European Journal of Pharmacology 799 (2017) 128–134

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Role of interleukin-10 in the neuroprotective effect of the Angiotensin Type 2 Receptor agonist, compound 21, after ischemia/reperfusion injury

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ARTICLE INFO

Keywords:
Stroke
Angiotensin Type 2 Receptor
Compound 21
Neuroprotection
And Interleukin 10

ABSTRACT

Introduction: We and others have shown that the angiotensin type 2 (AT2) receptor agonist, compound 21 (C21), provides neuroprotection and enhances recovery in rodent stroke models yet the mechanism involved is not known. Moreover, C21 treatment is associated with an anti-inflammatory response. Here we tested the hypothesis that C21 mediates neuroprotection by upregulating the neuroprotective and anti-inflammatory cytokine, interleukin (IL)–10.

Methods: Wistar rats were subjected to 3 h-middle cerebral artery suture occlusion and treated at reperfusion with C21 (0.03 mg/kg) ± IL-10 neutralizing antibody (0.1 mg/kg) both given i.p. Infarct size, behavioral outcomes, and molecular analysis were performed at 24 h post-injury. Primary rat neurons were used to test the direct neuroprotective effect of C21 in vitro.

Results: C21 treatment reduced infarct size, improved functional outcome and decreased the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) in the ischemic hemisphere compared to saline. Anti-IL-10 co-treatment blocked the C21-induced reduction in infarct size and inflammation, and the improvement in behavioral outcome. In vitro, C21 treatment increased neuron survival and reduced cell apoptosis after oxygen glucose deprivation (OGD) and OGD/reoxygenation. These effects were mediated through AT2R stimulation.

Conclusion: C21 provides direct neuroprotection as well as indirect protection through IL-10.

1. Introduction

Angiotensin type 2 (AT2R) receptor stimulation with the non-peptide agonist, compound 21 (C21), has been shown to provide neuroprotection and functional recovery after experimental ischemic stroke in rodents (Alhusban et al., 2015; Joseph et al., 2014; McCarthy et al., 2014; Min et al., 2014). C21 reduced infarct size after both permanent and temporary middle cerebral artery occlusion (MCAO). In addition, C21 reduced neuronal apoptosis and decreased mortality after stroke (Schwengel et al., 2016). Nevertheless, the mechanism underlying C21-mediated neuroprotection is still not known. In our hands, we reported an upregulation of the neuroprotective cytokine, interleukin (IL)–10 with C21 treatment at 24 h in the ischemic hemisphere after 3 h MCAO. Moreover, we reported an increase in the number of IL-10 positive cells at 7 days with a single dose of C21 after 90-min MCAO (Alhusban et al., 2015). Whether the increased IL-10 was causally related to the improved outcome remained unknown.

IL-10 is an anti-inflammatory cytokine that mediates its actions through activation of the JAK1-STAT3 (Janus Kinase 1 - Signal Transducer and Activator of Transcription 3) signaling pathway (Sabat et al., 2010). IL-10 has been shown to provide direct neuroprotection in vivo and in vitro. Administration of exogenous IL-10 centrally and systemically decreases the infarct size in rats after permanent focal ischemia (Spera et al., 1998), while IL-10 knockout mice show larger infarct volume following middle cerebral artery occlusion (Grilli et al., 2000). Moreover, post-ischemic IL-10 gene transfer attenuates brain infarction in rats subjected to focal and global ischemia (Ooboshi et al., 2005). Interestingly, neuroprotection by systemic immune cells such as regulatory T and B cells have also been shown to be mediated through IL-10 production (Bodhankar et al., 2013, 2014; Liesz et al., 2009; Liesz et al., 2013). In vitro, IL-10 protects murine cortical and cerebellar neurons from excitotoxic damage and oxygen glucose deprivation (OGD) by activating phosphatidylinositide 3-kinases (PI-3K) and signal transducer and activator of

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http://dx.doi.org/10.1016/j.ejphar.2017.02.016
Received 27 September 2016; Received in revised form 8 February 2017; Accepted 9 February 2017
Available online 10 February 2017

transcription 3 (STAT-3) pathways (Bachis et al., 2001; Grilli et al., 2000; Sharma et al., 2011).

Studies examining the protective role of AT2R stimulation using in vitro neuronal injury models are inconclusive. The peptide AT2R agonist, CGP-42112, but not C21, protected primary cortical neurons against glucose deprivation (Lee et al., 2012). In addition, Wu et al. showed neuroprotection with angiotensin type 1 (AT1R) receptor blockers (ARBs) but not CGP-42112 pretreatment against OGD/reoxygenation injury (Wu et al., 2010); these effects involved AT1R blockade and not indirect AT2R stimulation (Wang et al., 2014; Wu et al., 2010).

In this study, we aimed to determine the contribution of IL-10 to the neuroprotective effect of C21 using temporary MCAO in vivo. In addition, we tested the direct neuroprotective effect of C21 in vitro in an ischemia/reoxygenation injury model.

2. Materials and methods

Experiments were approved by the Care of Experimental Animal Committee of Augusta University/Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center, Augusta, GA.

2.1. Middle cerebral artery occlusion and treatment

Adult male Wistar rats (280–340 g) were subjected to 3 h-middle cerebral artery occlusion (MCAO) followed by reperfusion for 21 h to achieve ischemia/reperfusion injury in vivo as previously described (Alhusban et al., 2015). Animals were treated according to a 2×2 study design (Fig. 1.A). C21 was administered intraperitoneal (i.p.) at a dose of 0.03 mg/kg, which has previously shown AT2R-mediated neuroprotection in our hands using the same stroke model (Alhusban et al., 2015). Anti-IL-10 neutralizing antibody (Invitrogen) was given i.p. in a separate syringe (to avoid physical interaction) at a dose of (0.1 mg/kg) to block the effects of endogenous IL-10 (Cai et al., 2012). Immunoglobulin G (IgG) isotype control antibody was given to the rats that did not receive anti-IL-10. Animals were killed at 24 h and brains were collected. Behavioral outcome was assessed just before euthanasia. Sham animals were subjected to the same surgical procedure without actual MCAO occlusion.

2.2. Infarct size analysis

At 24 h, rats were killed after transcardial perfusion with ice cold PBS following ketamine/xylazine anesthesia. Brains were removed and sliced into seven 2 mm-thick coronal sections (A to G) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., Missouri, USA). Areas of the infarct, ischemic, and non-ischemic hemispheres were measured using ImageJ software (NIH) in a blinded fashion and infarct size was calculated with edema correction using the following formula: 100 X (non-stroked – (stroked – infarct))/non-stroked.

2.3. Western blotting

Ischemic hemispheres of B, C, D, and E brain sections were homogenized using a hand homogenizer. Protein was quantified using Pierce BCA protein assay kit (Thermo Scientific), and 50 µg protein aliquots were run on SDS-PAGE as described previously (Guan et al., 2011), transferred to nitrocellulose membranes and probed with mouse anti-TNF-α (Abcam) and rabbit anti-cleaved caspase 3 (Cell Signaling) antibodies (Ishrat et al., 2015). Rabbit anti-GAPDH (Cell Signaling) and mouse anti-β-actin (Sigma) were used as loading controls depending on the molecular weight and species of the probed proteins. Membranes were further probed with peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies. Optical densities

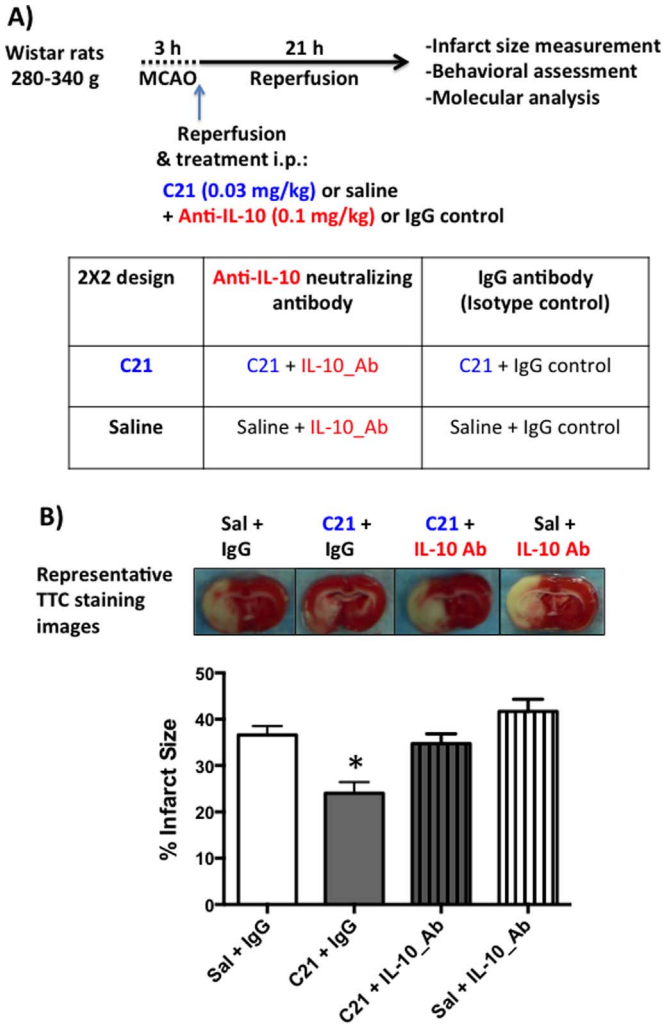

Fig. 1. IL-10 is involved in C21-mediated neuroprotection after ischemia/reperfusion injury in vivo. A: Schematic diagram representing the study outline and treatment groups. Wistar rats were subjected to 3 h MCAO using the suture model followed by reperfusion for 21 h. Treatments were administered i.p. at reperfusion in separate syringes. Animals were assigned to one of four treatment groups in a 2×2 study design as outlined in the table. **B:** Representative TTC stained brain sections and quantification of infarct size from the four treatment groups. C21 treatment reduced infarct size at 24 h after 3 h MCAO. Co-treatment with anti-IL-10 neutralizing antibody abrogated the neuroprotective effect of C21 treatment. N=7–9 per group. *P < 0.05 vs other groups (Two-way ANOVA).

of bands were quantified using ImageJ software (NIH), divided by the loading control, and normalized to the control group.

2.4. Behavioral testing

Motor behavior assessment was conducted in a blinded fashion.

2.4.1. Bederson score

This test examines animal behavior in an open field. Rats were scored from 0 to 3. One point is given for each of the following: forelimb flexion when suspended by tail; decreased resistance to lateral push; and contralateral circling (Bederson et al., 1986).

2.4.2. Beam walk score

This test examines animal balance on a horizontal beam. Rats were placed on a beam for 1 min and scored from 0 to 6 as follows: balances on the beam with a steady posture=0, grasps side of the beam=1, hugs the beam with 1 limb falling=2, hugs the beam with 2 limbs falling=3, falls off the beam within 40–60 s=4, falls off the beam within 20–