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Unfolded protein response and associated alterations in toll-like receptor expression and interaction in the hippocampus of restraint rats

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ABSTRACT

Recent evidence suggests that the cellular response to stress often elicits the unfolded protein response (UPR), which has an active role in major depression in emotionally relevant regions of the brain, such as the hippocampus. Much of the UPR activity has been found to be coalesced with the pro-inflammatory environment of the depressed brain. Specifically, downstream transcriptions of pro-inflammatory cytokines and increased regulation of candidate inflammatory mediators, such as toll-like receptors (TLRs), are promoted by the UPR. The present study examined the hippocampus associated expression profile of *Tlr* genes and their interaction with the UPR chaperone GRP94 in stress-induced rodent model of depression (restraint stress model). Also, the expression status of UPR related genes was evaluated in hippocampus using the same model. mRNA and protein levels of *Tlr* and UPR associated genes were examined by qRT-PCR and Western blot, respectively. Co-immunoprecipitation (Co-IP) method was used to determine the direct interaction between TLRs with GRP94 in depressed rat brain. The results showed that both UPR (*Xbp-1*, its spliced variant *sXbp-1*, *Atf-6*, *Chop*, and *Grp94*) and *Tlr* (2, 3, 4, 7 and 9) genes were significantly upregulated in the hippocampi of rats who were exposed to restraint stress. Similar upregulation was observed in the protein levels of the above-mentioned TLRs and the UPR chaperone protein GRP94 as well as total and phosphorylated forms of sensor proteins IRE1α and PERK. Further, a significantly increased interaction was observed between GRP94 and the activated TLR proteins. Since, increased inflammatory activity in vulnerable areas like hippocampus is coherently associated with depressed brain; our present data suggest that the UPR may be an integral part of increased activity of inflammatory regulations in depression.

1. Introduction

MDD is a psychiatric disorder that affects as much as 10% of the population with a lifetime incidence of 12% in men and 20% in women. Hallmarks of the disease include feelings of sadness, guilt, lethargy, changes in sleeping and eating patterns as well as subsequent weight gain or loss (Belmaker and Agam, 2008). Contributing factors of the depressive phenotype are thought to be environmental, genetic, and epigenetic in nature (Lopizzo et al., 2015); however underlying pathophysiological mechanisms associated with depression are still not clearly understood.

The unfolded protein response (UPR) can generally be described as the cellular response to environmental stressors (Khan and Schröder, 2008). Such stressors cause abnormalities in the quality of translated proteins in the endoplasmic reticulum. Consequently, the changes associated with misfolded proteins trigger the activation of three branches of the UPR first by dissociation of the chaperone protein GRP78 and then by subsequent release of activating transcription factor 6 (ATF6), oligomerization of inositol-requiring protein-1α (IRE1α), and/or the dimerization of protein kinase RNA-like endoplasmic reticulum kinase (PERK). The extent and duration of the stress determines which of these branches will become active. For example, acute stress may have ATF6 released with oligomerization of the IRE1α sensors, while a prolonged and extremely stressful environment would promote the activity of PERK and the C/EBP-homologous protein (CHOP)-mediated signaling pathway. These events initiate downstream signaling cascades which ultimately act on transcription, translation, and activation or repression of genes that can lead to inflammation, and even apoptosis (Kaufman, 2002; Ron, 2002).

We and other investigators have shown that the activity of UPR system may be responsible for some of the underlying pathophysiology of stress related disorders such as depression and have suggested that this response may intimately be involved in downstream pathways such as apoptosis, inflammation and dysfunctional cellular communication

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(Abrous et al., 2005; Calvano et al., 2005; Timberlake and Dwivedi, 2016). In the learned helpless model, which is a well-established rodent model of stress-induced behavioral depression (Henn and Vollmayr, 2005; Maier, 1984), we found that gene expression from each of the three branches of the UPR were upregulated in the hippocampus (Timberlake and Dwivedi, 2016); the three branches of activity being the events downstream of the activation of the sensors ATF6, IRE1α, and PERK (Wang and Kaufman, 2014). These data are consistent with the previous preclinical studies in hippocampus of restraint rats (Zhang et al., 2014) and clinical studies in temporal cortex of MDD brain (Bown et al., 2000), which showed expression related changes of UPR chaperones GRP78 and GRP94 as well as hippocampal shrinkage (Bremner et al., 2000) and hippocampal apoptosis (Zhang et al., 2014).

As mentioned earlier, changes in inflammation are intimately linked to depression (Miller and Raison, 2016; Raison et al., 2006). When in a state of cellular stress, one of the first responses of the UPR is to increase the recruitment of chaperones to the membrane that are responsible for folding, or aiding in the folding of proteins. Previously, we showed that *Grp78* and *Grp94* were significantly upregulated at the transcriptional level in hippocampus of learned helpless rats (Timberlake and Dwivedi, 2016). GRP94, specifically, is important in folding toll-like-receptors (TLRs) which are closely involved in mediating the inflammatory response (Garg et al., 2012; Raison et al., 2006; Staron et al., 2010; Takeda and Akira, 2005; Yang et al., 2007; Zacharowski et al., 2006). In the context of depression, TLRs 2–5, 7 and 9 have been shown to be upregulated in peripheral blood or brain of depressed individuals (Hung et al., 2014; Pandey et al., 2014). Interestingly, these TLRs are folded by GRP94 except TLRs 3 and 5 (Yang et al., 2007). Downstream of these phenomena can lead to production of pro-inflammatory cytokines which have been shown to be elevated in depression (Takeda and Akira, 2005). How altered UPR system is linked to inflammatory changes in depressed brain, however, is not clearly understood.

The purpose of the present study, therefore, is several folds: 1) to confirm our previous finding of activated UPR in depression using a different animal model of depression (chronic restraint); 2) whether TLRs are altered in depressed rat brains; and 3) whether altered expression of TLRs are associated with a specific chaperone molecule of the activated UPR system. In this study we have used the chronic restraint model as it is well established model of stress and has shown to impact both behavior (like the forced swim test and EPM) and physiology (changes in body weight, food intake, and water intake as well as sucrose preference) of rats that undergo the stress paradigms (Chiba et al., 2012; Klenerová et al., 2007; Torres et al., 2002; Wang et al., 2016). More specifically, we examined the expressions of *Grp94*, *Xbp-1* (including transcript variant s*Xbp-1*), *Atf6*, *Atf4*, and *Chop* of the UPR pathway and *Tlrs* 2, 3, 4, 7 and 9 in the hippocampus of restraint rats, all of which have implications in depression (Bown et al., 2000; Green et al., 2008; Hung et al., 2014; Timberlake and Dwivedi, 2016; Zhang et al., 2014). We also examined the expression of GRP94 as well as TLRs 2, 4, 7 and 9 at translational levels. As part of activated UPR system we tested the expression levels of total (T) and phosphorylated (P) forms of both IRE-1 and PERK proteins. In addition, we studied the *in-vivo* interaction of the TLRs with GRP94 to show the relationship between the UPR and inflammatory system in the context of depression.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (250–300 g body weight) were obtained from Envigo (Indianapolis, IN, USA) and housed in similar cages (2 rats/cage) within the same room under standard laboratory conditions (temperature 21 ± 1 °C, humidity 5 ± 5%). Animals were given free access to food and water and adapted to the laboratory environment for 1 week prior to the experiment. Rats were randomly assigned to a naïve control group and chronic restraint stress group. Restraint stress was given to rats during the light cycle (08:00–12:00). All the experiments were carried out according to the National Institutes of Health (NIH) guide for the care and use of Laboratory animals and were approved by the Animal Care Committee (IACUC) of the University of Alabama at Birmingham.

2.2. Chronic restraint stress

Rats were placed individually in clear acrylic tubes (21.59 cm long, 6.35 cm internal diameter, air vents in the cap and along the tube) with the tail extending from the rear of the tube. The cap was placed inward enough to prevent the rat from moving forward or backward inside the tube. Rats were restrained for 2 h/day for 14 consecutive days. Control rats were handled daily but not restrained. All the studies were done in 7–9 controls and 7–9 restraint rats.

2.3. Tissue collection

Twenty-four hours after the final restraint session, rats were decapitated and brains removed. The brains were immediately flash frozen in liquid nitrogen and stored at −80 °C. Brains were sectioned using a cryostat (Leica CM 1950) set at −20 °C. The samples were suspended in a 1:1 mix of optimal cutting temperature compound OCT (Tissue-Tek; Sakura Finetek USA) and 30% sucrose solution (50 mL NaPO4 [1000 nm], 150 mL 3% saline, 300 mL 50% sucrose) and sliced into 300 μm slices. Whole-hippocampal tissue was dissected from brain slices. Hippocampal and remaining brain tissue samples were returned to −80 °C storage until analysis.

2.4. RNA isolation

RNA was isolated using TRIzol® (Life Technologies, USA) as described earlier (Timberlake and Dwivedi, 2016). Initially the RNA samples were screened based on their purity (260/280 nucleic acid quantification; cut-off ≥1.8) as determined with NanoDrop spectrophotometer (ThermoScientific, Waltham, MA, USA). Afterwards, the RNA quality was further assessed using denaturing agarose gel electrophoresis and evaluating the 28S and 18S rRNA band integrity. Finally, only those samples were selected for analyses which showed 260/280 ≥1.8 and 28S:18S rRNA = 2:1.

2.5. QPCR based gene expression assay

M-MLV based reverse transcription of hippocampal RNA was performed following oligo (dT) priming method. One microgram (1 ug) total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, Grandsland, NY, USA) and oligo $(dT)_{18}$ primer (Invitrogen, Grandsland, NY, USA). The oligo $(dT)_{18}$ primer annealing step was carried out at 5 μM concentration in presence of 1 mM dNTPs (Invitrogen, Grandsland, NY, USA) by incubating the reaction at 65 °C for 5 min. The reaction was quenched by holding at 4 °C for 2 min. The reaction was mixed with 1X first strand synthesis buffer (Invitrogen, Grandsland, NY, USA), 0.01 mM DTT (Invitrogen, Grandsland, NY, USA), 2 U of RNaseOut (Invitrogen, Grandsland, NY, USA) and 200 U of M-MLV Reverse Transcriptase and incubated at 37 °C for 50 min. Finally, the reaction was inactivated at 70 °C.

Relative abundance of transcripts was measured with a quantitative real time PCR machine (AriaMx Real-Time PCR System; Agilent Technologies, USA) using 1X EvaGreen qPCR mastermix (Applied Biological Material Inc., Canada) in combination with 0.8 μM each of gene specific forward and reverse primers (Table 1). Forty-fold diluted raw cDNA was used as template for qPCR amplification using a thermal parameter of initial denaturation at 95 °C for 10 min followed by a repeating 40 cycles of denaturation at 95 °C for 10 s, primer annealing at 60 °C for 15 s and an extension of amplicon at 72 °C for 20 s. Possibility