Endothelin Confers Protection Against High Glucose-induced Neurotoxicity via Alleviation of Oxidative Stress

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Abstract

Recent findings linked the inhibition in the neuromodulator peptide endothelin-1 (ET-1) level to the high glucose-evoked neurotoxicity. However, definitive neuroprotective role for ET-1, and the major neuronal ET (ET-3), against high glucose-evoked toxicity, and the implicated neurochemical responses triggered by their ET-A and ET-B receptors remain unknown. Here, we tested the hypothesis that ET-B activation alleviates high glucose-evoked oxidative stress and cell death. High glucose (100 mM for 48 hrs.)-evoked cell death was associated with elevation in reactive oxygen species, inhibition of catalase activity, and a paradoxical upregulation of hemeoxygenase-1 expression along with ET-A and ET-B receptors were down-regulated and up-regulated, respectively. ET-1 or ET-3, in concentrations that had no effect on PC12 cell viability in normal glucose medium, alleviated all high glucose-evoked neurochemical responses, except for the reduction in ET-A receptor expression. Prior (4 hrs.) incubation with a selective ET-A (BQ123) or ET-B (BQ788) receptor blocker abrogated the neuroprotection conferred by ET-1 or ET-3. However, the ET-B receptor played a greater role because BQ788 abrogated the favorable ET-1 or ET-3 mediated reversal of the ERK1/2 phosphorylation and the inhibition in catalase activity caused by high glucose. These findings suggest that endothelin exerts ET-B receptor dependent favorable redox and neuroprotective effects against high glucose-evoked oxidative damage and neurotoxicity.
Key words

Endothelin, PC12 cells, Neuroprotection, High glucose, Oxidative stress, ET-receptors.
Introduction

High glucose associated with diabetes mellitus (DM) causes central and peripheral nervous system complications (Holder et al., 1997; Edwards et al., 2008) due to cellular oxidative stress (Radi et al., 2014; Yan et al., 2014). Specifically, oxidative stress, due to glucose oxidation or reduced capacity of the antioxidant defense system (Edwards et al., 2008; Kamboj et al., 2010), can trigger neuronal cell death, a major cause of the neuropathology associated with DM (de la Monte and Tong, 2014). Therefore, local neuromodulators serve important roles in mediating or guarding against exacerbation of these neurotoxic effects of high glucose.

Endothelin (ET), a neuromodulator peptide that exists in three isoforms ET-1, 2 and 3 (Inoue et al., 1989), activates two ET receptor subtypes, ET-A and ET-B, with varying degrees of selectivity (Arai et al., 1990). Although high glucose increases ET level in vivo (Schneider et al., 2002) or decreases its level in in-vitro models (Ward and Ergul, 2016), the role of ET in high glucose-evoked neurotoxicity remains elusive due to controversial reports on ET effects on the nervous system. For example, ET activation of the ET-A receptor is implicated in cerebral ischemia/reperfusion injury (Matsuo et al., 2001), in other reports ET confers neuroprotection through both ET-A and B receptors (Laziz et al., 2011). It is possible, therefore, that ET alterations of the functions of redox enzymes and cell survival depend on the pathological conditions.

Catalase is generally recognized as antioxidant enzyme that confers cellular protection (Spitz et al., 1987). Similarly, hemeoxygenase (HO) exerts protective effect against cellular injury (Maines, 1997). Of the 3 HO isozymes, HO-1 is inducible by noxious stimuli and oxidative stress (Hartsfield et al., 1997). It is imperative to note, however, that chronic HO-1 induction may contribute to deleterious iron aggregation and mitochondrial dysfunction in neurodegenerative disorders (Schipper, 2000). Despite a shared ability of ET and HO-1 to guard against or contribute to cellular derangements under different settings, it is not known if both molecules will exert the
same function in high glucose-induced neurotoxicity. Interestingly, both ET and HO-1 share the ability to activate a common downstream signaling product, the extracellular signal-regulated kinases (ERK1/2), and to modulate reactive oxygen species (ROS) and apoptosis (Wu-Wong et al., 2000; Chen et al., 2016). Despite this knowledge, there are no studies on the effect of ET on HO-1 expression in neurons exposed to high glucose concentrations. Notably, a very recent correlative link between a reduction in ET-1 and high glucose-evoked neuroinflammation in vitro inferred a neuroprotective role for ET-1 (Ward and Ergul, 2016). Collectively, current evidence does not unequivocally support a neuroprotective role for ET-1 or ET-3 against high glucose evoked toxicity, and the implicated neurochemical mechanisms remain unknown.

The first aim of the present study was to determine if ET contributes to, or guards against, high glucose-induced neurotoxicity in cultured PC12 cells. Next, we tested the hypothesis that ET alleviates high glucose-evoked neurotoxicity via an ET-B receptor dependent up-regulation of HO-1 and enhancement of catalase activity. PC12 cells were used in our studies because they assume neuronal phenotype when exposed to nerve growth factor (Tischler and Greene, 1978), and constitute an established model for studying mechanisms of adverse neuronal effects caused by high glucose levels such as apoptosis and ROS generation (Lelkes et al., 2001). Cell viability, redox status and neurochemical responses were investigated in PC12 cells exposed to high or normal glucose level in the presence or absence of ET-1 or ET-3 and additional studies were conducted in the presence of selective ET-A or ET-B receptor blockade under high glucose condition.
Materials and Methods

Drugs

ET-1, ET-3, BQ123, BQ788 were purchased from sigma-Aldrich (St. Louis, MO).

Cell culture and treatment

Rat pheochromocytoma cells (PC12 cells) (ATCC, Rockville, MD) were cultured in RPMI 1640 medium supplemented with horse serum (15%), fetal bovine serum (2.5%), penicillin (100 U/ml) and streptomycin (100 U/ml), at 37°C with saturated air containing 5% CO₂. Cells were passaged in Corning Cell Bind flasks for proper adherence then differentiated by treating with NGF (50 ng/ml) for 48 hrs. to initiate neuronal differentiation, and culture media was changed every 2 days as in our previous studies (Zhang et al., 2001). The differentiated cells were examined microscopically and the differentiated cells were used in subsequent experiments.

Determination of cell viability

In order to establish the concentration-dependent neurotoxicity caused by glucose in our model system, PC12 cells were seeded at 50,000 cells/ml in 96-well plate in RPMI 1640 medium with serum for 48 hrs. (90% confluence), then treatments were started in low serum RPMI 1640 (0.5% FBS and 1% antibiotic-antimycotic) with normal (11.11 mM) or escalated (25, 50,100,150 mM) glucose concentrations for another 48 hrs. At the end of the incubation period, cell viability was measured by MTS cell proliferation assay kit (Promegra, Madison, WI) with absorbance measured at 495 nm in accordance with manufacturer’s instructions. The 100 mM glucose concentration was selected for the subsequent studies. Second, to investigate the effect of ET on cell viability, cells were treated with ET-1 (10⁻¹⁴ - 10⁻⁶ M), ET-A blocker (BQ123, 10⁻¹²-10⁻⁶ M) or ET-B blocker (BQ788, 10⁻¹²-10⁻⁶ M) in low serum RPMI 1640 with normal (11.11 mM) or high (100
mM) glucose concentrations for 48 hrs. Subsequently, a 1 µM dose of these pharmacological interventions was selected for conducting the mechanistic studies detailed below.

**Caspase 3/7 activity assay**

The same experimental protocol described above was followed under this experiment. Cultured cells were plated in white-walled 96-well plate and incubated for 48 hrs. Media containing the appropriate concentration of different agents was added to the cells for 48 hrs. After that, 100 µl of Caspase-Glo 3/7 reagent (Promega, Madison, WI) was then added to each well as directed by the manufacturer and luminescence was measured in a luminometer. Luminescence is proportional to the caspase activity.

**ROS measurement**

We used two methods to measure oxidative stress in cultured cells, the chloromethyl-2’, 7’ dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Life Technologies, Grand Island, NY) and the dihydroethidium methods. Under oxidative stress conditions, CM-H2DCFDA is oxidized to 2’, 7’ dichlorofluorescein (DCF). Cells were loaded with 5 mM CM-H2DCFDA (Molecular Probes, Invitrogen) in phenol red-free, low serum medium containing 11.11, 100 and 150 mM glucose for 48 hrs. Cells were then trypsinized and DCF fluorescence was measured using an Accuri C6 flow cytometer (BD Accuri Cytometers, Ann Arbor, MI) at an excitation wavelength of 488 and a 533 nm emission filter.

Moreover, the effect of ET-1 or ET-3 (1 µM for 48 hrs.) on ROS levels was studied in cultured PC12 cells grown in normal (11.11 mM) or high (100 mM) glucose medium using the DCFH-DA method. The oxidation- sensitive fluoroprobe DCFH-DA was used as a substrate for measuring intracellular ROS. Quantification was conducted by examining fluorescence intensity using a microplate fluorescence reader at excitation 485 nm/emission 530 nm. Kinetic readings were
recorded for 60 min at 37°C. ROS level was calculated by relative DCF fluorescence per microgram of protein. Positive and negative controls were used to validate the assay as in our previous studies (McGee and Abdel-Rahman, 2012). Further, we used mannitol (100 mM) as osmotic control for high glucose (100 mM) in accordance with reported studies (Sharifi et al., 2009).

**Dihydroethidium staining for superoxide detection**

To validate the findings with the DCF method and in accordance with current recommendations that at least 2 different methods are needed for quantifying ROS generation (Griendling et al., 2016), PC12 cells were incubated with 10 µM dihydroethidium (DHE) (Molecular Probes, Grand Island, NY) at 37°C in the presence of 5% CO₂ in a moist chamber for 30 min. Positive and negative controls were used to validate the assay. Images were visualized with a Zeiss LSM 510 microscope. Quantification was conducted using Image J Software (National Institutes of Health) and changes in total fluorescence intensity, normalized to control, were calculated as reported (Collin et al., 2007).

We elucidated the implicated ET receptor subtype by investigating the effects of 48-hrs. treatment with ET-1 or ET-3 (1 µM) on PC12 cell viability (MTS) and apoptosis (caspase-3/7) and ROS level, under high (100 mM) glucose conditions, in the absence or presence of a selective ET-A (BQ123, 1 µM) or ET-B (BQ788, 1 µM) blocker. The selective blocker or its vehicle was added to the incubation medium 4 hrs. before ET-1, ET-3 or their vehicle.

**Western blot analysis**

The method described in our previous studies was employed to determine the effect of high (100 mM) glucose level expression level of ET-A, ET-B, HO-1, nNOS and ERK1/2 (Rezq and Abdel-Rahman, 2016) in the absence or presence of ET-1 or ET-3. In these experiments, PC12
cells were cultured at 50,000 cells/ml in 6-well plate in triplicates in RPMI with serum for 48 hrs. (90% confluence). Treatment started with low serum RPMI with NG (11.11 mM) or HG (100 mM) for 48 hrs. with or without ET-1 or ET-3 (1 µM) in presence or absence of ET-A (BQ123, 1 µM) or ET-B (BQ788, 1 µM) blockade. At the end of each experiment, plates were put on ice and cells were washed with cold sterile PBS twice then stored at -80 ºC for later use.

Cells were harvested by scrapping on ice with lysis buffer containing 20 mM TRIS, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton x-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate and 1 µg/ml leupitin with protease inhibitor cocktail (Roche diagnostics, Indianapolis, IN). After homogenization and centrifugation, proteins in the supernatant were quantified by using Bio-Rad protein assay system (Bio-Rad laboratories, Hercules, CA). Equivalent amounts of proteins (20 µg/lane) were applied to 4-12% SDS/PAGE gel (Invitrogen, Carlsbad, CA) and then transfer was done using nitrocellulose membranes which were then incubated with anti ET-A, ET-B receptors, HO-1 or p-ERK1/2 polyclonal antibodies (1:500; Abcam) at 4 ºC overnight. Nitrocellulose membranes were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 then incubated for 60 min with mixture containing IRDye680-conjugated goat anti-mouse and IRDye800-conjugated goat anti-rabbit (1:15000; LI-COR Biosciences). Bands representing the proteins were detected simultaneously by using Odyssey Infrared Imager and analyzed with Odyssey application software version 5.2 (LI-COR Biosciences). Data represents mean values of integrated density ratio of ET-A, ET-B receptors, HO-1 or p-ERK1/2 normalized to the corresponding housekeeping GAPDH or β-actin protein and expressed as percentage of control. Negative controls were run to validate the specificity of the antibodies used in our studies.
Catalase activity measurements

PC12 cells were seeded into 6-well plate at a density of $1 \times 10^5$ cells/well. After the treatments, cells were harvested and homogenized in 50 µl of 0.1 M phosphate buffer (pH 7.4). The mixture was centrifuged at 3000 rpm for 5 min at 4 °C. The supernatant was separated and assayed for protein content (Bradford assay, Bio-Rad). Catalase activity was determined calorimetrically in 10 µg protein using the Catalase Assay Kit (catalog no. CAT-100, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions by spectrophotometry.

Data analysis and statistics

Data were collected from three independent experiments, and are expressed as mean ± standard error of mean (SEM). Statistical analysis was conducted by using one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey's post hoc test. Prism 5.0 software (Graphpad software Inc., San Diego, CA) was used to perform statistical analysis, P < 0.05 was considered significant.
Results

ET-1 and ET-3 abrogated high glucose-induced neurotoxicity.

Exposure of PC12 cells to higher than normal glucose for 48 hrs. caused concentration dependent reductions in cell viability (Fig. 1A). While incubation with ET-1 (10^{-14}-10^{-6} M) or ET-3 (10^{-6} M) for 48 hrs. had no effect on net cell number of PC12 cells cultured in normal glucose (Fig. 1B and 1C), ET-1 (10^{-6} M) abrogated the high (100 mM) glucose-evoked reduction in cell viability (Fig. 1D). Therefore, we selected the 100 mM glucose and the 10^{-6} M ET-1 or ET-3 concentrations for subsequent experiments. Neither selective ET-A (BQ123) nor ET-B (BQ788) antagonist (10^{-12} to 10^{-6} M) influenced PC12 cells viability in normal or in high (100 mM) glucose medium (Fig. 1E and 1F). However, BQ123 (10^{-6} M) or BQ788 (10^{-6} M) abrogated the neuroprotective effect of ET-1 (10^{-6} M) against high glucose (100 mM)-evoked neurotoxicity (Fig. 1E). Similarly, BQ788 (10^{-6} M) abrogated the neuroprotective effect of ET-3 (10^{-6} M) in high (100 mM) glucose medium (Fig. 1F).

Flow cytometry (CM-H2DCFDA) findings showed that PC12 cells grown for 48 hrs. in high glucose (100 and 150 mM) concentration exhibited approximately two-fold increases in ROS levels (Fig. 2). Two additional assays, DCF kinetics (Fig. 3) and DHE fluorescence staining intensity (Fig. 4), confirmed and extended this finding by showing that the significant (P < 0.05) high (100 mM) glucose-evoked increase in ROS level was significantly (P < 0.05) attenuated by ET-1 or ET-3 (10^{-6} M) (Figs. 3A and 4G). The latter neuroprotective effect was abrogated by prior ET-A (BQ123, 10^{-6} M) or ET-B (BQ788, 10^{-6} M) blockade (Fig. 3B). Exposure to high glucose significantly (P < 0.05) enhanced caspase-3 activity, and this effect was abrogated by co-incubation with ET-1 (10^{-6} M), but not with ET-3 (10^{-6} M) (Fig. 5A). This neuroprotective anti-apoptotic effect of ET-1 was negated by selective ET-A (BQ123, 10^{-6} M) or ET-B (BQ788, 10^{-6} M) blockade (Fig. 5B). Importantly, ET-1 (10^{-6} M) or ET-3 (10^{-6} M) significantly (P < 0.05) increased
ROS level (Figs. 3A and 4G) and apoptosis (Fig. 5A) when PC12 cells were cultured in normal glucose, and prior selective ET-A (BQ123, 10^{-6} M) or ET-B (BQ788, 10^{-6} M) blockade abrogated the ET-1 or ET-3 evoked increase in ROS level (Fig. 3A).

**ET reversed HO-1 induction and catalase inhibition caused by high glucose.**

High glucose (100 mM) concentration significantly decreased ET-A receptor expression and this inhibition was not affected by ET-1 or ET-3 in presence or absence of ET-A or ET-B antagonist (Fig. 6A). On the other hand, ET-1 or ET-3 reversed high glucose induced elevation in ET-B receptor expression (Fig. 6C) and selective ET-B blockade (BQ788) abrogated the effect of ET-1 (Fig. 6C). Also, high glucose (100 mM) caused significant ($P < 0.05$) HO-1 induction (Fig. 7), ERK1/2 phosphorylation (Fig. 8) and inhibition of catalase activity (Fig. 9A). Notably, the parallel increases in p-ERK1/2 and total ERK1/2 expression under high glucose conditions (Table 1) precluded appropriate elucidation of the role of ET-1 or ET-3 in ERK1/2 phosphorylation in our model system. Therefore, we normalized p-ERK1/2 to GAPDH in these studies (Fig. 8). These neurochemical effects were reversed by ET-1 or ET-3 in an ET-B dependent manner because selective ET-B, but not ET-A, blockade abrogated the favorable effects of ET-1 or ET-3 (Figs. 7, 8 and 9B). Further, under high glucose conditions, ET-1 or ET-3 enhanced ($P < 0.05$) n-NOS phosphorylation and this effect was abrogated by ET-A or ET-B blockade (Fig. 10).
Discussion

The current study is the first to present evidence for, and elucidate the mechanism of, ET alleviation of high glucose-induced neurotoxicity based on the following main findings in PC12 cells: (i) ET-1 or ET-3 protected against high glucose-induced ROS generation and cell death. (ii) High glucose down-regulated ET-A, and up-regulated ET-B, receptors. (iii) ET-1 or ET-3 reversed the high glucose-evoked induction of HO-1 and ERK1/2 phosphorylation and the reduction in catalase activity. (iv) Selective ET-B blockade abrogated the favorable effects of ET-1 or ET-3 on the cell survival and the associated neurochemical responses. Together, these data suggest that ET-1 and ET-3 alleviate high glucose-evoked neurotoxicity, at least partly, via ET-B dependent reversal of the compromised catalase activity and the paradoxical induction of HO-1.

We adopted the PC12 cell model system for the following reasons. First, reported studies including ours show that PC12 cells assume neuronal phenotype when incubated with nerve growth factor (Tischler and Greene, 1978; Zhang et al., 2001). Second, high glucose causes oxidative stress and cell death in PC12 cells (Koshimura et al., 2002; Sharifi et al., 2009). Third, PC12 cells express ET-1, ET-3 and their receptors, ET-A and ET-B (Martin et al., 1990). Despite this knowledge, there are no studies on whether ET-A/ET-B signaling modulates the high glucose evoked neurotoxicity in any neuronal model system. As an important foundation, we showed that glucose caused concentration-dependent oxidative stress (ROS) and cell death in our model system (Figs. 1-5), which agrees with findings in different in vitro preparations (Koshimura et al., 2002; Ward and Ergul, 2016). We utilized three ROS and two cell death/survival assays in accordance with recent guidelines that at least two different assays are needed for ROS (Griendling et al., 2016), and cell death (Mills et al., 1995). Collectively, our initial findings are consistent with an important role for oxidative stress in the high glucose-evoked neurotoxicity in cultured neuronal cells (Lelkes et al., 2001; Vincent et al., 2005; Shi et al., 2015). It should be noted that mannitol (100 mM) used as osmotic control for high glucose had no effect on ROS.
generation (Fig. 3), cell viability or apoptosis (Sharifi et al., 2009), which rules out hyperosmolar conditions as a cause for high glucose-evoked oxidative stress and cell toxicity in PC12 cells.

While several lines of evidence implicate ET in diabetes related vascular complications (Ergul, 2011), few controversial findings are reported on ET role in diabetes-induced neurological impairment (Demir et al., 2015; Ward and Ergul, 2016). Given the lack of studies on ET effects on PC12 cell function, and the paucity of such studies in other neuronal model systems, we considered the possibility that ET-1 exerts a biphasic effect on the redox status and cell survival. To address this question, we investigated the effect of ET-1 on the viability of cells incubated in normal or high glucose media in concentrations (10^{-14} to 10^{-6} M) used in studies on ET receptor signaling in PC12 cells cultured in normal glucose medium (Takekoshi et al., 2002; Gardner et al., 2005); no data on cell survival or ROS level were generated in these studies.

We show, for the first time, that consistent with findings in vascular smooth muscle cells (Dong et al., 2005), ET-1 or ET-3 increased ROS level (Figs. 3 and 4) and apoptosis (Fig. 5) in PC12 incubated in normal glucose. By marked contrast, we present a novel ET-1 concentration-dependent protection against cell death (Fig. 1D), and the ability of ET-1 or ET-3 (1 µM) to abrogate high glucose-evoked ROS generation (Figs. 3 and 4). These findings are consistent with ET-1 preservation of cell viability, via limiting apoptosis, in serum-deprived neurons (Laziz et al., 2011). However, the effect of ET-1 or ET-3 on cell viability of neurons cultured in normal glucose was not investigated in the latter study (Laziz et al., 2011). Collectively, while ET-1 or ET-3 produces oxidative stress in PC12 cells cultured in normal glucose medium, it protects against neuronal death caused by high glucose or other stressful conditions.

While the opposite effects of ET-1 on neuronal apoptosis in normal and high glucose media remains to be elucidated, it is important to note that ROS production might serve as a protective adaptive mechanism against cell death (Dong et al., 2005). For example, at different levels, ROS

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may elicit variable biological responses depending upon the cell type and conditions, including cell proliferation enhancement (Wedgwood et al., 2001; Martindale and Holbrook, 2002). The latter might explain the preservation of the net cell number of PC12 cells despite ROS generation and apoptosis caused by ET-1 in cells cultured in normal glucose medium (Figs. 3-5). Another plausible explanation for the contrasting effects of ET on the redox status and outcomes under normal and stressful (high glucose) conditions is the modulation of some redox enzymes.

We investigated the effect of ET-1 or ET-3 on catalase activity and HO-1 expression because of their antioxidant roles (Spitz et al., 1987). First, consistent with catalase antioxidant biology (Chen et al., 2005), the inhibition of its activity (Fig. 9A) might explain, at least partly, the high glucose-evoked neurotoxicity. This notion is supported by the ET-1 or ET-3 restoration of catalase activity (Fig. 9A) and the alleviation of the neurotoxicity (Fig. 1D). Second, an unexpected induction of the antioxidant enzyme HO-1 (Fig. 7) might be explained by HO-1 mediation of chronic oxidative stress and neurodegeneration in Alzheimer and Parkinson's diseases (Schipper et al., 1995; Frankel et al., 2000; Schipper, 2004; Calabrese et al., 2006). Further, the ability of ET-1 or ET-3 to enhance n-NOS phosphorylation (Fig. 10) might contribute to the suppression of ROS level in PC12 cells (Kasamatsu et al., 2014). Mechanistically, HO-1 induction of ERK1/2 phosphorylation (Chen et al., 2016) contributes to oxidative stress (Myhre et al., 2004) via activation of NADPH oxidase (Shichiri et al., 1998) or phospholipase A2 (Sellmayer et al., 1996). Here, we present the first evidence that high glucose caused HO-1 induction and ERK1/2 phosphorylation, and that ET-1 or ET-3 reversed these neurochemical responses (Figs. 7 and 8). Nonetheless, it was important to determine if the ET-1 or ET-3 reversal of the divergent effects of high glucose on catalase and HO-1 were ET receptor-mediated and to determine the implicated ET receptor subtype(s).

Our data suggest that the ET-B, with a lesser contribution of the ET-A, receptor mediates the neuroprotective effect of ET-1 or ET-3 because: (i) Selective ET-A (BQ123) or ET-B (BQ788)
blockade abrogated the ET-1 or ET-3 alleviation of high glucose-evoked HO-1 induction, ROS production and cell death. (ii) ET-1 or ET-3 reversed the ET-B receptor upregulation, but had no effect on ET-A receptor downregulation, caused by high glucose. (iii) Selective ET-B receptor blockade (BQ788) abrogated the ET-1 or ET-3 mediated reversal of high glucose-evoked ERK1/2 phosphorylation and catalase inhibition. These findings are consistent with the widespread expression of ET-A and ET-B receptors in the brain (Naidoo et al., 2004), a neuroprotective and anti-apoptotic role of ET (Shichiri et al., 1998; Dong et al., 2005; Laziz et al., 2011), and a neurorescuer potential for selective ET-B agonists against stroke and cerebral ischemia (Nishikawa et al., 2011; Leonard and Gulati, 2013). Further, a very recent study showed that non-selective ET receptor blockade abolished the indirectly deduced neuroprotective effect of ET-1 against high glucose-evoked inflammation in hippocampal cells (Ward and Ergul, 2016). To overcome the limitations of the latter study (Ward and Ergul, 2016), we utilized both ET-1 and ET-3 (the prevalent brain ET) besides selective ET-A and ET-B blockers under normal and high glucose conditions.

It is important to acknowledge a limitation of our study that pertains to a lack of support of a causal role for HO-1 induction in the high glucose-evoked neurotoxicity. We considered determining if HO-1 inhibition protects against high glucose evoked neurotoxicity. However, this approach was deemed unfeasible because the accumulation of HO-1 neurotoxic substrate, hemin (Levy et al., 2002; Robinson et al., 2009), which may explain a counterintuitive elevation in blood pressure following pharmacological inhibition of brainstem HO-1 in our earlier studies (Nassar et al., 2011). Despite this limitation, our findings that ET-B blockade abrogated the induction of HO-1 and protected against high glucose evoked neurotoxicity highlight the importance of the down-regulatory role of ET-B receptor signaling on HO-1 expression in our model system. Future studies are needed to understand the mechanism of the drastic suppression and elevation in ET-B receptor and HO-1, respectively, caused by high glucose in our model system.
In conclusion, our study provides the first evidence for neuroprotective roles for ET-1 and ET-3 against high glucose-evoked neurotoxicity. While dual ET receptors blockade protects against diabetes-evoked cognitive impairment by improving vascular function via ET-A blockade (Abdelsaid et al., 2014), a deeper insight is needed to determine if concurrent blockade of neuronal ET-B receptor somehow dampens the neuroprotection conferred by neuronal ET-1 and ET-3. Our findings are relevant to diabetic patients treated with nonselective ET receptor blockers, for the treatment of vascular anomalies, because they might lose the ET-B mediated protection against high glucose-evoked neuronal complications.
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Authorship contributions

Participated in research design: Fouda, Abdel-Rahman.

Conducted experiments: Fouda.

Performed data analysis: Fouda.

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References


Footnotes:

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Figure legends

**Fig. 1.** PC12 cells viability measured by MTS assay after 48 hrs. incubation showing (A) Effect of gradual increasing of glucose concentration (11.11, 25, 50, 100, 150 mM). (B) Effect of ET-1 (10^{-14}-10^{-6} M) on cells incubated in normal glucose medium (11.11 mM). (C) Effect of ET-1 (10^{-6} M) or ET-3 (10^{-6} M) or their vehicle (sterile distilled water) on cells incubated in normal glucose medium (11.11 mM). (D) Effect of ET-1 (10^{-12}-10^{-6} M) or its vehicle on high glucose (100 mM) induced cytotoxicity. (E) Effect of co-incubation of selective ET-A antagonist (BQ123, 10^{-6} M) or selective ET-B antagonist (BQ788, 10^{-6} M) with ET-1 (10^{-6} M) on cells incubated in high glucose concentration (100 mM) compared to vehicle-treated cells incubated in normal (11.11 mM) or high (100 mM) glucose concentration. (F) Effect of co-incubation of selective ET-B antagonist (BQ788, 10^{-6} M) with ET-3 (10^{-6} M) on the viability of cells incubated in high glucose concentration (100 mM) compared to vehicle-treated cells incubated in normal (11.11 mM) or high (100 mM) glucose concentration. Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle” values; #P < 0.05 versus corresponding “100 mM/vehicle”. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 2.** The chloromethyl-2', 7' dichlorodihydrofluorescein diacetate (CM-H2DCFDA) probe assay of intracellular ROS measurement for PC12 after 48 hrs. incubation in normal (11.11 mM) or high (100 or 150 mM) glucose concentration (A). Quantification of DCF fluorescence by the flow cytometry is shown (B). Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.
**Fig. 3.** The 2′,7′-dichlorofluorescein biochemical assay of the generation of ROS in PC12 cells showing the slopes (regression coefficients) of the regression lines representing the rate of ROS production elicited by ET-1 (10⁻⁶ M) or ET-3 (10⁻⁶ M) or their vehicle in normal or high (100 mM) glucose concentrations are shown (A). Effect of co-incubation of selective ET-A antagonist (BQ123, 10⁻⁶ M) or selective ET-B antagonist (BQ788, 10⁻⁶ M) or their vehicle with ET-1 (10⁻⁶ M) or ET-3 (10⁻⁶ M) or ET-vehicle on ROS production in PC12 incubated in high glucose (100 mM) is shown (B). Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle” values; †P < 0.05 versus corresponding “100 mM/vehicle”; ‡P < 0.05 versus corresponding “vehicle/ET-1” or “vehicle/ET-3” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 4.** Confocal images showing superoxide level indicated by dihydroethidium (DHE) staining (red) in PC12 cells incubated for 48 hrs. with ET-1 (10⁻⁶ M) or ET-3 (10⁻⁶ M) or their vehicle in normal (11.11 mM) or high (100 mM) glucose concentration (A to F). Group data showing ROS level expressed as mean fluorescence intensity of DHE staining measured using NIH ImageJ analysis of confocal images (G). Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle” values; †P < 0.05 versus corresponding “100 mM glucose/vehicle” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 5.** Caspase 3/7 activity for apoptosis measurement shows the effect of ET-1 (10⁻⁶ M) or ET-3 (10⁻⁶ M) or their vehicle in normal or high (100 mM) glucose concentrations (A). Effect of co-incubation of selective ET-A antagonist (BQ123, 10⁻⁶ M) or selective ET-B antagonist (BQ788, 10⁻⁶ M) or their vehicle with ET-1 (10⁻⁶ M) or ET-3 (10⁻⁶ M) or ET-vehicle in PC12 incubated in high glucose (100 mM) (B). Values are expressed as means ± SEM of three independent experiments.
Data are presented as a percentage increase from untreated control cells. Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle” values; #P < 0.05 versus corresponding “100 mM glucose/vehicle”. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 6.** Western blots analysis showing the effect of ET-1 (10^{-6} M), ET-3 (10^{-6} M) or their vehicle in normal or high (100 mM) glucose concentrations on protein expression of ET-A receptor ratio to GAPDH protein (housekeeping protein) in presence or absence of selective ET-A antagonist (BQ123, 10^{-6} M) or selective ET-B antagonist (BQ788, 10^{-6} M) or their vehicle (A). Western bands depicting the protein expression are shown below the bar graphs (B). Western blots analysis showing the effect of ET-1 (10^{-6} M) or ET-3 (10^{-6} M) or their vehicle in normal or high (100 mM) glucose concentrations on protein expression of ET-B receptor ratio to GAPDH protein (housekeeping protein) in presence or absence of selective ET-A antagonist (BQ123, 10^{-6} M) or selective ET-B antagonist (BQ788, 10^{-6} M) or their vehicle (C). Western bands depicting the protein expression are shown below the bar graphs (D). Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle” values. #P < 0.05 versus corresponding “100 mM glucose/vehicle”; $P < 0.05 versus corresponding “vehicle/ET-1” or “vehicle/ET-3” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 7.** Western blots analysis showing the effect of ET-1 (10^{-6} M), ET-3 (10^{-6} M) or their vehicle in normal or high (100 mM) glucose concentrations on protein expression of HO-1 receptor ratio to β-actin protein (housekeeping protein) in presence or absence of selective ET-A antagonist (BQ123, 10^{-6} M) or selective ET-B antagonist (BQ788, 10^{-6} M) or their vehicle (A). Western bands depicting the protein expression are shown below the bar graphs (B). Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle”
values; $^\# P < 0.05$ versus corresponding “100 mM glucose/vehicle”. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 8.** Western blots analysis showing the effect of ET-1 ($10^{-6}$ M), ET-3 ($10^{-6}$ M) or their vehicle in normal or high (100 mM) glucose concentrations on protein expression of phosphorylated extracellular signal-regulated kinases (p-ERK1/2) to GAPDH protein (housekeeping protein) in presence or absence of selective ET-A antagonist (BQ123, $10^{-6}$ M) or selective ET-B antagonist (BQ788, $10^{-6}$ M) or their vehicle (A). Western bands depicting the protein expression are shown below the bar graphs (B). Values are expressed as means ± SEM of three independent experiments. *$P < 0.05$ versus corresponding “NG/vehicle” values; $^\# P < 0.05$ versus corresponding “100 mM glucose/vehicle”; $^\$ P < 0.05$ versus corresponding “vehicle/ET-1” or “vehicle/ET-3” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 9.** Effect of ET-1 ($10^{-6}$ M) or ET-3 ($10^{-6}$ M) or their vehicle in normal or high (100 mM) glucose concentrations on catalase activity is shown (A). The influence of co-incubation of selective ET-A antagonist (BQ123, $10^{-6}$ M) or selective ET-B antagonist (BQ788, $10^{-6}$ M) or their vehicle with ET-1 ($10^{-6}$ M) or ET-3 ($10^{-6}$ M) or ET-vehicle in PC12 incubated in high glucose (100 mM) (B). Values are expressed as means ± SEM of three independent experiments. *$P < 0.05$ versus corresponding “NG/vehicle” values; $^\# P < 0.05$ versus corresponding “100 mM glucose/vehicle”; $^\$ P < 0.05$ versus corresponding “vehicle/ET-1” or “vehicle/ET-3” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.

**Fig. 10.** Western blots analysis showing the expression of phosphorylated neuronal nitric oxide synthase (p-nNOS) normalized to total nNOS in PC12 cultured in high glucose (100 mM) medium
in the absence or presence of ET-1 (10^{-6} \text{M}), ET-3 (10^{-6} \text{M}) as well as selective ET-A (BQ123, 10^{-6} \text{M}) or selective ET-B (BQ788, 10^{-6} \text{M}) or blockade (A). As a control, data collected under normal glucose conditions is also shown. Western bands depicting the protein expression are shown below the bar graphs (B). Values are expressed as means ± SEM of three independent experiments. *P < 0.05 versus corresponding “NG/vehicle” values; #P < 0.05 versus corresponding “100 mM glucose/ET-1”; &P < 0.05 versus corresponding “100 mM glucose/ET-3” values. Statistical analysis was conducted by one-way or repeated measures analysis of variance for multiple comparisons followed by Tukey’s post hoc test.
Table 1. Western blot data showing the effect of ET-1 (10^{-6} M), ET-3 (10^{-6} M), compared to their vehicle, in normal (NG) or high (100 mM) glucose concentrations on the expression of phosphorylated extracellular signal-regulated kinases (p-ERK1/2), total extracellular signal-regulated kinases protein (t-ERK1/2) and p-ERK1/2 to t-ERK1/2 ratio in presence or absence of selective ET-A (BQ123, 10^{-6} M) or ET-B (BQ788, 10^{-6} M) receptor blockade or their vehicle.

<table>
<thead>
<tr>
<th>Group</th>
<th>p-ERK (Pixels)</th>
<th>t-ERK (Pixels)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG (Veh/Veh)</td>
<td>100.00 ± 7.24</td>
<td>100.00 ± 5.61</td>
<td>1.01 ± 0.12</td>
</tr>
<tr>
<td>100 mM Glucose (Veh/Veh)</td>
<td>356.62 ± 28.53</td>
<td>318.34 ± 14.56</td>
<td>1.12 ± 0.06</td>
</tr>
<tr>
<td>100 mM Glucose (Veh/ET-1)</td>
<td>101.25 ± 5.33</td>
<td>86.21 ± 5.88</td>
<td>1.19 ± 0.14</td>
</tr>
<tr>
<td>100 mM Glucose (BQ123/ET-1)</td>
<td>171.55 ± 21.67</td>
<td>153.11 ± 2.67</td>
<td>1.12 ± 0.15</td>
</tr>
<tr>
<td>100 mM Glucose (BQ788/ET-1/)</td>
<td>337.98 ± 33.29 $</td>
<td>284.37 ± 3.74 $</td>
<td>1.19 ± 0.12</td>
</tr>
<tr>
<td>100 mM Glucose (Veh/ET-3)</td>
<td>98.34 ± 7.64</td>
<td>93.57 ± 5.41</td>
<td>1.05 ± 0.02</td>
</tr>
<tr>
<td>100 mM Glucose (BQ788/ET-3)</td>
<td>245.64 ± 24.56 $</td>
<td>206.55 ± 5.20 $</td>
<td>1.19 ± 0.12</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM of three independent experiments, and normalized to values obtained under normal glucose conditions. *P < 0.05 versus corresponding “NG (veh/veh)” values; #P < 0.05 versus corresponding “100 mM glucose (veh/veh)” values; $P < 0.05 versus corresponding “100 mM glucose (ET-1/veh)” or “100 mM glucose (ET-3/veh)” values.
Figure 1

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Figure 3

A

ROS production (μM/mg protein/min)

Mannitol  Veh/veh  Veh  BQ123  BQ788  Veh  BQ788  Veh  ET-1  ET-3

NG  100 mM glucose

0  50  100  150

ET-1  ET-3

B

ROS production (μM/mg protein/min)

Veh/veh  Veh  BQ123  BQ788  Veh  BQ788  Veh  ET-1  ET-3

100 mM glucose

0  50  100  150

ET-1  ET-3
Figure 4

A  Veh  
Normal Glucose

B  ET-1  

C  ET-3  

D  
100 mM Glucose

E  

F  

G  NG  
100 mM glucose

Fluorescence Intensity ($\times 10^x$)

Ven  ET-1  ET-3  
Ven  ET-1  ET-3

*  #  #

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Figure 5

A

NG

100 mM glucose

Caspase-3 activity (% of NG)

Veh  ET-1  ET-3

Veh  ET-1  ET-3

B

100 mM glucose

Veh  Veh  BQ123  BQ788

Veh  BQ788

ET-1  ET-3
Figure 7

**A**

![Graph showing the expression of HO-1/β-Actin (in % of NG) under different conditions.](image)

**B**

![Western blot images of HO-1 and β-Actin.](image)
Figure 9

A

Catalase activity (mMoles/µg protein/min)

Veh  ET-1  ET-3  Veh  ET-1  ET-3

NG  100 mM glucose

B

100 mM glucose

Veh  Veh  BQ123  BQ788  Veh  BQ788

ET-1  ET-3

#  #  *  #  #  $
Figure 10

![Graph showing p-nNOS/t-nNOS (% of NG) under different conditions.](image)

- **NG**
- 100 mM glucose

**Y-axis:**
- p-nNOS/t-nNOS (% of NG)
- 0 to 350

**X-axis:**
- Veh/veh
- Veh/veh
- Veh
- BQ123
- BQ788
- Veh
- BQ788

**Annotations:**
- ET-1
- ET-3
- *p < 0.05 compared to NG
- #p < 0.05 compared to Veh
- $p < 0.05 compared to Veh/veh

**Images below graph:**
- p-nNOS
- t-nNOS
- 160 kDa