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Research report

Inhibition of the mitochondrial calcium uniporter inhibits Aβ-induced apoptosis by reducing reactive oxygen species-mediated endoplasmic reticulum stress in cultured microglia

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ABSTRACT

Amyloid-beta (Aβ) has been shown to induce microglial apoptosis, which is itself sensitive to disturbed mitochondrial calcium (Ca^{2+}) homeostasis. The mitochondrial calcium uniporter (MCU) plays an important regulatory role in mitochondrial Ca^{2+} homeostasis, but its role in Aβ-induced microglia apoptosis is unknown. In this study, we found increased mitochondrial Ca^{2+} concentration in Aβ-treated primary microglia and BV-2 cells; also, the MCU inhibitor Ru360 significantly attenuated Aβ-induced microglial apoptosis, whereas the MCU activator spermine augmented it. In addition, Ru360 significantly attenuated Aβ-induced mitochondrial reactive oxygen species (ROS) production, as well as endoplasmic reticulum (ER) stress characterized by glucose-regulated protein 78 (GRP78) and C/-EBP homologous protein (CHOP) expression. Spermine, however, exerted the opposite effects on mitochondrial ROS production and ER stress. We also found that mitochondria-targeted antioxidant (Mito-TEMPO) treatment decreased GRP78 and CHOP expression in Aβ-treated microglia. Moreover, blocking endogenous CHOP expression using a CHOP small interfering RNA (siRNA) attenuated Aβ-induced cell death. Altogether, our data suggested that 1) inhibition of MCU exerts a neuroprotective effect on Aβ-induced microglia apoptosis, and 2) that the underlying mechanism may be related to reducing mitochondrial ROS-mediated ER stress.

1. Introduction

Neuroinflammation is a hallmark of Alzheimer's disease (AD), the progressive neurodegenerative disorder affecting elderly people worldwide that is characterized by amyloid-beta (Aβ) deposition in the brain. AD-related neuroinflammation is largely mediated by microglia, the brain's main immune cells (Wes et al., 2016). Normally active microglia clear up aggregated proteins such as Aβ, but sustained activation ("overactivation") causes release of cytotoxins that lead to neurotoxicity. Alternatively, persistent activation of microglia ultimately culminates in apoptosis, contributing to a subsequent uncontrolled inflammatory response (Hao et al., 2013). However, the underlying mechanisms regulating Aβ-induced microglial apoptosis remain obscure.

Mounting evidence suggests that functions of activated microglia like phagocytosis and the release of proinflammatory factors such as tumor necrosis factor-α (TNF-α) and nitric oxide (NO) are Ca^{2+} dependent(Farber and Kettenmann, 2006; Ikeda et al., 2013). The endoplasmic reticulum (ER) is a major intracellular calcium storage pool, and is sensitive to changes in intracellular homeostasis. ER-mitochondria Ca^{2+} transfer is thought to be involved in Aβ-induced apoptotic neuronal cell death (Ferreira et al., 2015). The mitochondrial calcium uniporter (MCU) is a selective Ca^{2+} ion channel localized in the inner mitochondrial membrane that is required for Ca^{2+} buffering (Baughman et al., 2011). Excessive uptake of Ca^{2+} into mitochondria through MCU is detrimental to mitochondrial function and leads to mitochondrial reactive oxygen species (ROS) production in AD (Toglia et al., 2016). Oxidative stress can disrupt ER function, and induces ER stress, in which the ER activates an unfolded protein response (UPR) to promote

Abbreviations: Aβ, amyloid-beta; Ca^{2+}, calcium; MCU, mitochondrial calcium uniporter; ROS, reactive oxygen species; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; CHOP, C/-EBP homologous protein; AD, Alzheimer's disease; TNF-α, tumor necrosis factor-α; NO, nitric oxide; UPR, unfolded protein response; siRNA, small interfering RNA; RCA-1, recinus communis agglutinin-1; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphe-nyltetra zolium bromide.

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correct protein folding and degrade abnormally folded proteins. However, if the stress is prolonged, it may lead to apoptosis. Recent studies suggest that Aβ leads to ER stress, and that mitochondrial dysfunction can enhance the Aβ-induced ER stress response (Costa et al., 2013). However, the role of MCU in Aβ-induced ER stress in microglia has never been examined.

In the current study, we investigated the role of MCU in Aβ-induced apoptosis in primary microglia and BV-2 cell cultures; we analyzed its possible mechanism using the MCU activator spermine (Zhang et al., 2006) and inhibitor Ru360 (Zazueta et al., 1999). In addition, we used Mito-TEMPO treatment to determine whether mitochondrial ROS production is linked to ER stress, and C/-EBP homologous protein (CHOP) small interfering RNA (siRNA) inhibition to confirm the role of ER stress in Aβ-induced microglial apoptosis.

2. Results

2.1. Effect of Ru360 and spermine on Aβ-induced microglia apoptosis

To determine the effect of Ru360 and spermine on Aβ-treated microglia, BV-2 and primary microglia were pre-incubated with Ru360 and spermine, respectively, at different concentrations for 1 h, prior to 24 h of exposure to Aβ (20 μM). Cell viability was detected using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Fig. 1A, Aβ significantly reduced cell viability, and incubation with Ru360 (1–10 μM) concentration-dependently reversed Aβ-induced cell death in primary microglia. However, incubation with spermine (2–20 μM) concentration-dependently enhanced Aβ-induced cell death in primary microglia. In addition, Ru360 and spermine themselves had no detectable effects on cell viability (Fig. 1C, D). Similar results were observed in BV-2 cells treated with Aβ (data not shown).

To determine whether apoptosis played a role in Aβ-induced cell death, we pretreated primary microglia and BV-2 cells for 1 h with 5 μM Ru360 or 10 μM spermine, and then treated them with 20 μM Aβ for 24 h. Apoptotic cells were analyzed either via a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Fig. 2A) and flow cytometric analysis (Fig. 2B). We showed that Ru360 significantly decreased microglia apoptosis, while spermine increased microglial apoptosis compared to cells treated with Aβ alone.

2.2. Effect of Ru360 and spermine on mitochondrial Ca^{2+} concentration change

As shown in Fig. 3, mitochondrial Ca^{2+} concentration increased with Aβ treatment compared with the control group in primary microglia, and pretreatment with Ru360 significantly decreased mitochondrial Ca^{2+} concentration compared with Aβ treatment alone. However, pretreatment with spermine augmented the Aβ-induced mitochondrial Ca^{2+} concentration increase. Similar results were observed in BV-2 cells treated with Aβ (data not shown).

2.3. Effect of Ru360 and spermine on mitochondrial ROS production

Our results showed that the level of mitochondrial ROS formation increased with Aβ treatment relative to that of the control group in primary microglia, and pretreatment with Ru360 significantly decreased the levels of mitochondrial ROS compared with Aβ treatment alone (Fig. 4). However, pretreatment with spermine increased Aβ-induced mitochondrial ROS production versus Aβ treatment alone (Fig. 4). Similar results were observed in BV-2 cells treated with Aβ (data not shown).

2.4. Effect of Ru360 and spermine on glucose-regulated protein (GRP78) and CHOP expression

Our results showed that the levels of GRP78 and CHOP increased after Aβ treatment compared with the control group in primary microglia, and pretreatment with Ru360 significantly decreased the expression of GRP78 and CHOP. However, pretreatment with spermine increased the expression of GRP78 and CHOP compared to Aβ treatment alone (Fig. 5). Similar results were observed in BV-2 cells treated with Aβ (data not shown).

2.5. Mitochondrial ROS production modulated activation of ER stress in Aβ-treated microglia

Mito-TEMPO has been recently reported as a mitochondria-targeted antioxidant with low toxicity, making it a potential candidate for mitochondrial ROS experiments (Trnka et al., 2009). Our results showed that the elevated level of Aβ-induced mitochondrial ROS was effectively reduced by treating primary microglia with Mito-TEMPO at a concentration of 200 μM (Fig. 6A). In addition, Mito-TEMPO treatment led to a decrease in the expression of GRP78 and CHOP (Fig. 6B). Similar results were observed in BV-2 cells treated with Aβ (data not shown). Altogether, these results indicated that mitochondrial ROS production modulated the activation of ER stress in Aβ-treated microglia.

2.6. ER stress played an important role in Aβ-induced toxicity in microglia

Having determined that ER stress was upregulated following Aβ exposure, we attempted to link the ER stress with Aβ-induced toxicity in microglia. Since CHOP is critical in ER stress-induced apoptosis, we next sought to determine its role in Aβ-induced cell death using siRNA to block endogenous CHOP expression in BV-2 cells. We found that there was a significant reduction in CHOP protein in BV-2 cells transfected with CHOP siRNA compared to the cells treated with scrambled (non-specific) siRNA (Fig.7A). Cell viability was examined in BV-2 cells transfected with CHOP or scrambled siRNA, and exposed to Aβ. As shown in Fig. 7B, the viability of cells transfected with CHOP siRNA was significantly higher, suggesting that ER stress was a key mechanism in regulating Aβ-induced microglial cell death.

3. Discussion

Previous studies have shown that ER-mitochondria crosstalk is involved in Aβ-induced neuronal apoptosis, and that mitochondrial dysfunction enhances the ER stress response induced by Aβ (Costa et al., 2012). ER stress can activate the UPR to restore homeostasis. The UPR is initiated by activation of three ER-resident transmembrane proteins: PERK, IRE1 and ATF6. Under physiological conditions, the luminal domains of the three proteins are occupied by the ER chaperone GRP78, which inactivates them. When the UPR is initiated, GRP78 dissociates from the three transmembrane proteins, leading to their activation. Our results demonstrated that the expression of GRP78 was increased in Aβ-treated microglia. Ru360 significantly decreased the mitochondrial Ca^{2+} concentration and prevented increased expression of GRP78, while spermine increased the mitochondrial Ca^{2+} concentration and augmented the expression of GRP78. In addition, Ru360 and spermine themselves had no effects on the levels of mitochondrial Ca^{2+} concentration under normal conditions. We suspected that Ru360 and spermine exerted effects based on MCU activation stimulated by Aβ treatment (de Jesus Garcia-Rivas et al., 2005; Yu et al., 2016). These results suggest that ER stress occurred upon microglial expo-