DNA. Taqman universal mastermix and Taqman primers for Cox2 and beta actin (Rn01483828 and Rn00667869, respectively) were used for the qPCR analysis. Samples were analyzed in triplicates in 96-well plates on the StepOne Plus Real time PCR system from Applied Biosystems. The comparative threshold cycle method was used for calculation of qPCR results (Livak and Schmittgen, 2001).

2.6. Statistical analysis

Data are expressed as mean ± SEM for 3 or more litters of pups or as indicated. *t*-tests or one-way analysis of variance with Dunnett's Post-test was used to compare Ang II treatment groups with controls. To compare Wistar and SHR samples, two-way analysis of variance with Bonferroni's Post-test was used. Statistical significance was set at $p < .05$.

3. Results

3.1. Cox2 basal expression in Wistar and SHR astrocytes

We determined the basal protein and mRNA expression of Cox2 in both Wistar and SHR astrocytes isolated from the cerebellum and brainstem. As shown in (Fig. 1A), the basal protein levels of Cox2 in brainstem astrocytes were lower in Wistar compared to SHR samples. For the cerebellum (Fig. 1B), Wistar astrocytes had higher basal Cox2 protein compared to SHR astrocytes. For both brainstem and cerebellum astrocytes, basal Cox2 mRNA expression was higher in SHR compared to Wistar astrocytes. The differences in the basal Cox2 mRNA levels between the two animal models were significantly different for both brain regions (Fig. 1C).

3.2. Effect of Ang II on Cox2 protein expression in astrocytes

Ang II increased Cox2 protein levels relative to untreated controls in a time-dependent manner in brainstem (Fig. 2A), and cerebellum (Fig. 2B) astrocytes isolated from both Wistar and SHRs. Increases in Cox2 expression were evident within 4 h, with a decrease to almost basal levels by 48 h. There were significant differences in Ang II-induced Cox2 protein levels between Wistar and SHR astrocytes in brainstem. The Wistar brainstem astrocytes had significantly higher Cox2 protein levels compared to SHR brainstem astrocytes. The pattern of Cox2 elevation induced by Ang II for cerebellar astrocytes was similar to that of brainstem. The Wistar cerebellar astrocytes had higher

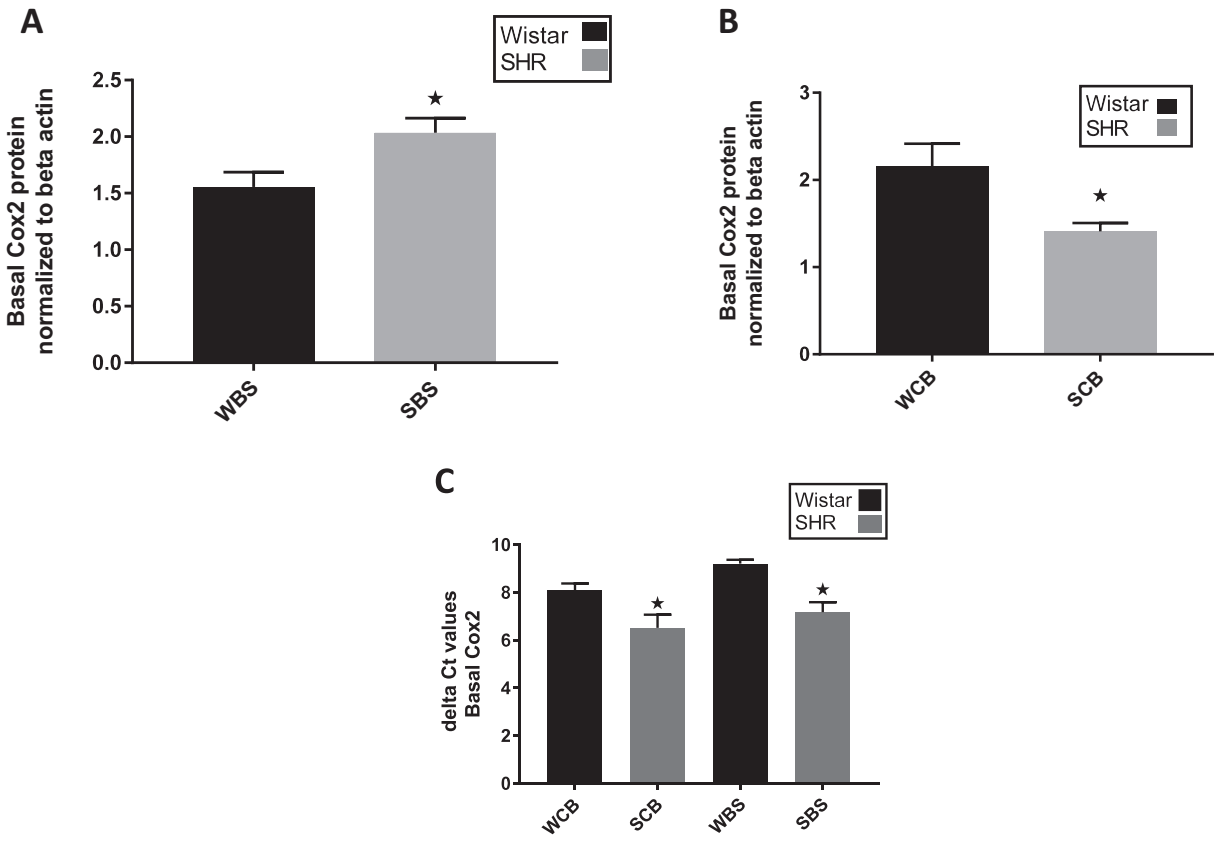

Fig. 1. Cox2 basal protein in brainstem (Fig. 1A) and cerebellum (Fig. 1B). Cox2 mRNA expression (Fig. 1C) in brainstem and cerebellum astrocytes isolated from SHRs and Wistar rats. Cox2 protein and mRNA levels were measured by Western blotting and qPCR, respectively as described. Protein and mRNA levels were normalized to beta actin, the internal control. The Y-axis represents arbitrary units after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 3 or more litters of neonatal rat pups. *represents $p < .05$ for SHR versus Wistar astrocytes using two-way analysis of variance. WBS: Wistar brainstem; WCB: Wistar cerebellum; SBS: SHR brainstem; SCB: SHR cerebellum.

Cox2 protein expression compared to SHR astrocytes at most time points examined.

For Ang II-induced Cox2 protein expression, protein levels were significantly greater in brainstem compared to cerebellum at most time points examined for Wistar astrocytes (Fig. 3A). For SHR astrocytes (Fig. 3B), there were no significant differences between Ang II effects in the brainstem versus cerebellum.

3.3. Effect of Ang II receptor blockers on Cox2 protein expression in astrocytes

Brainstem and cerebellar astrocytes were pre-treated with AT1R and AT2R receptor blockers with and without Ang II. The AT2R receptor blocker failed to inhibit Ang II elevation of Cox2 protein (Figs. 4 and 5). The AT1R blocker, losartan, significantly inhibited ($p < .05$) Ang II elevation of Cox2 protein in Wistar and SHR brainstem and cerebellum astrocytes. Thus, Ang II actions to induce Cox2 were mediated by the AT1R.

3.4. Effect of Ang II on Cox2 mRNA expression in astrocytes

Ang II increased Cox2 mRNA levels relative to untreated controls in a time-dependent manner in brainstem (Fig. 6A), and cerebellum (Fig. 6B) Wistar and SHR astrocytes. Ang II rapidly increased Cox2 levels within 4 h followed by a decrease to almost basal levels. In brainstem, for most of the time points examined, SHR astrocytes had higher Ang II-induced Cox2 elevation compared to Wistar astrocytes. For the cerebellum, Ang II-induced Cox2 levels were significantly higher in SHRs compared to Wistar rat astrocytes for the earlier time points.

For Ang II-induced Cox2 mRNA expression, there were no significant differences in Cox2 mRNA expression between the brainstem and cerebellum for Wistar rat astrocytes (Fig. 7A). For the SHR astrocytes (Fig. 7B), Cox2 mRNA levels were higher in brainstem astrocytes compared to cerebellum astrocytes at the 8 and 24 h time points.

3.5. Effect of Ang II receptor blockers on Cox2 mRNA expression in astrocytes

Brainstem and cerebellar astrocytes were pre-treated with AT1R and AT2R blockers with and without Ang II for 4 h. The AT2R blocker failed to inhibit Ang II elevation of Cox2 mRNA levels (Tables 1 & 2). The AT1R receptor blocker inhibited Ang II elevation of Cox2 mRNA in Wistar and SHR astrocytes for both brainstem and cerebellum. These findings suggest that Ang II actions on Cox2 mRNA levels were mediated by the AT1R.

4. Discussion

It is well established that Ang II stimulates pro-inflammatory actions in astrocytes and other cell types. In addition, an exaggerated brain-RAS has a role in the pathogenesis of hypertension and other cardiovascular diseases. The association between an exaggerated brain-RAS and neuroinflammation is gaining interest. It is believed that neuroinflammation may exacerbate or induce the hypertensive state. As a result, an exaggerated brain-RAS may contribute to hypertension by a number of mechanisms. The Cox system interacts with the RAS in the periphery to regulate blood pressure (Jaiswal et al., 1993; Meune et al.,

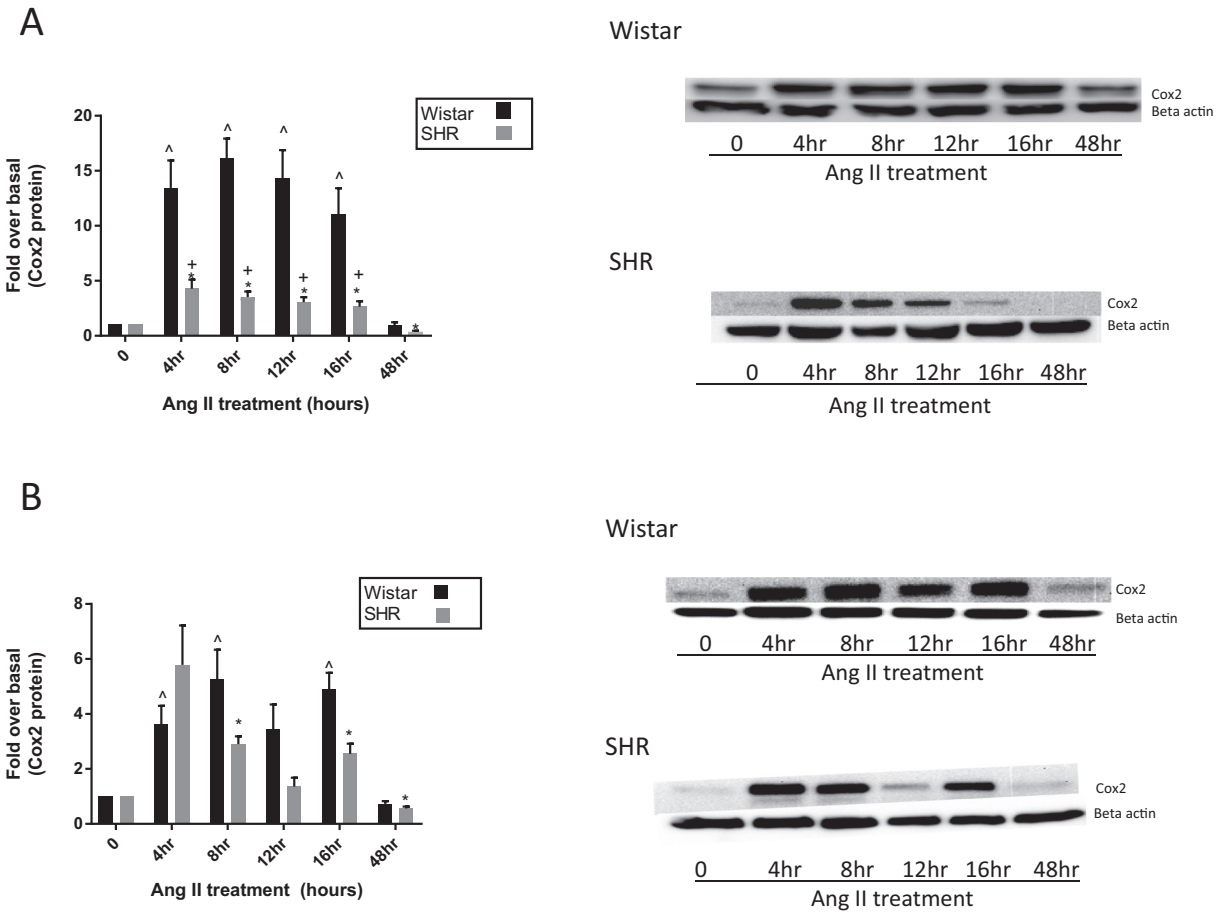

Fig. 2. Effect of time on Ang II-induced Cox2 protein expression in brainstem (Fig. 2A) and cerebellum (Fig. 2B) astrocytes isolated from SHRs and Wistar rats. Quiescent astrocytes were treated with 100 nM Ang II for 4 to 48 h. Cox2 protein was measured by Western blotting as described. Protein levels are expressed as fold change over basal after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 4 or more litters of neonatal rat pups. ^represents $p < .05$ compared to basal Wistar astrocytes. *represents $p < .05$ compared to basal SHR astrocytes. $^{+}$represents $p < .05$ for SHR compared to Wistar rat astrocytes using two-way analysis of variance.

2003; Gawrys et al., 2018). This interaction in the brain is not well studied and requires further examination. Moreover, astrocytes are known to be a major source of prostaglandins in the brain (Arimura et al., 1989). We have previously shown that Ang II stimulated pro-inflammatory cytokines and enzymes in the brainstem and cerebellum of rat astrocytes (Alanazi et al., 2014; Gowrisankar and Clark, 2016). Also, we recently showed that the reactive oxygen species inhibitor YCG063 and the NFκb inhibitor BAY 11–7082 abolished Ang II-mediated IL-6 expression in brainstem and cerebellar astrocytes (Gowrisankar and Clark, 2016). This highlights a role for NFκb in Ang

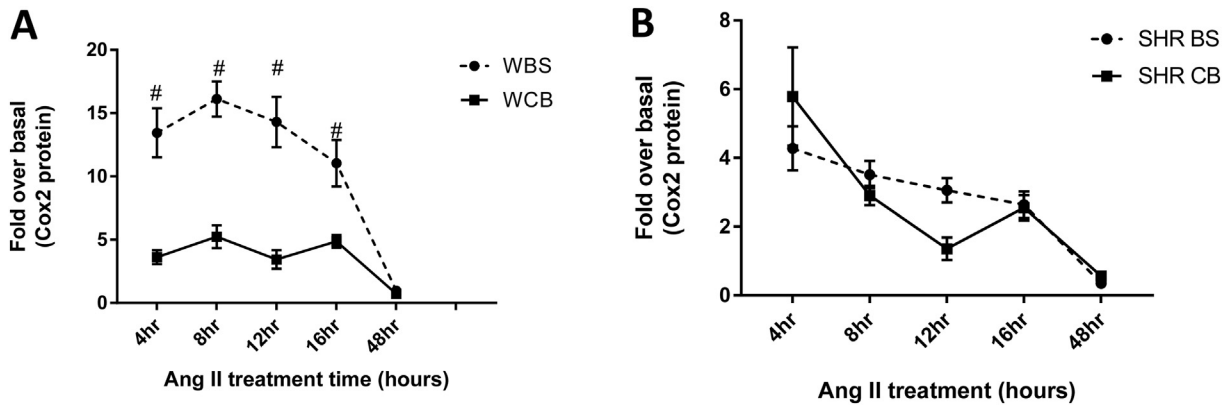

Fig. 3. Comparison of Ang II-induced Cox2 protein expression patterns in distinct brain regions in astrocytes isolated from Wistar rats (Fig. 3A) and SHRs (Fig. 3B). Quiescent astrocytes were treated with 100 nM Ang II for 4 to 48 h. Cox2 protein levels were measured by Western blotting as described. Cox2 protein levels are expressed as fold change over basal after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 4 or more litters of neonatal rat pups. #represents $p < .05$ for brainstem versus cerebellum using two-way analysis of variance. WBS: Wistar brainstem; WCB: Wistar cerebellum; SHR BS: SHR brainstem; SHR CB: SHR cerebellum.

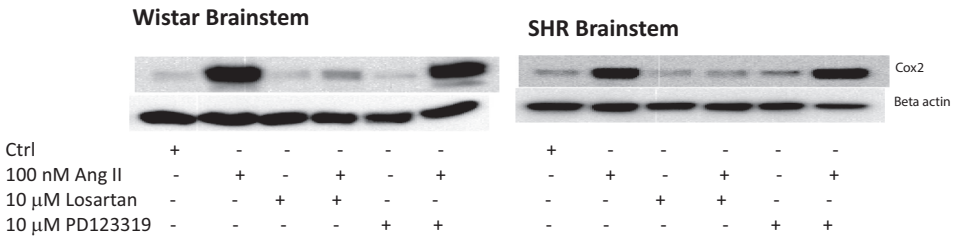

Fig. 4. Effect of Ang II receptor blockers on Cox2 protein expression in brainstem astrocytes from Wistar rats and SHRs. Quiescent brainstem astrocytes were treated with AT1R (10 μM Losartan) and AT2R (10 μM PD123319) receptor blockers with and without 100 nM Ang II for 4 h. Cox2 protein was measured by Western blotting as described. Cox2 protein levels were determined as fold change over basal after normalization to beta actin. Preparations of brainstem astrocytes from 3 or more litters of neonatal rat pups were used in the study. Statistical significance was set at $p < .05$.

II-mediated pro-inflammatory effects in astrocytes. Ang II activates NFκb in numerous cell types including VSMCs. NFκb is a ubiquitous transcription factor that regulates immune and inflammatory responses (Barnes and Karin, 1997) including the expression of Cox2. Blockade of NFκb activity similar to inhibition of Cox2 has harmful effects because both have essential physiological functions that become disrupted (Barnes and Karin, 1997). Elucidating the cell specific responses and actions of Ang II on these inflammatory mediators, may provide insight on alternative molecular targets that may inhibit their deleterious actions.

The primary objectives of this study were to determine the effect of Ang II on Cox2 expression in astrocytes from distinct brain regions in the SHR model and, to further compare these effects to the normotensive control rat model. In addition, we sought to determine the Ang II receptor involved in this effect. The main finding of our study is that Ang II potently increases Cox2 in a time-dependent manner in astrocytes from the brainstem and cerebellum. These effects were elicited via the AT1R. These results are consistent with other findings that Cox2 is potently induced by mitogenic and inflammatory stimuli in a time-dependent manner (Kujubu et al., 1993; Font-Nieves et al., 2012). Ang (1–7), a counterregulatory peptide for Ang II elicits its effects by inhibiting Cox2 in cerebral ischemia (Jiang et al., 2013). In addition, Ang (1–7) reduces proliferation of cancer cells and tumors by reducing Cox2 expression (Tallant and Gallagher, 2015). Previous studies have shown constitutive expression of Cox2 in tissue homogenates from mice brainstem and cerebellum (Kirkby et al., 2016). We determined the basal Cox2 expression in our samples, and we found that Wistar brainstem astrocytes had lower levels of basal Cox2 protein compared to SHR brainstem astrocytes. Cox2 basal protein expression in cerebellum astrocytes showed a tendency towards higher levels in Wistar astrocytes compared to SHR astrocytes. Cox2 mRNA was more highly expressed in SHR astrocytes compared to Wistar astrocytes in both the brainstem and cerebellum. Previous reports showed higher basal Cox2 gene expression in mice brainstem compared to the cerebellum (Kirkby et al., 2016). We did not find any significant differences in constitutive Cox2 mRNA expression between brainstem and cerebellum astrocytes. Interestingly, basal prostaglandins were higher in Wistar Kyoto (WKY) rat VSMCs compared to SHR VSMCs (Jaiswal et al., 1993). Not only were the prostaglandin levels higher, but the ratio of basal anti-inflammatory to pro-inflammatory prostaglandins was several folds higher in WKY compared to SHRs (Jaiswal et al., 1993). This basal profile however, could be attributed to either Cox isoform.

In VSMCs, Hu et al. showed that Ang II regulated Cox2 expression at the transcriptional level (Hu et al., 2002). In their study in VSMCs, Ang II-induced Cox2 mRNA levels increased within 3 h followed by a decrease. In contrast, the Cox2 protein levels increased within 3 h and remained elevated for up to 24 h (Hu et al., 2002). Our data showed that Ang II increased Cox2 protein levels relative to untreated controls in a time-dependent manner, in brainstem and cerebellum astrocytes for both Wistar and SHRs. Increases in Cox2 protein expression were evident within 4 h, with a decrease to almost basal levels by 48 h. The pattern of Cox2 elevation that we observed was similar to that observed by Hu and colleagues in VSMCs (Hu et al., 2002). The Cox2 protein levels remained elevated for up to 16 h; by 48 h the levels declined to almost basal levels. For both the brainstem and cerebellum astrocytes, Wistar Cox2 protein expression was generally higher than SHRs, however this was statistically significant for only the brainstem. A similar Cox2 expression profile was observed for cultured astrocyte exposed to LPS by Font-Nieves and colleagues (Font-Nieves et al., 2012). In their study, LPS-induced Cox2 protein remained elevated up to 24 h in contrast to LPS-induced Cox2 mRNA, which peaked at about 4 h then decreased to almost basal levels by 24 h (Font-Nieves et al., 2012). The levels of prostaglandins were also elevated in response to LPS. In contrast to the other prostaglandins measured, the synthesis of PGE_2 mirrored the Cox2 protein expression pattern (Font-Nieves et al., 2012). These findings confirmed the ability of inflammatory stimuli to significantly alter Cox2 expression and prostanoid synthesis.

In our study, the significantly higher Ang II-induced Cox2 protein levels in Wistar compared to SHR astrocytes is unexpected. In neuronal cultures, Ang II receptors were more highly expressed in SHRs compared to WKY (Raizada et al., 1984). This suggests a higher sensitivity to Ang II in SHRs (Raizada et al., 1984). It is possible that the profile of prostaglandins generated by the overexpressed Cox2 may differ in both animal models. Elucidating the profile of prostaglandins generated by the Ang II-induced Cox2 in both models may provide insight on the balance of the inflammatory versus anti-inflammatory prostaglandins produced. Cox2 was more elevated in SHRs compared to normotensive rats in VSMCs and aortic fibroblasts exposed to inflammatory stimuli (Álvarez et al., 2007; Beltrán et al., 2009). It would be easy to assume that in SHR astrocytes, an increased sensitivity to Ang II would translate

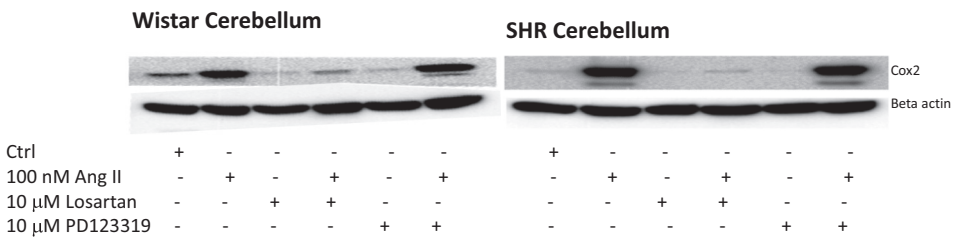

Fig. 5. Effect of Ang II receptor blockers on Cox2 protein expression in cerebellum astrocytes from Wistar rats and SHRs. Quiescent cerebellum astrocytes were treated with AT1R (10 μM Losartan) and AT2R (10 μM PD123319) receptor blockers with and without 100 nM Ang II for 4 h. Cox2 protein was measured by Western blotting as described. Cox2 protein levels were determined as fold change over basal after normalization to beta actin. Preparations of cerebellum astrocytes from 3 or more litters of neonatal rat pups were used in the study. Statistical significance was set at $p < .05$.

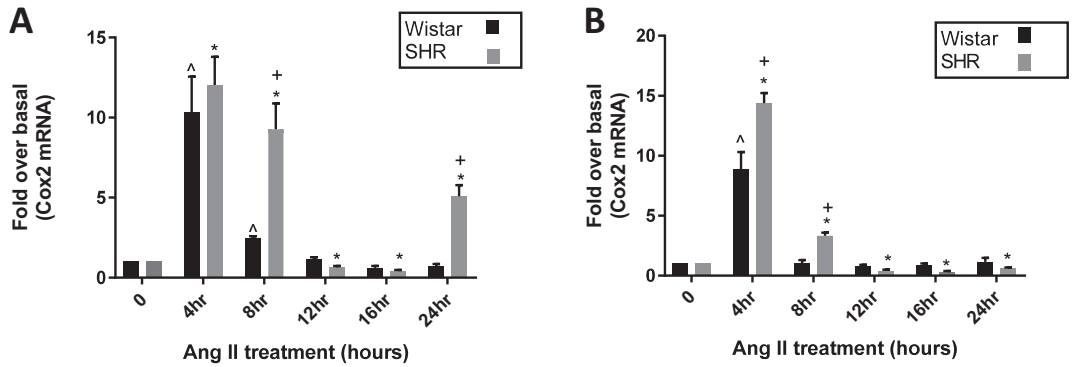

Fig. 6. Effect of time on Ang II-induced Cox2 mRNA levels in brainstem (Fig. 6A) and cerebellum (Fig. 6B) astrocytes isolated from SHRs and Wistar rats. Quiescent astrocytes were treated with 100 nM Ang II for 4 to 24 h. Cox2 mRNA was measured by qPCR as described. Cox2 mRNA levels are expressed as fold change over basal after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 4 or more litters of neonatal rat pups. ^represents $p < .05$ compared to basal Wistar astrocytes. *represents $p < .05$ compared to basal SHR astrocytes. $^{+}$ represents $p < .05$ for SHR compared to Wistar astrocytes using two-way analysis of variance.

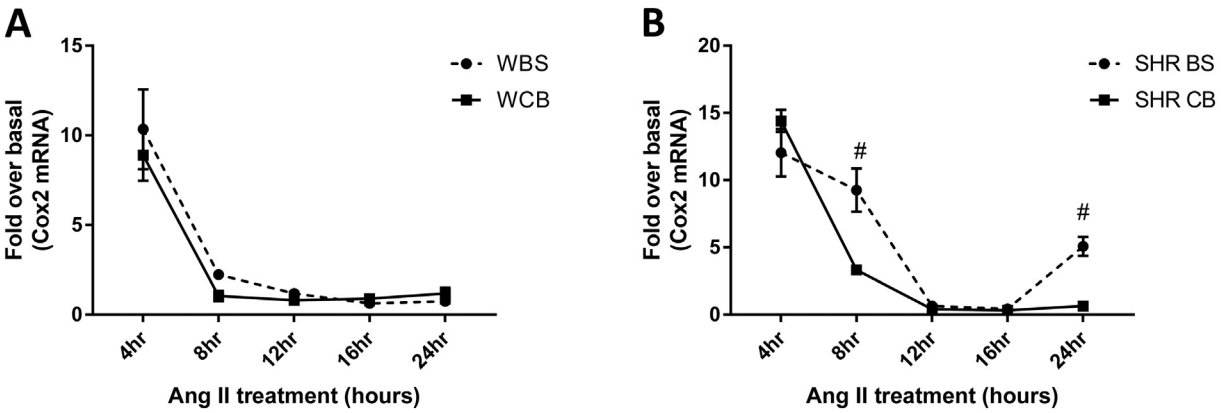

Fig. 7. Comparison of Ang II-induced Cox2 mRNA expression patterns in distinct brain regions in astrocytes isolated from Wistar rats (Fig. 7A) and SHRs (Fig. 7B). Quiescent astrocytes were treated with 100 nM Ang II for 4 to 24 h. Cox2 mRNA levels were measured by qPCR as described. Cox2 mRNA levels are expressed as fold change over basal after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 4 or more litters of neonatal rat pups. #represents $p < .05$ for brainstem versus cerebellum of the same model using two-way analysis of variance. WBS: Wistar brainstem; WCB: Wistar cerebellum; SHR BS: SHR brainstem; SHR CB: SHR cerebellum.

Table 1
Effect of Ang II receptor blockers on Cox2 mRNA expression in brainstem.

Treatment	Fold over basal mRNA	Fold over basal mRNA
	Wistar brainstem	SHR brainstem
Ang II	10.8 ± 4.6*	12.6 ± 1.1*
Losartan	1.1 ± 0.3	1.5 ± 0.4
Losartan + Ang II	1.0 ± 0.4	1.1 ± 0.2
PD123319	1.1 ± 0.4	1.3 ± 0.2
PD123319 + Ang II	10.7 ± 4.0*	12.5 ± 2.6*

Quiescent brainstem astrocytes were treated with AT1R (10 μM Losartan) and AT2R (10 μM PD123319) receptor blockers with and without 100 nM Ang II for 4 h. Cox2 mRNA was measured by qPCR as described. Cox2 mRNA levels are expressed as fold change over basal after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 3 or more litters of neonatal rat pups.

* Represents $p < .05$ for treatment versus basal.

Table 2
Effect of Ang II receptor blockers on Cox2 mRNA expression in cerebellum.

Treatment	Fold over basal mRNA	Fold over basal mRNA
	Wistar cerebellum	SHR cerebellum
Ang II	9.1 ± 0.9*	14. 6 ± 2.7*
Losartan	1.1 ± 0.5	1.1 ± 0.1
Losartan + Ang II	0.9 ± 0.4	1.1 ± 0.1
PD123319	1.0 ± 0.2	1.1 ± 0.2
PD123319 + Ang II	8.7 ± 4.9*	15.0 ± 4.4*

Quiescent cerebellum astrocytes were treated with AT1R (10 μM Losartan) and AT2R (10 μM PD123319) receptor blockers with and without 100 nM Ang II for 4 h. Cox2 mRNA was measured by qPCR as described. Cox2 mRNA levels are expressed as fold change over basal after normalization to beta actin. Each value represents the mean ± SEM of preparations of brainstem and cerebellum astrocytes from 3 or more litters of neonatal rat pups.

* Represents $p < .05$ for treatment versus basal.

to elevated inflammatory mediators compared to normotensive strains. However, similar to previous observations in astrocytes (Gowrisankar and Clark, 2016), we have found the opposite effect. The explanation for this could lie in activation of compensatory mechanisms or the stage of hypertension (Jaiswal et al., 1993).

In addition to their role as inflammatory mediators, Cox-derived prostaglandins have direct effects on blood pressure. Furthermore, previous studies have shown that in contrast to systemic PGE_2, centrally administered PGE_2 increases sympathetic activity and blood pressure (Yang and Du, 2012). The diverse effects of PGE_2 on blood pressure is mediated by different EP receptors. As such, the effects of PGE_2 on central blood pressure regulation will also largely depend on the distribution of the EP receptors (Yang and Du, 2012). The implication of this is that the increased Cox2 expression observed will require

additional studies on the tissue distribution of the derived prostaglandins and the localized EP receptors in that tissue. It may well be that despite increased Cox2 expression in both animal models, the prostaglandins that are produced differ, and different EP receptors are being activated. It is accepted that prostaglandins may independently regulate blood pressure or may mediate RAS actions (Jaiswal et al., 1991). As such, the role of Ang II-induced Cox2 expression in blood pressure regulation may involve multiple molecular mechanisms.

In the current study, there was a rapid elevation in Ang II-induced Cox2 mRNA levels followed by a sharp decline to almost basal levels for Wistar and SHR astrocytes in both brainstem and cerebellum. Ang II increased Cox2 mRNA levels relative to untreated controls in a time-dependent manner, in brainstem and cerebellum for both Wistar and SHR astrocytes. Ang II rapidly increased Cox2 levels within 4 h followed by a decrease to almost basal levels by 24 h except for SHR brainstem astrocytes, which showed an increase at 24 h. The Ang II-induced Cox2 mRNA elevation is similar to the results observed for the studies in VSMCs (Hu et al., 2002). For Ang II-induced Cox2 protein levels, Wistar astrocytes generally had higher levels than SHR astrocytes. This is in striking contrast to the Cox2 mRNA expression that showed lower Cox2 mRNA levels in Wistar compared to SHR astrocytes at early time points except for brainstem SHR astrocytes at 24 h. These differences between the protein and mRNA results could be attributed to a number of factors including greater Cox2 protein stability compared to the mRNA stability. It is widely accepted that mRNA expression shows how a gene is regulated and does not necessarily correlate with protein expression. There are numerous modifications following transcription or translation that might contribute to this. In addition, increased Cox2 mRNA could be attributed to either increased transcription and/or reduced degradation (Hu et al., 2002).

In the current study, when the Ang II-induced astrocyte Cox2 expression was compared in distinct brain regions, the general trend was significantly higher levels in brainstem compared to cerebellum astrocytes or no significant differences between the two. The implications of this are not fully known. We suspect that higher AT1R expression in the brainstem compared to the cerebellum might contribute to these differences (Tallant et al., 1996).

In VSMCs of hypertensive rats, increased levels of Cox2 expression was associated with AT1R activation and increased oxidative stress (Álvarez et al., 2007). Other studies in human VSMCs have also shown that Ang II induced Cox2 expression via AT1R activation (Hu et al., 2002). In our study, we determined the Ang receptor mediating Ang II-induced Cox2. Blockade of the AT1R showed significant reduction in Cox2 expression to almost basal levels. Blockade of the AT2R had no effect on Ang II-induced Cox2 expression. These results confirm that the Ang II effects on Cox2 expression are elicited by the AT1R. Previous studies in other cell types have also shown AT1R-mediated Cox2 elevation (Slice et al., 2005). In aortic fibroblast cells, sustained expression of Cox2 in SHRs was linked to Ang II-induced p38 MAPK activation, an effect that was independent of the reactive oxygen species pathway (Beltrán et al., 2009). We have previously shown that Ang II induces p38 MAPK in astrocytes and that these effects were AT1R-mediated (Alanazi et al., 2014).

The goal of this study was to better understand the role of astrocytes in inflammatory conditions, and how astrocytes might play a role in the pathogenesis of hypertension. An overactive RAS is known to contribute to hypertension through both vasoactive actions and inflammatory contributions. Inflammation in brain regions regulating cardiovascular control such as the brainstem, contributes to the pathogenesis of hypertension by increasing sympathetic outflow (Shi et al., 2010). Astrocytes have emerged as important contributors to neuroinflammation (Haspula and Clark, 2018a, 2018b) leading to increased interest in the molecular mechanisms underlying their inflammatory actions. Cox2 is a widely studied inflammatory mediator linked to numerous disease conditions marked by pain and inflammation. However, selective inhibition of Cox2 by non-steroidal anti-inflammatory drugs had harmful side effects including increased blood pressure and increased risk of cardiovascular events. The results of our study confirm that Ang II induces the inflammatory mediator Cox2 in both normotensive and hypertensive rat models. The general trend was higher Ang II-induced Cox2 protein levels in Wistar astrocytes compared to SHR astrocytes. This effect was unexpected and further corroborates the need to elucidate the profile of the Cox2 products and prostaglandin receptors following Ang II stimulation. Additionally, our studies showed that brainstem astrocytes were more sensitive to Ang II effects on Cox2. This could be attributed to higher levels of AT1R in brainstem compared to cerebellum astrocytes (Tallant et al., 1996). The brainstem is responsible for cardiovascular control and elevated Cox2 in this region might have implications in central blood pressure regulation. Overall, our study findings suggest differential Cox2 expression in SHR compared to Wistar brainstem astrocytes. Differing prostaglandin profiles may be associated with the Cox2 expression observed and will be the focus of future studies. Since the brainstem is responsible for cardiovascular actions, an altered prostaglandin profile therein may affect central regulation of blood pressure by more than one mechanism.

Declaration of Competing Interest

None.

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