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MAPK activation patterns of AT1R and CB1R in SHR versus Wistar astrocytes: Evidence of CB1R hypofunction and crosstalk between AT1R and CB1R

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ABSTRACT

Background: Angiotensin (Ang) II and cannabinoids regulate physiologically relevant astroglial functions via receptor-mediated activation of Mitogen-activated protein kinases (MAPKs). In this study, we investigated the consequences of astroglial Ang II type 1 receptor (AT1R) and Cannabinoid type 1 receptor (CB1R) activation, alone and in combination, on MAPK activation in the presence and absence of hypertensive states. In addition, we also investigated a novel unidirectional crosstalk mechanism between AT1R and CB1R, that involves PKC-mediated phosphorylation of CB1R.

Methods: Astrocytes were isolated from the brainstem and cerebellum of Spontaneously hypertensive rats (SHRs) and normotensive Wistar rats. The cells were treated with either 100 nM Ang II or 10 nM Arachidonyl-2′-chloroethylamide (ACEA), both alone and in combination, for varying time periods, and the extent of phosphorylation of MAPKs, ERK and p38, and the phosphorylated forms of CB1R (p-CB1R), were measured using western blotting.

Results: Ang II treatment resulted in a greater activation of MAPKs in SHR brainstem astrocytes, but not SHR cerebellar astrocytes when compared to Wistar rats. ACEA-mediated MAPK activation was significantly lower in brainstem astrocytes of SHRs when compared to Wistar rats. ACEA negatively modulates AT1R-mediated MAPK activation in both cerebellar and brainstem astrocytes of both models. The effect however was diminished in brainstem astrocytes. Ang II caused a significant increase in phosphorylation of CB1R in cerebellar astrocytes, while its effect was diminished in brainstem astrocytes of both models.

Conclusion: Both Ang II and ACEA-induced MAPK activation were significantly altered in SHR astrocytes when compared to Wistar astrocytes. A possible reduction in CB1R functionality, coupled with a hyperfunctional AT1R in the brainstem, could well be significant factors in the development of hypertensive states. AT1R-mediated phosphorylation of CB1R could be critical for impaired cerebellar development characterized by a hyperactive RAS.

1. Introduction

Astrocytes play critical roles in several pathological conditions. Their ability to serve as mediators of communication between neurons [1], to alter neuroinflammatory states [2], to be a major source for Angiotensinogen in the brain [3], and to regulate energy stores [4], lends these cells to be an ideal model for studying cardiovascular and neurological disorders. The Spontaneously hypertensive rat (SHR) is a well-established animal model for essential hypertension [5]. In addition to being a model of choice to study hypertension and several other cardiovascular disorders, they have also been used to study neurodevelopmental disorders such as Attention Deficit Hyperactivity Disorder (ADHD) [6]. While astrocytes from the brainstem have been implicated in augmenting sympathoexcitatory drive [7], increased astrogliosis has

Abbreviations: ADHD, Attention Deficit Hyperactivity Disorder; MAPK, Mitogen-activated protein kinases; ALAAC, Association for Assessment and Accreditation of Laboratory Animal Care International; Ang, Angiotensin; AT1R, Ang type 1 receptor; AT2R, Ang type 2 receptor; BCA, Bicinchoninic acid; CB1R, Cannabinoid Type 1 Receptor; p-CB1R, phospho-CB1R; RAS, Renin Angiotensin System; SHR, spontaneously hypertensive rat; ACEA, Arachidonyl-2′-chloroethylamide; ERK, extracellular signal regulated kinase; PKC, Protein kinase C; DAGL, Diacylglycerol lipase; BIM I, Bisindolylmaleimide I, Hydrochloride; DMEM/F12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; FBS, Fetal Bovine Serum; NaCl, Sodium chloride; NaF, Sodium fluoride; $NaVO_4$, Sodium orthovanadate; PMSF, Phenylmethylsulfonyl fluoride; TBS, Tris buffered saline

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been reported in the SHR cerebellum [8] which further illustrates the necessity of investigating the potential dysregulated molecular machinery in these cells.

An overactive brain renin angiotensin system (RAS) is one of the most prominent characteristics of SHRs [9]. Astroglial Ang II type 1 receptor (AT1R) in the brainstem has a critical role in elevating sympathetic activity in cardiovascular diseases [10]. In astrocytes from the brainstem and cerebellum, Angiotensin (Ang) II via activation of the AT1R results in an increase in pro-hypertensive and a decrease in anti-hypertensive markers [11,12]. Several reports of crosstalk between the angiotensin system and the endocannabinoid system have emerged in the recent past [13,14]. Cannabinoid Type 1 receptor (CB1R) is one of the most abundant G protein–coupled receptors (GPCRs) in the brain, and its impairment has been linked to a multitude of neurological disorders [15]. CB1R antagonism has been demonstrated to both potentiate [16], as well as neutralize Ang II-mediated effects [14]. In the brain, both exogenous and endogenous cannabinoids (endocannabinoids) are claimed to have prominent neuroprotective and anti-inflammatory effects via activation of the CB1R [15]. Interestingly, astroglial AT1Rs and CB1Rs have seemingly opposing roles in the regulation of several astroglial functions. For instance, while astroglial AT1R can cause astrocyte senescence [17] and an increase in proinflammatory cytokines [18], astroglial CB1R activation confers protection against apoptosis [19] and an elevation in anti-inflammatory cytokines [20]. An increase in pro-inflammatory cytokines from glial cells has been shown to elevate sympathetic activity [21], and hence relevant to hypertensive states.

Activation of cell surface receptors, such as AT1Rs and CB1Rs, evokes cellular responses that are tightly regulated by key signal transduction pathways which fall under the umbrella of Mitogen-activated protein kinases (MAPKs). MAPKs serve as critical linking points between receptor activation and cellular functions. MAPK activation in astrocytes is associated with a diverse array of functions, which have both physiological and pathophysiological consequences. Activation of astroglial MAPKs such as extracellular signal regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) regulate proliferation [22], astrogliosis and mobilization of neuroinflammatory cytokines [23,24]. These functions make them important targets of neurological and cardiovascular diseases. Studies from our laboratory and others have shown that MAPKs are involved in Ang II-mediated proliferative [25] and pro-inflammatory effects [26]. Cannabinoids also employ the ERK MAPK pathway in mediating their protective effects in astrocytes [27]. In brainstem neurons, ERK and other signal transduction pathways are crucial for CB1R-mediated regulation of cardiovascular parameters [28]. While AT1R signaling mechanisms have been studied in SHR neurons [29], both AT1R- and CB1R-mediated effects on MAPK activation has not been well investigated in SHR astrocytes.

Several studies have reported alterations in CB1R levels in SHR cells when compared to their normotensive counterparts [6,30]. A change in CB1R expression levels has also been demonstrated to correlate strongly with an alteration in CB1R-mediated downstream effects [15]. Previously, we have reported a reduction in the CB1R expression levels in brainstem, but not cerebellar, astrocytes of SHRs when compared to Wistar rats [31]. Whether this reduction translates into a dampened CB1R tone, has not been investigated. MAPK activation is governed by many factors, one of the primary factors being receptor density and trafficking [32]. Comparing signal transduction pathways of the CB1R and the AT1R, between the two strains would enable us to have a better understanding of potential endocannabinoid or Ang receptor dysregulation in pathological conditions, respectively. Crosstalk between the two receptors was also reported at the level of ERK in a neuroblastoma cell line [33]. Co-treatment of Ang II with a CB1R agonist, HU-210, led to an increase in AT1R-mediated activation of ERK.

Therefore, the focus of our study was three-fold. First to investigate the effects of Ang II and a potent CB1R agonist, ACEA, individually on MAPK activation in SHR and Wistar rat astrocytes. This would enable us to understand the patterns of MAPK activation by RAS and endocannabinoid systems in astrocytes. Secondly, we investigated crosstalk between the two systems. This was assessed by investigating the effect of co-treatments with Ang II and ACEA on MAPKs. Whether ACEA treatment neutralizes or potentiates Ang II-mediated activation of MAPKs, when compared to Ang II alone would then help us to understand the mode of crosstalk that exists between these two systems in these primary cells. Lastly, we explored a novel mode of crosstalk between AT1R and CB1R, which involves PKC activation. Garcia et al. [34] has earlier demonstrated that PKC activation by Phorbol 12-myristate 13-acetate (PMA) results in phosphorylation of the CB1R in the third intracellular loop, which diminishes its ability to function. As the AT1R is a Gq GPCR, Ang II is functionally capable of activating PKC and thereby phosphorylating CB1Rs. Hence, both basal and Ang II-mediated phosphorylation of CB1R, was also investigated in this study.

2. Materials and methods

2.1. Materials

Ang II was obtained from Bachem (Torrance, CA). PD123319, the selective AT2R antagonist was obtained from Sigma (St. Louis, MO), and Losartan (AT1R antagonist) was kindly provided by Du Pont Merck (Wilmington, DE). ACEA, the specific CB1R agonist, was purchased from Tocris (Bristol, UK) and Bisindolylmaleimide I, Hydrochloride #9841 (BIM I), the potent PKC inhibitor, was obtained from Cell Signaling Technology (Beverly, MA). Orlistat, the diacylglycerol lipase (DAGL) inhibitor and PMA, the PKC activator, were purchased from Sigma (St. Louis, MO). Western blotting supplies were purchased from Bio-Rad Laboratories (Hercules, CA) or VWR International (Suwanee, GA). The polyclonal antibody that detects the phosphorylated forms of CB1R, p-CB1 Antibody (Ser 316) [sc-17555], was purchased from Santa Cruz (Dallas, TX), and the monoclonal phospho-CB1R antibody, Anti-Cannabinoid Receptor I (phospho S316) antibody [EPR2223(N)], was purchased from Abcam (Cambridge, MA). The phospho-p38 (P-p38) and the phospho-ERK (P-ERK) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Beta-actin antibody (A2066) was purchased from Sigma (St. Louis, MO). The BCA protein kit was obtained from Pierce Biotechnology (Rockford, IL). All other chemicals were purchased from either VWR International (Suwannee, GA), Fisher Scientific (Waltham, MA) or Sigma (St. Louis, MO).

2.2. Isolation and culture of primary astrocytes

Timed pregnant Wistar rats and SHRs were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the ALAAC-accredited animal facility of Nova Southeastern University. 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. The brainstem and cerebellar astrocyte cultures were prepared using mechanical dissociation as previously described [35]. Briefly, brains from 3-day old rat pups were isolated and the cerebellum and brainstem were carefully separated from each brain. These regions are visible to the naked eye and can be clearly differentiated from each other. Astrocyte cultures were then prepared from the pooled brainstem and the pooled cerebellum by mechanical dissociation. The cells were grown in DMEM/F12 culture media containing 10% FBS, 10,000 I.U/mL penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin B at 37 °C in a humidified incubator (5% CO_2 and 95% air). The cell cultures were fed every 3–4 days.

On attaining confluency, the cells were subjected to vigorous shaking overnight which resulted in the detachment of microglia, oligodendrocytes and cell debris. Subsequently, the cell cultures were detached with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA) and replated at a ratio of 1:10. The astrocyte enriched cultures were fed once