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Relationship of Endothelin-1 and NLRP3 Inflammasome Activation in HT22 Hippocampal Cells in Diabetes

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Abstract

Diabetes increases the risk and worsens the progression of cognitive decline. Diabetic rats treated with the dual endothelin receptor antagonist bosentan, have been shown to improve hippocampal-based cognitive deficits. The NLRP3 inflammasome has been implicated in vascular complications of diabetes. We hypothesized that diabetes-mediated increase in endothelin-1 (ET-1) in hippocampal cells causes NLRP3 activation and inflammation. An *in vitro* model was employed by exposing HT22 hippocampal cells to normal (25 mM), low (5.5 mM) and high (50 mM) glucose conditions with and without palmitate (200 μ M) in the presence and absence of 10 μ M bosentan for 24 hours. NLRP3 activity was measured by western blotting for cryopyrin and caspase-1. ET-1 and IL-1 β expression was determined by ELISA. HT22 cells synthesize high levels of ET-1 in normal conditions, which was reduced with palmitate and bosentan as well as low and high glucose conditions. Decreased ET-1 levels were associated with greater activation of NLRP3 and IL-1 β in normal glucose. High glucose increased NLRP3 markers and activation compared to normal and low glucose. These data suggest that ET-1 may be protective to neurons. Although endothelin antagonism may be beneficial in improving vascular dysfunction and cognitive impairment, its impact on hippocampal neurons should be further explored.

Keywords

Diabetes; Endothelin; NLRP3 inflammasome; Hippocampus

Introduction

Diabetes, which affects nearly 29 million people in the United States alone, has been associated with a wide array of complications including cognitive impairment, stroke, cardiovascular disease, retinopathy and nephropathy (Kodl and Seaquist, 2008). Learning

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and memory deficits are the most prominent forms of cognitive impairment seen in type 2 diabetes (McCrimmon et al., 2012). It has been suggested that longer duration of diabetes is linked to poorer cognitive functioning (Gorelick et al., 2011). Diabetes-mediated microvascular dysfunction is believed to trigger neuronal, glial and vascular injury pathways contributing to pathological neovascularization, blood brain barrier (BBB) disruption, neuroinflammation and white matter damage. While the mechanisms are multifactorial, cerebrovascular dysfunction and reduced cerebral blood flow is believed to precede the negative changes in cognitive function observed in patients and experimental models. Our lab has shown cognitive deficits in Goto-Kakizaki (GK) rats, a lean model of type 2 diabetes, which is associated with cerebrovascular dysfunction and pathological neovascularization (Prakash et al., 2013). We recently reported similar changes in a high fat diet plus low dose streptozotocin-induced model of diabetes (Qu et al., 2014; Valenzuela et al., 2015). Activation of microglia in male GK rats is elevated and is thought to contribute to cognitive dysfunction and neurodegeneration (Hussain et al., 2014).

Diabetes is a chronic inflammatory disease. Increased glial activation in the hippocampus, an important domain in learning and memory, has been correlated with cognitive deficits in diabetic rats (Nagayach et al., 2014). Induction of cytokines and their downstream effectors cause astrocytic reactivity and increases BBB permeability due to endothelial injury. Nod-like receptor family pyrin domain-containing (NLRP) inflammasomes are important in the development of acute and chronic inflammatory responses through production of mature IL-1 β and IL-18. NLRP3, the most widely studied inflammasome, is comprised of the cytosolic NLR containing a pyrin domain, the adaptor protein apoptotic-speck containing a CARD (ASC) and caspase-1. NLRP3 has been implicated in diabetic retinopathy, a microvascular complication of diabetes (de Rivero Vaccari et al., 2014; Mohamed et al., 2014; Shi et al., 2015), but its regulation within the hippocampus in diabetes remains elusive.

Endothelin-1 (ET-1), a potent vasoconstrictor, stimulates inflammation through increased NF κ B activation, oxidative stress and pro-inflammatory cytokines via the activation of the ET_A receptor (Loomis et al., 2005; Yeager et al., 2012) while the activation of the ET_B receptor subtype may exert anti-inflammatory actions (Juergens et al., 2008). Elevation of ET-1 has been well documented in patients and experimental models in diabetes (Matsumoto et al., 2004; Takahashi et al., 1990). Dual endothelin receptor blockade prevents cerebrovascular dysfunction and improper neovascularization as well as improvement in hippocampal-based cognitive tasks in diabetes (Abdelsaid et al., 2014a, 2014b; Singh et al., 2014). Overexpression of endothelial ET-1 impairs BBB function and worsens cognitive dysfunction after ischemic injury (Zhang et al., 2013). Although ET-1 has been implicated in cognitive impairment in numerous studies, ET-1 effects on vascular dysfunction has been the focus. The role of ET-1 on neuronal function, specifically in the hippocampus, remains unclear. Thus, we proposed to examine the relationship between ET-1 and NLRP3 and hypothesized that diabetes-mediated increase in ET-1 in hippocampal neurons causes NLRP3 activation and inflammation.

Methods

HT22 Hippocampal Cell Culture and Treatment

Immortalized mouse HT22 hippocampal cells, a mouse hippocampal cell line that was spontaneously immortalized (Alliot et al., 1996) were provided by Dr. William Hill's laboratory, Augusta University, Augusta, GA. HT22 cells were cultured in DMEM media, supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin cocktail in a humidified incubator at 37°C with 5% CO₂. Because of the high metabolic needs, HT22 cells require higher glucose in normal growth medium. All experiments were completed in cells passaged no more than four times. Since we use a diet-induced model of diabetes, high glucose (50 mM) plus palmitate (200 µM in 50% ethanol) was used for all *in vitro* experiments. Palmitate is the most abundant saturated fatty acid and has been used in models of diabetes, such as diabetic retinopathy in endothelial cells (Mohamed et al., 2014). Cells were grown in low glucose (5.5 mM) to mimic low nutrient state, which may occur during endothelial dysfunction. Since we have previously shown bosentan treatment to attenuate vascular dysfunction in diabetic rats, bosentan (10 µM in water), a dual-endothelin receptor antagonist, was used to inhibit ET-1 action in HT22 cells. All treatments were incubated for 24 hours. Exogenous ET-1 (1 µM) was examined in control and high glucose plus palmitate for 24 hours. Cells and media were collected for the following analyses.

Western Blot Analysis

Cells were harvested after treatment and lysed in a modified RIPA buffer (Millipore Billerica, MA). Equal protein loads were boiled in sample buffer and separated on a 4–15% Mini-PROTEAN TGX gel by electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed for pro-caspase-1, caspase-1 p10 and cryopyrin (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. Primary antibodies were detected using a horseradish peroxidase-conjugated antibody and enhanced with chemiluminescence (GE Healthcare, NJ). Expression was standardized using β-actin (Abcam, Cambridge, MA). Immunoreactivity was analyzed using densitometry software (Alpha Innotech, Protein Simple, San Jose, CA).

Enzyme-Linked Immunosorbent Assay (ELISA)

ET-1 and IL-1β levels in the cell supernatant were assessed by enzyme-linked immunosorbent assay (R&D, Minneapolis, MN) according to the manufacturer protocol. Absorbance was read on a spectrophotometer with a wavelength of 450 nm.

Statistical Analysis

One-way ANOVA was performed on the ranks of each data set to compare treatments in each growth condition. A Tukey's adjustment for multiple comparisons was used for all post-hoc mean comparisons for significant ANOVAs. Data were expressed as mean ± SEM, with $p < 0.05$ denoted significant differences.

Results

Effects of dual endothelin receptor blockade on HT22 cells

HT22 cells grown in normal conditions synthesize high levels of ET-1 (n=3). Levels of ET-1 had a decreasing trend with palmitate or bosentan treatment (n=4). Dual-treatment with palmitate and bosentan significantly reduced ET-1 expression from no treatment (Fig. 1A; n=3). To study the relationship between ET-1 and inflammasome activation, NLRP3 markers and IL-1 β were measured. Palmitate in the presence (n=3) or absence (n=4) of bosentan decreased cryopyrin expression compared to baseline (n=4) and bosentan alone (n=3). Although dual endothelin receptor blockade with bosentan seemed to elevated cryopyrin expression, no difference was found (Fig. 1B). Pro-caspase-1, the inactive form of caspase-1, had no change in expression from baseline (n=3) with palmitate treatments (n=4), yet treatment with bosentan decreased expression (n=3). Treatment with palmitate and bosentan decreased pro-caspase-1 (n=3) compared to no treatment and palmitate alone (Fig. 1C). Active caspase-1 was measured by expression of the p10 subunit. HT22 cells treated with palmitate and bosentan, individually or together, decreased caspase-1 p10 subunit compared to baseline (n=4 all groups except bosentan alone where n=3). Bosentan treatment alone was significantly reduced from both groups treated with palmitate (Fig. 1D). Bosentan increased active IL-1 β in the presence or absence of palmitate (n=3), whereas palmitate treatment reduced IL-1 β expression compared to control (Fig. 1E; n=4).

Effects of dual endothelin receptor blockade on HT22 cells in low glucose

Palmitate decreased ET-1 levels and in combination with bosentan nearly abolishes expression in low glucose (Fig 2A; n=4). Cryopyrin expression increased in bosentan treated cells (n=4) compared to baseline and palmitate treatment (n=5), but no other significance was detected (Fig 2B). Inactivated pro-caspase-1 had higher expression with bosentan treatments compared to no treatment and palmitate (n=4 in all except palmitate treatments which n=5). Yet cells exposed to both palmitate and bosentan significantly decreased levels when compared to bosentan alone (Fig. 2C). Active caspase-1 expression in low glucose treated cells only showed significant decrease in palmitate plus bosentan treated cells when compared to bosentan alone (Fig. 2D; n=4). Bosentan heightened IL-1 β expression alone, although no significant difference was observed in the presence of palmitate (Fig. 2E; n=4).

Effects of dual endothelin receptor blockade on HT22 cells in high glucose

High glucose plus palmitate, used to model diabetic conditions, decreased ET-1 expression when compared to control growth conditions (p<0.05; n=4). Bosentan did not further reduce ET-1 levels, while palmitate decreased expression in the presence and absence of bosentan (Fig 3A; n=4). High glucose induced higher cryopyrin expression compared to control (p<0.01). Palmitate with (n=3) or without bosentan (n=4) decreased the expression of cryopyrin in high glucose conditions compared to non-treated cells (n=3) (Fig. 3B). High glucose in the presence or absence of palmitate reduced pro-caspase-1 expression compared to control glucose levels (p<0.01; n=4). Although neither palmitate nor bosentan (n=3) alone showed significant difference in pro-caspase-1 expression, combined treatment (n=3) increased expression from palmitate treatment alone (Fig. 3C). All treatments with palmitate and/or bosentan decreased caspase-1 p10 expression (Fig. 3D; n=4 except in bosentan alone

where n=3). NLRP3 inflammasome activation was measured through expression of IL-1 β . High glucose in the presence and absence of palmitate was higher than cells grown in control glucose conditions ($p<0.01$; n=3 no treatment; n=4 palmitate). Although bosentan had no difference from baseline, in the presence of palmitate, IL-1 β declined compared to all other treatment groups (Fig. 3E; n=3 bosentan; n=4 bosentan plus palmitate).

Effects of exogenous ET-1 on HT22 cells

To explore the impact of ET-1 directly on HT22 hippocampal cells, 1 μ M of ET-1 was added to normal growth conditions and diabetic growth conditions, mimicked by high glucose and palmitate. NLRP3 inflammasome markers and activation was measured. Cryopyrin expression was elevated in high glucose and palmitate compared to both control groups (Fig. 4A; n=3). Although no differences were observed in expression of pro-caspase-1 (Fig. 4B; n=3), there was a trend towards reduced expression in ET-1 treated diabetic (high glucose plus palmitate) cells. Activated caspase-1, measured by expression of the p10 subunit, was elevated in high glucose plus palmitate compared to control conditions, but treatment with ET-1 had no effect on caspase-1 p10 expression (Fig. 4C; n=3). Although no other statistical differences were seen, much like pro-caspase-1 in high glucose and palmitate treated cells, introduction of ET-1 to growth conditions had a trend towards reducing caspase-1 p10 subunit expression. High glucose plus palmitate was significantly higher in IL-1 β expression than control cells in the presence and absence of ET-1. Upon treatment with ET-1, IL-1 β levels decreased from baseline in high glucose plus palmitate treated cells, although the difference was not significant (Fig. 4D; n=4 control glucose; n=3 high glucose).

Discussion

The current study provides novel information for ET-1 and NLRP3 interaction in hippocampal cells. Our results showed that 1) HT22 hippocampal cells synthesize high levels of ET-1, which is reduced in low and high glucose conditions; 2) decreases in ET-1 expression is correlated with elevated levels of NLRP3 inflammasomes markers; 3) dual-endothelin receptor antagonism increases inflammatory markers in control and hypoglycemic condition; and 4) exogenous ET-1 reduces inflammasome activation in diabetic growth conditions.

Numerous studies have implicated endothelin in cognitive impairment in patients and experimental models (Calderón-Garcidueñas et al., 2013; Rodriguez et al., 2008; Singh et al., 2014; Zhang et al., 2013). A study in urban children showed treatment with flavonol-rich dark chocolate reduced ET-1 levels and individual improvement on short-term memory tasks, suggesting an association between ET-1 and cognition (Calderón-Garcidueñas et al., 2013). In experimental models, overexpression of ET-1 was correlated to cognitive decline following an ischemic/reperfusion injury (Zhang et al., 2013). These studies suggested that ET-1-mediated vascular dysfunction contributes to cognitive decline, which has been expanded to diabetic complications. Patients with type 2 diabetes show cognitive deficits including decreased verbal and nonverbal memory, attention, processing speed and executive function (Kodl and Seaquist, 2008). Diabetes is associated with elevated ET-1 plasma levels in patients and experimental models (Matsumoto et al., 2004; Takahashi et al., 1990). While

our lab and others have previously shown treatment with bosentan in diabetic rats prevents cerebrovascular dysfunction (Abdelsaid et al., 2014a) and subsequent cognitive impairment (Singh et al., 2014), vascular dysfunction has been the emphasis. Here we focus on neuronal impact of ET-1, specifically in HT22 hippocampal cells. We showed HT22 cells synthesize high levels of ET-1, while expression was reduced with treatment of bosentan in hypo- and hyperglycemic conditions. This reduction was associated with greater activation of NLRP3 and subsequent IL-1 β cleavage in control conditions. Growth conditions using high glucose plus palmitate to mimic high fat diet-induced diabetes in hippocampal cells with treatment of exogenous ET-1 showed decreased NLRP3 inflammasome activation. These results suggest that ET-1 may be protective to hippocampal neurons. Indeed, Park and Lee showed exogenous ET-1 protected murine HN33 hippocampal neurons from serum deprivation-induced apoptosis (Park and Lee, 2008). Mice lacking the endothelin converting enzyme-2 displayed deficiency in learning and memory tasks, such as Morris water maze (Rodriguez et al., 2008), supporting the potential neuroprotective role endothelin may play in cognition.

ET-1 receptors ET_A and ET_B are to be widely expressed in the brain, including in the neurons located in the hippocampus (Naidoo et al., 2004). We chose to use the dual endothelin receptor antagonist bosentan due to our previous work reporting prevention of cerebrovascular dysfunction and remodeling in diabetes (Abdelsaid et al., 2014a, 2014b). Others have reported improvement of hippocampal-based tasks through bosentan treatment in diabetic rats (Singh et al., 2014). Use of ET_B agonist IRL-1620 or ET_A selective antagonists BQ123 and BMS182874 prevented elevated oxidative stress and cognitive impairment induced by amyloid beta in rats (Briyal et al., 2014, 2011), suggesting that stimulation of ET_B is protective. We did not explore differential expression of ET-1 receptors in HT22 cells, which is a limitation of this study. Quantification of the ET_A/ET_B ratio may provide a better understanding of protective effects of ET-1. Dysregulation of ET receptors may provide an explanation for elevation of inflammatory markers seen in HT22 hippocampal cells. Future studies with specific ET_A and ET_B inhibitors are needed to elucidate the mechanism of ET-1 in regulation of inflammation in hippocampal cells.

The relationship between inflammasome activation and ET-1 has not been extensively studied. In systemic sclerosis patients, there is a positive correlation between NLRP3 activation and ET-1 levels (Martinez-Godinez et al., 2015). The role of ET-1 in NLRP3 activation in the brain and in diabetes has not been reported. Here we showed lower ET-1 levels in hippocampal cells correlated with an elevation in IL-1 β , but without concomitant increases in NLRP3 markers. Activation of IL-1 β can occur from multiple inflammasome complexes, including NLRC4, NLRP1, NLRP3 and AIM2 inflammasomes, although NLRP3 is the most extensively studied. NLRP1 can interact with caspase-1, -4 or -5, which then cleaves IL-1 β into its active form and is expressed in neurons and glial cells (Singhal et al., 2014). The lack of concomitant increases of active and inactive caspase-1, while IL-1 β was elevated, suggests that another mechanism for activation is taking place, perhaps through NLRP1. Indeed, it has been reported that NLRP1 mediates injury in cortical neurons exposed to high glucose (Meng et al., 2014). Further studies should explore other pathways of IL-1 β activation in order to better elucidate a mechanism for ET-1 and inflammasome activation.

The current study has several limitations that should be identified. First, cell survival and viability was not measured. It has been shown that high glucose leads to accumulation of reactive oxygen species leading to decreased cell viability in hippocampal cells as early as 24 hours, with major changes at 72 hours in 50 mM concentrations (Liu et al., 2014). Secondly, it has been reported that bosentan does not cross the BBB. While in diabetes BBB integrity is compromised, to better mimic the impact of dual-receptor antagonism on hippocampal neurons, a treatment that readily crosses the BBB should be further explored. Third, we used only one cell line. In a recent review, it was noted that cell lines derived from males and females behave differently. Hippocampal cells have been seen to be more sensitive to oxidative stress in male-derived cells than females (Shah et al., 2014). HT22 cells were derived from male mice. Future studies should explore other hippocampal cell lines, such as HN33, or primary rat hippocampal neurons, which would allow us to identify sex differences in hippocampal neurons. Lastly, we solely used hippocampal neurons and did not explore the interaction between neurons, endothelial cells and glial cells. Nevertheless, we conclude that in contrast to our hypothesis, both low and high glucose conditions decreased ET-1 levels in HT22 cells. The fact that decreased ET-1 levels are associated with greater activation of NLRP3 and IL-1 β in control conditions suggests that ET-1 may be protective in hippocampal neurons. Moreover, exogenous ET-1 suppresses inflammatory markers. Although endothelin antagonism may be beneficial in improving vascular dysfunction and preventing cognitive impairment, these results warrant further exploration in the relationship between ET-1 and inflammasome activation in diabetes and cognitive deficits.

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Fig. 1. HT22 hippocampal cells synthesize high levels of ET-1

Murine HT22 hippocampal cells were grown in 25 mM glucose media in the presence or absence of palmitate and/or bosentan for 24 hours. ET-1 levels were high in control conditions (n=3), while both the dual-receptor antagonist bosentan and palmitate treatment (n=4) reduced expression (A). NLRP3 inflammasome complex components cryopyrin (B) pro-caspase-1 (C) and p10 subunit of caspase-1 (D) were measured using western blot. IL-1 β levels increased in bosentan treated cells (E) without concomitant increases in caspase-1 levels (n=3). Results are expressed at mean $\pm$ SEM, *p<0.05 vs no treatment; #p<0.05 vs palmitate; \$p<0.05 vs bosentan; &p<0.05 vs palmitate and bosentan.

Fig. 2. Reduction of ET-1 is exacerbated in bosentan-treated cells in low glucose
 HT22 hippocampal cells were grown in low (5.5mM) glucose conditions to model nutrient deprivation which might occur with endothelial dysfunction. ET-1 levels decreased at baseline (n=4), and were further reduced by bosentan treatment (n=4) (**A**). Bosentan alone increased inflammatory markers cryopyrin (**B**), pro-caspase-1 (**C**) and IL-1 β (**E**), but not caspase-1 p10 (**D**) (n=4). Results are expressed at mean $\pm$ SEM, *p<0.05 vs no treatment; #p<0.05 vs palmitate; \$p<0.05 vs bosentan.

Fig. 3. High glucose reduces ET-1 expression and elevates inflammation

HT22 hippocampal cells grown in high glucose (50mM) were exposed to palmitate and/or bosentan for 24 hours. High glucose reduced ET-1 concentrations (n=4) compared to control, which was further decreased by palmitate (n=4) (A). Inflammatory markers were all elevated compared to control (B–E). While all treatments reduced cryopyrin (palmitate n=4; bosentan/bosentan plus palmitate n=3) (B) and caspase-1 p10 subunit (palmitate/bosentan plus palmitate n=4; bosentan n=3) (D), palmitate increased IL-1 β expression (n=4) (E). Results are expressed at mean $\pm$ SEM, *p<0.05 vs no treatment; #p<0.05 vs palmitate; §p<0.05 vs bosentan; &p<0.05 vs palmitate plus bosentan.

Fig. 4. Exogenous ET-1 reduces inflammasome markers in high glucose and palmitate treated group

HT22 hippocampal cells were grown in control glucose (25 mM) or high glucose (HG, 50 mM) plus palmitate in the presence and absence of exogenous ET-1 for 24 hours. Cryopyrin, although elevated in high glucose plus palmitate conditions, was reduced from baseline in ET-1 treated cells (A). Neither pro-caspase-1 (B) nor caspase-1 p10 subunit (C) showed significance with ET-1 treatments, although there was a trend towards reducing levels with ET-1. IL-1 β expression was elevated in high glucose plus palmitate treated groups, which was reduced back to control levels with ET-1 treatment (D). Results are expressed at mean $\pm$ SEM, n=3 in all groups except control and control + ET-1 in IL-1 β graph where n=4, *p<0.05 vs control; #p<0.05 vs control plus ET-1.