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Coupling corticotropin-releasing-hormone and angiotensin converting enzyme 2 dampens stress responsiveness in male mice

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ABSTRACT

This study used mice to evaluate whether coupling expression of corticotropin-releasing hormone (CRH) and angiotensin converting enzyme 2 (ACE2) creates central interactions that blunt endocrine and behavioral responses to psychogenic stress. Central administration of diminazene aceturate, an ACE2 activator, had no effect on restraint-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis; however, mice that ubiquitously overexpress ACE2 had reduced plasma corticosterone (CORT) and pituitary expression of POMC mRNA. The Cre-LoxP system was used to restrict ACE2 overexpression to CRH synthesizing cells and probe whether HPA axis suppression was the result of central ACE2 and CRH interactions. Within the paraventricular nucleus of the hypothalamus (PVN), mice with ACE2 overexpression directed to CRH had a ≈2.5 fold increase in ACE2 mRNA, which co-localized with CRH mRNA. Relative to controls, mice overexpressing ACE2 in CRH cells had a decreased CORT response to restraint as well as decreased CRH mRNA in the PVN and CEA and POMC mRNA in the pituitary. Administration of ACTH similarly increased plasma CORT, indicating that the blunted HPA axis activation that accompanies ACE2 overexpression in CRH cells is centrally mediated. Anxiety-like behavior was assessed to determine whether the decreased HPA axis activation was predictive of anxiolysis. Mice with ACE2 overexpression directed to CRH cells displayed decreased anxiety-like behavior in the elevated plus maze and open field when compared to that of controls. Collectively, these results suggest that exogenous ACE2 suppresses CRH synthesis, which alters the central processing of psychogenic stress, thereby blunting HPA axis activation and attenuating anxiety-like behavior.

1. Introduction

Anxiety disorders are comorbid with hypertension (Sandstrom et al., 2016) and common pathophysiology may contribute to each disease. Augmentation of the renin-angiotensin-system (RAS) increases the synthesis of angiotensin II (Ang-II) and its activation of the angiotensin type- 1a receptor (AT1aR) is established to promote hypertension; however, emerging evidence suggests that the RAS also contributes to the onset of anxiety disorders. Psychogenic stress increases Ang-II and interventions that inhibit AT1aR(s) attenuate anxiety-like behavior and hypothalamic-pituitary-adrenal (HPA) axis activity (Saavedra et al., 2005). Angiotensin-converting-enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs) alleviate hypertension. Interestingly, patients with posttraumatic stress disorder (PTSD) taking ACEi or ARBs have fewer traumatic stress symptoms relative to PTSD patients taking other types of blood pressure lowering medications (Khoury et al., 2012). Dysregulation of the HPA axis frequently occurs in patients with anxiety disorders (Abelson et al., 2007; Brand et al., 2011; Vreeburg et al., 2010) and chronic administration of an ARB corrects this dysregulation and improves affect (Pavlatou et al., 2008). These results implicate the RAS in the etiology of affective disorders and suggest it may serve as a viable target for therapeutic

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interventions.

The relatively recent discovery of angiotensin converting enzyme 2 (ACE2) revealed a 'protective limb' of the RAS that opposes many of the deleterious consequences of AT1aR activation (Xu et al., 2011). ACE2 metabolizes Ang-II into angiotensin 1-7 (Ang1-7) which promotes cardio-protection by activating the Mas receptor (MasR) (Xia and Lazartigues, 2010). Levels of ACE2 and Ang1-7 are elevated in patients taking ACEi and ARBs (Furuhashi et al., 2015; Luque et al., 1996), suggesting that blocking the synthesis of Ang-II or its actions at the AT1aR augments ACE2 activity (Ferreira et al., 2010). We recently discovered that increasing ACE2 activity in the brain is potently anxiolytic in mice (Wang et al., 2016a) but whether increasing ACE2 activity alters activation of the HPA axis, another indicator of stress responsiveness, is unknown.

Activation of the HPA axis is initiated by neurons in the paraventricular nucleus of the hypothalamus (PVN) that secrete corticotropin-releasing-hormone (CRH) into the median eminence to stimulate the release of adrenocorticotropic hormone (ACTH), which drives adrenal glucocorticoid (CORT) secretion. Anxiety disorders are associated with HPA axis dysfunction (Abelson et al., 2007; Brand et al., 2011; Vreeburg et al., 2010) and we discovered that the majority of CRH neurons in the PVN express AT1aR(s) and their deletion down-regulates CRH mRNA (de Kloet et al., 2013; de Kloet et al., 2017; Wang et al., 2016b). Neuropsychiatric illnesses are associated with impaired CRH signaling in the brain (Banki et al., 1987; Nemeroff et al., 1984, 1988) and coupling CRH transcription and ACE2 overexpression may inhibit the stimulation of AT1aR expressed on CRH neurons and down-regulate its production. Increasing ACE2 activity also has the beneficial effect of elevating levels of Ang1-7, which promotes anxiolysis by activating MasR(s) (Kangussu et al., 2017; Moura Santos et al., 2017; Wang et al., 2016a). Probing CRH and ACE2 interactions within the CNS may reveal novel strategies for reducing stress responsiveness by dampening HPA axis activation and attenuating anxiety.

This study used mice to evaluate whether central CRH and ACE2 interactions dampen endocrine and behavioral responses to psychogenic stress. We administered mice diminazene aceturate (DIZE) or engineered mice that ubiquitously overexpress ACE2 in order to evaluate whether pharmacological up-regulation of endogenous ACE2 or genetic overexpression of exogenous ACE2 alters HPA axis activity. Next, we used the Cre-LoxP system to direct ACE2 overexpression to CRH transcription to probe whether altered HPA axis activation was the result of a CRH and ACE2 interaction. Anxiety-like behavior was assessed to determine whether any differences in HPA axis activation were associated with altered behavioral responses to psychogenic stressors. The results suggest that directing ACE2 overexpression to CRH transcription creates central interactions that inhibit stress-induced HPA axis activation and attenuate anxiety-like behavior.

2. Materials and methods

2.1. Animals

All mice were male and 8-12 weeks-old at the initiation of the study. Mice were given *ad libitum* access to pelleted rodent chow and water and were individually-housed on a 12 h/12 h light-dark cycle. The light phase started at 0700 h and the dark phase started at 1900 h. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

C57BL/6 mice. We used C57BL/6 mice obtained from the Harlan Laboratories to evaluate the effects of centrally administered DIZE, an ACE2 activator (De Maria et al., 2016; Qi et al., 2013a), on stress-induced activation of the HPA axis.

ACE2 KI mice. We have previously described the generation of ACE2 KI mice and their wild-type littermate controls (WT) (Wang et al., 2016a). Briefly, ACE2 KI mice have the expression of ACE2 driven by ROSA26. Both ACE2 KI mice and WT are maintained on a 129/B6 mixed background. We have previously validated that ACE2 KI mice have significantly increased ACE2 mRNA expression and ACE2 activity in central and peripheral tissues (Qi et al., 2016; Wang et al., 2016a).

CRH ACE2 KI mice. To overexpress ACE2 specifically in CRH cells, we first generated mice homozygous for floxed STOP ACE2 (ACE2 gene was preceded by a stop codon that was flanked by two loxP sites) after the ROSA26 promoter (floxed STOP ACE2 mice). These floxed STOP ACE2 mice were bred to mice heterozygous for Cre recombinase driven by the gene encoding for CRH (CRH-Cre mice, Jackson Laboratory Stock # 012704) to generate mice carrying both floxed STOP ACE2 and CRH-Cre (CRH ACE2 KI mice) as well as littermate controls carrying only the floxed STOP ACE2 gene (CON mice).

CRH-Cre mice. To evaluate whether Cre recombinase expression, in and of itself, affects HPA axis activity, we bred heterozygous CRH-Cre mice to C57BL/6J mice (Jackson Laboratory) to generate CRH-Cre mice and littermate wild-type controls (littermate WT CON).

2.2. Intracerebroventricular (ICV) infusion of DIZE

To determine whether activating endogenous ACE2 in the brain influences HPA axis activity, we chronically administered normal saline or DIZE (low dose group: 0.11 μg/h, medium dose group: 1.1 μg/h, high dose group: 11 μg/h) into the lateral ventricles of C57BL/6 mice for 2 weeks. The icv infusions were conducted using micro-osmotic pumps (Model 1004, flow rate: 0.11 μl/h, ALZET, Cupertino, CA, USA). Prior studies determined that central delivery of DIZE, at the medium and high doses used here, significantly decreases anxiety-like behavior (Wang et al., 2016a). The procedures for preparation and implantation of micro-osmotic pumps have been described previously (Wang et al., 2016a). Two weeks after implantation of micro-osmotic pumps, the plasma corticosterone (CORT) response to restraint stress was assessed.

2.3. Assessment of plasma CORT

We examined plasma CORT concentrations before, during and after a 30 min restraint challenge in DIZE-infused mice, ACE2 KI mice, CRH ACE2 KI mice, and their respective controls. Between 0800 h and 0900 h, we restrained mice in clear plastic ventilated tubes and collected tail vein blood samples (40 μl) within 3 min to measure morning basal levels of CORT. Thirty-minutes after the onset of restraint, we collected another set of blood samples, and mice were released and returned to home cages. Additional blood samples were collected at 60 and 120 min after the onset of restraint. Blood samples were centrifuged at 3500 rpm for 15 min at 4 °C to isolate plasma. Plasma samples were stored at −80 °C.

To assess afternoon basal CORT levels in CRH ACE2 KI mice and CON mice, blood samples were taken during the light phase between 1800 h and 1900 h and plasma was isolated and stored at −80 °C.

Plasma concentration of CORT was measured using an I^{125} radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) as previously described (Krause et al., 2008, 2011).

2.4. Assessment of pro-opiomelanocortin (POMC) expression in the pituitary gland

We used semiquantitative real-time polymerase chain reaction (PCR) to measure mRNA expression of POMC in the pituitary.