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The ER chaperone, BIP protects Microglia from ER stress-mediated Apoptosis in Hyperglycemia

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Abstract

A major endoplasmic reticulum (ER) chaperone, binding of Immunoglobulin heavy chain protein (BIP) facilitates the assembly of newly synthesized proteins in the ER. Microglia vigorously respond to brain injuries and eliminate the damaged neuronal and apoptotic cells through phagocytosis in the central nervous system. However, the mechanism of BIP-mediated microglial function is not clear in hyperglycemia. We explored the molecular mechanism of BIP in microglial function during hyperglycemic conditions. Hyperglycemia was induced in mice by two consecutive intraperitoneal injections of streptozotocin (STZ 100/kg) and confirmed by measuring the blood glucose from day 2 to day 14. After 14 days of experimental hyperglycemia, mice were sacrificed and brains were collected for ER chaperone expression. In-vitro hyperglycemia was induced by exposing HMC3 cells to 25mM glucose for 5 days and proteins involved in ER stress, apoptosis, and autophagy were analyzed. In hyperglycemic conditions, BIP protein expression was dramatically reduced in HMC3 cells, which led to increased apoptosis through the

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Declaration of competing interest

The authors declare no competing interests.

Ethics Approval

All animal protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC) of the University of Toledo, Ohio, USA, under the National Institute of Health guidelines. The authors declare that they have no conflict of interest.

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activation of CHOP and mitochondrial pro-apoptotic proteins (Bax, Bad, and cleaved caspase-3). The flow cytometry results indicate hyperglycemia-induced apoptosis and reactive oxygen species (ROS) production. Interestingly, the BIP inducer X restored the apoptosis in HMC3 cells by derepressing BIP expression and inhibiting ER stress. These results suggest that the ER chaperone BIP is required for the microglial function and protects from apoptosis in hyperglycemia. A better understanding of BIP's molecular mechanism and role in microglial function may contribute to developing novel therapies for microglia dysfunction-associated neurodegenerative diseases.

Keywords

ER stress; Hyperglycemia; Reactive oxygen species; Apoptosis; ER chaperone; BiP inducer-X

1. Introduction

The binding of Immunoglobulin heavy chain Protein (BIP) is one of the major endoplasmic reticulum (ER) chaperones and is a highly conserved member of the heat shock protein family (Lee et al., 1992; Lee et al., 2001). BIP plays a crucial role in protein refolding when unfolded or misfolded proteins are accumulated in the ER (Kuznetsov et al., 1997; Klausner et al., 1990). BIP protein expression is regulated through the binding of X-box protein (XBP1) on the ER stress-responsive element (ERSE) located in the promoter of BIP (Raghubir et al., 2011). It also serves as an ER sensor, and its expression is profoundly increased in ER stress. Protein kinase R-like ER kinase (PERK) phosphorylates the eukaryotic translation initiation factor-2 alpha (eIF2α) and activates the active transcription factor-4 (ATF4) and CHOP, which further activates the pro-apoptotic signal leading to cell death or apoptosis (Marciniak et al., 2006; Hardinget al., 2003; Marciniak et al., 2004). ER is the unique and dynamic compartment involved in the protein, lipid synthesis, and post-translation modifications of secretary proteins through glycosylation. Protein glycosylation defect causes the accumulation of unfolded or misfolded proteins in the ER leading to ER stress (James et al 2016; William James et al., 2019). The accumulation of unfolded protein in the ER activates the unfolded protein response (UPR) as an adaptive response to protect the cells from ER stress-mediated apoptosis (Helenius et al., 1994). The previous study showed that BIX induces the BiP protein expression and protects neurons from apoptosis induced by ER stress (Y. Oida et al., 2008). However, whether the BIX protects the microglia from apoptosis induced by ER stress in hyperglycemia is unknown.

Glucose fuels the brain's physiological functions and cells, including neurons, astrocytes, and microglia. The brain derives energy from glucose, glutamate, lactate, and ketone bodies because these metabolites have specialized carriers that can cross the blood-brain barrier (BBB). Glucose is essential for ATP synthesis, neurotransmitters precursor synthesis and maintaining ionic concentration gradients across the membrane, neuronal plasticity, learning, and memory. Dysregulation in glucose homeostasis leads to hyperglycemia or hypoglycemia, which is associated with impaired brain functions (Seaquist et al., 2015). Recent studies show that dysregulation of brain glucose metabolism and hyperglycemia is associated with neurodegenerative diseases. Hyperglycemia and insulin resistance are risk factors that can increase the incidence of stroke (Pugazhenthi et al., 2017; Sridhar et

al., 2015). Chronic hyperglycemia leads to diabetic neuropathy, retinopathy, and dementia, which are also associated with glucotoxicity conditions (Lee et al., 2018; Chatterjee et al., 2018). Microglia dysfunction or self-degradation is associated with enhanced neurodestructive effects, a major concern in diabetic brains. Microglia play a crucial role in brain development and contribute to neural cell genesis and synaptic development (Kettenmann et al., 2011; Fan et al., 2017). Microglia form the innate defensive system of the central nervous system and eliminate the damaged cells and dysfunctional synapses through interaction with neurons during pathological conditions (Fan et al., 2017). Microglia have bifunctional characteristic features and their function might vary based on the activation state because they rapidly respond to chronic disease and acute insults affecting the CNS. Activated microglia release neurotrophic factors, which promote neuronal survival and differentiation (Nakajima et al., 2007). Recent studies reported that microglia benefit neurogenesis, progenitor proliferation, survival, migration, and differentiation in the early stages of Alzheimer's disease (AD). The initial microglial activation is protective in clearing amyloids in AD (Hamelin et al., 2016).

In the present study, We selected the chemical compound BIX (BiP inducer-X) to investigate whether BIP protects microglia from apoptosis induced by ER stress in hyperglycemic conditions. We explored the mechanism of the BIP repression-mediated ER stress-induced microglia dysfunction in *in-vitro* hyperglycemia and STZ-induced diabetes mouse model. The potential effect of BIX on ER stress, ER chaperone, apoptosis, and autophagy was comprehensively evaluated by immunocytochemistry and flow cytometry analysis.

2. Materials and Methods

2.1. Cell Culture

The Human microglia clone 3 cell line (HMC3) was cultured in eagle's minimum essential medium (EMEM) (ATCC, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air/5% CO2. HMC3 cells were seeded at a density of 2×10^5 cells in normal glucose (1 g/L, 5.5mM) and high glucose medium (4.5 g/L, 25mM), cultured for 5 days, and cells were harvested for protein expression, RNA isolation, and subcellular organelles isolation. For BIX treatment, cells were cultured in control and hyperglycemia conditions for 48hr. After 48 hr, fresh medium was replaced with 25μM of BIX (MedChem Express LLC, NJ, USA) for 72 hr, and immunocytochemistry and Western blotting were performed as described in the method section.

2.2. Animals

We used 10 weeks old, 23 – 25gms weight male C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in a pathogen-free barrier facility under a 12-hours dark/light cycle at 21°C temperature and 40–60% humidity in the Department of Laboratory Animal Resources (DLAR), University of Toledo, Health Science Campus (HSC). All animal protocols were approved by the Institutional Animal Care and

Utilization Committee (IACUC) of the University of Toledo under the National Institute of Health guidelines.

2.3. Streptozotocin-induced type-I diabetes model

Age and weight-matched mice were subdivided into two groups: normoglycemic (n=5) and hyperglycemic (n=5). Streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 10 mM citrate buffer (pH=4.5). The hyperglycemic group was injected with STZ (100 mg/Kg) intraperitoneally over two consecutive days, whereas the normoglycemic group was injected with an equal volume of citrate buffer as a vehicle (Ghaith A Bahader et al., 2021). Hyperglycemia was confirmed by measuring blood glucose levels (>300mg/dl) from the tail vein using a Contour Next EZ glucometer (Ascenia Diabetic Care, NJ, USA). After 14 days of experimental conditions, mice were sacrificed and the striatum region was isolated from the brain using cryostats (Minux FS800A, RWD Life Sciences, Delaware, USA).

2.4. Real-time PCR (RT-qPCR)

Cells were cultured in EMEM with normal glucose 1 g/L, 5.5 mM) and high glucose (4.5 g/L,25 mM) for 5 days. The experiment was carried out at different times as biological triplicates and samples were stored at −80°C. Total RNA was isolated using the TRIzol reagent (Life Technologies, California, USA) and the RNA concentration and quality were measured using NanoDrop™ (Thermo Fisher Scientific, MA 02451, USA). Complementary DNA (cDNA) was synthesized using the high-capacity cDNA reverse transcription kit (Thermo Scientific, USA) with 1x RT buffer, 1x random primers, 4 mM dNTP mix, 50 U/ml reverse transcriptase, and 1 μg of total RNA. The primers were designed using Primer Express® 3.0 (Applied Biosystems), and the primer sequences are listed in Figure S5. Relative mRNA expressions were determined by quantitative real-time RT-PCR using SYBR Green real-time PCR master mix (Applied Biosystems) by following the manufacturer's instructions. PCR was initiated with denaturation at 95 °C for 10 min, which was followed by 40 cycles of 95 °C for 15 s, 58 °C for 60 s, and 95 °C for 15 s, and a final extension was performed at 72 °C for 5 min. Amplification was measured using an ABI 7500 instrument (Applied Biosystems). The mRNA expression levels were analyzed in triplicate, and the results were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The β-actin was used as an endogenous control for normalization. The q-RTPCR data are presented as the mean values, and the data were analyzed using Student's t-test.

2.5. Western blot analysis

Cells were grown in EMEM medium with normal glucose (1 g/L, 5.5 mM) and high glucose (4.5 g/L, 25 mM) for 5 days, washed with 1x PBS. The experiment was carried out at different times as biological triplicates and samples were stored at −80°C. Cell lysates were prepared in ice-cold lysis buffer (20 mM/L Tris (pH 8.0), 137 mM/L NaCl, 1% NP40,10% glycerol) supplemented with protease inhibitors (1mM phenylmethyl sulfonyl fluoride (PMSF), 10μg/ml aprotinin, 10μg/ml leupeptin), and 3mM Na_3VO_4. The lysate was cleared by centrifugation and the protein concentration was determined using the Bradford assay (Bio-Rad; Hercules, CA, USA). The total protein sample (25μg) was resolved using 10 to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 30 min with blocking

buffer (5%BSA, PBS, 0.05% Tween-20) membranes were incubated with the primary antibodies, anti-BIP (1:1000), anti-eIF2α (1:1000), anti-CHOP (1:1000), anti-Bax (1:1000), anti-Bad (1:1000), anti-caspase-3 (1:1000), anti-cleaved caspase-3 (1:1000), anti-caspase-1 (1:1000), anti-cytochrome-C (1:1000), anti-Bcl2 (1:1000), calreticulin (1:1000), anti-ATG12 (1:1000), anti-LC-3(1:1000), anti-Beclin 1(1:1000) and β-actin (1:5000) at 4° C overnight. Anti-rabbit and anti-mouse HRP IgG (1:5000) antibodies were used as secondary antibodies and β-actin (1:3000) (Cell Signaling Technology, MA, USA) was used as a loading control.

2.6. Flow cytometry analysis

Cells were cultured, trypsinized (0.05%), washed with serum-containing medium, and resuspended in 0.5ml of ice-cold 1X binding buffer. A 5μl of Annexine V5-FITC (fluorescein isothiocyanate-annexin-V (Thermo Fisher Scientific) was added and incubated at room temperature (18 to 24°C) for 15mins in the dark. Cells were centrifuged at 1000xg for 5mins at room temperature and the supernatant was removed and the cells were resuspended in 1X binding buffer and analyzed via Annexin binding by flow cytometry (Ex=488; Em=518 nm) using FITC signal detector (FL1) according to manufacturer instructions.

2.7. H_2DCFDA- ROS staining

Cells were trypsinized (0.05%) and washed with serum-containing medium and resuspended in 1.0 ml of 1X buffer. Cells were stained with 20μM DCFDA in final concentration (2',7'-dichlorodihydrofluorescein diacetate, DCFDA Assay Kit, ab113851) for 30mins at 37°C in the dark, and the fluorescence intensity was measured by flow cytometry (Ex=485; Em=535nm) using a FITC signal detector (FL1) according to manufacturer instructions. The fluorescence intensity was determined by the changes as a percentage of control after background subtraction.

2.8. Immunocytochemistry

Coverslips were pre-coated with poly-D-lysine (50μg/ml) overnight and dried in a CO_2 incubator. Cells were cultured for 5 days on the coverslips placed in the 6-well plates containing normal glucose and high glucose medium, then washed in wash buffer (1XPBS+0.1%tween-20) and fixed with 4% paraformaldehyde for 10mins at room temperature. Then cells were washed three times for 5 mins in ice-cold PBS (pH=7.4) and incubated in PBS containing 0.25% TritonX-100 for 10 mins to increase the membrane permeability. Cells were incubated in PBST blocking buffer (1% BSA, 22.52 mg/ml glycine, 0.1% Tween-20) for 30 mins. Cells were incubated in the primary antibody in a blocking buffer (1% BSA in PBST) overnight at 4°C and washed three times in PBS for 5 min. Then cells were incubated with the secondary antibody in 1% BSA for 1 h at room temperature and washed three times with PBS for 5 min in the dark. Coverslips were removed from 6 well plates and wiped, another side of the coverslips was placed on the microscope slide and mounted with a drop of Fluoromount-G, with DAPI (Thermo Fisher Scientific, Lot; E140588). Coverslips were sealed with nail polish to prevent drying and movement under a microscope. Imaging was performed using a fluorescence microscope at 40x magnification and fluorescence intensity.

2.9. Statistical analysis

Statistical analyses were performed using Prism6 (GraphPad Software). Data were analyzed using unpaired Student's t-test with Welch's correction and one-way ANOVA followed by Tukey's post hoc tests for multiple comparisons. All values are presented as the mean ± standard error of the mean and significant differences $P < 0.05$, $P< 0.01$, and $P< 0.001$, are symbolized as *, **, and ***, respectively.

3. Results

3.1 ER chaperone BIP repression increases ER stress in HMC3 cells

To explore the chronic effect of hyperglycemia on ER chaperones in microglia, we used HMC3 cells. Cells were cultured in normal glucose (5.5mM) and hyperglycemia (25mM) for 5 days and analyzed for BIP expression on days 2, 3, 4, and 5. BIP protein expression was significantly downregulated on day 5 (Fig.1A), whereas the ER stress key marker proteins p-eIF2α and CHOP levels were upregulated in hyperglycemia than control (Fig.1B, C). There was no change observed in the expression of total eIF2α. Next, we analyzed the mRNA expression of genes involved in the ER stress and found BIP mRNA expression ~ 50% reduced, and CHOP and ATF4 upregulated to ~ 3 fold than the control (Fig.1D).

3.2. ER stress is increased in STZ-induced mouse model

To further validate the results of BIP downregulation, we used streptozotocin (100 mg/Kg) induced type-1 diabetes mouse model. Blood glucose levels (>300mg/dl) were increased on days 1, 7, and 14 in STZ-injected mice (S4). The ER chaperone, BIP expression was significantly downregulated, whereas p-eIF2α, CHOP, and cleaved caspase-3 expression levels were significantly upregulated in the STZ-injected mice brain striatum than the control group (Fig 2A, B). However, the ER chaperone calreticulin, total eIF2α, and caspase-3 protein expression levels were not changed in the STZ-injected mice. These results suggest that hyperglycemia induces ER stress and apoptosis.

3.3. Hyperglycemia-induced apoptosis in HMC3 cells

Since the BIP expression was reduced and ER stress increased in both *in-vitro* and *in-vivo* models, we further explored whether BIP repression could induce apoptosis in HMC3 cells during hyperglycemic conditions. Astonishingly, the pro-apoptotic markers Bax and Bad protein expression were significantly increased, and mitochondria-mediated apoptosis marker cytochrome-C and cleaved caspase-3 increased. However, no significant change was observed in the anti-apoptosis marker, BCL2, and total caspase-3 expression in hyperglycemia (Fig. 3A). Next, we tested the expression of proteins involved in autophagy as apoptosis was increased in hyperglycemia. In hyperglycemic conditions, LC3-BII expression was significantly reduced, and no significant change was detected in the expression of Atg12 and Beclin-1 (Fig. 3A). Furthermore, to validate our findings, we analyzed the apoptosis and ROS production by annexin-V5-FITC and DCFDA staining. Flow cytometry showed that hyperglycemia caused 44.30% of apoptotic cells than control (Fig. 3B, S2). Also, the DCFDA staining result showed dramatically increased ROS

production in hyperglycemia (Fig. 3C, S3). These results suggest that hyperglycemia induces apoptosis in HMC3 cells.

3.4. BIX mitigated the ER stress through the derepression of BIP expression in HMC3 cells

The ER stress and apoptosis were increased in HMC3 cells when cultured in hyperglycemic conditions. Therefore, we speculated that increased apoptosis might be mediated through the down-regulation of ER chaperone BIP. To comprehensively validate and prove the hypothesis, we used BiP inducer-X (BIX) to derepress the BIP expression. The HMC3 cells were cultured in 25mM glucose, treated with 25μM BIX on day 3, and cultured until day 5 followed by the immunocytochemistry and Western blot analysis. As expected, we found significantly reduced fluorescence of BIP protein in hyperglycemic conditions than the control and interestingly, the BIP protein expression was restored in BIX-treated cells (Fig. 4A).

The BIP protein expression was significantly increased in BIX-treated cells than in untreated hyperglycemia control, but its expression was not induced as equal to normoglycemia control. Subsequently, the CHOP, p-eIF2α, and total eIF2α expression were reduced in BIX-treated cells than in control (Fig. 4B). These results suggest that hyperglycemia increased the ER stress through repression of the ER chaperone BIP and alleviated the ER stress through derepression of BIP protein expression when cells were treated with BIX.

3.5. BIX protects HMC3 cells from apoptosis

The BIP protein expression was enhanced and ER stress was reduced in BIX-treated cells. Therefore, we investigated whether inhibiting ER stress and derepression of BIP protein expression will reduce apoptosis in microglia cells. We evaluated the expression of proteins involved in apoptosis in BIX-treated cells. Interestingly, the pro-apoptotic marker proteins, Bax, Bad, cytochrome-C, and caspase-1, cleaved caspase-3 expression levels were significantly reduced in BIX-treated cells, whereas caspase-3 expression was not changed (Fig. 5A). Furthermore, we investigated whether the autophagy process could play a role on the protection of microglia from apoptosis or ER stress. Nevertheless, the BIX-treated cells did not show increased LC3-BII in hyperglycemia than control (Fig.5B), suggesting that BIX does not alter the autophagy through the induction of BIP in hyperglycemia. These results indicate that BIX protects microglia from apoptosis by derepressing the BIP protein expression and inhibiting ER stress.

4. Discussion

In the present study, we reported that hyperglycemia reduced the BIP protein expression and subsequently increased ER stress in HMC3 cells partly due to a defect in protein folding and accumulation of unfolded or misfolded proteins in the ER. Similarly, BIP expression was downregulated, whereas p-eIF2α, CHOP, and cleaved caspase-3 expression levels were upregulated in the STZ-injected mice brain striatum. The *in-vitro* and *in-vivo* results suggest that hyperglycemia induces ER stress and apoptosis.

Numerous studies suggested that inhibition of ER stress is most productive for neurodegenerative disease. The activation of UPR is an ER adaptive mechanism to protect the cells, but prolonged UPR activation is also detrimental and causes cell death. The UPR adaptive mechanism comprises three principal signaling pathways, inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). In addition, the UPR adaptive mechanism is mediated by molecular chaperones and luminal enzymes, including the BIP/GRP78, GRP94, calreticulin, and lectin calnexin, disulfide isomerase (PDI) Erp57, thiol-disulfide oxidoreductase (Szegezdi et al., 2006). Previous studies have shown that BIP protein expression was increased in ER stress and caused more than 70% cell death during glucose deprivation (Selene et al., 2014). In contrast, we observed that BIP protein expression was reduced, and p-eif2α protein expression and ER stress were increased in hyperglycemia conditions, possibly due to the disassociation of BIP from PERK in hyperglycemic conditions. The disassociation of BIP from the PERK complex or reduced expression of BIP possibly increased the phosphorylation of eIF2α through PERK (Harding et al., 1999). The increased expression of ATF4 in hyperglycemia due to PERK-mediated increased phosphorylation of eIF2α. The increased expression of CHOP in hyperglycemic conditions is possibly due to derepression of ATF4, and activation of transcription of genes involved in apoptosis (Szegezdi et al., 2006).

We investigated the expression of ER chaperone, ER stress, and apoptosis marker protein in the striatum region of STZ-injected mice brains. Previous studies reported that microglia proliferation is increased in the striatum after ischemia, producing ischemic tolerance in the brain (Liu et al., 2001). The streptozotocin-induced diabetes mouse model also revealed that BIP repression induced the ER stress through the activation of CHOP and hyperphosphorylation of eIF2α. The increased expression of CHOP further activated the caspase-3 through interaction with Bax, leading to apoptosis in STZ-injected mice. Previous studies have also shown that the knockdown of BIP increased phosphorylation of eIF2α, and increased CHOP expression in both *in-vitro* and *in-vivo* models (Rangel et al., 2021). Inhibition or suppression of BIP induces the compensatory mechanism by upregulating ER chaperones. However, no compensatory ER chaperone expression was detected (calreticulin) when BIP protein expression was reduced in STZ-injected mice.

Caspases are critical mediators of apoptosis in mammalian cells. The BCL2 is an anti-apoptotic protein that plays an essential role in cell proliferation. In hyperglycemia, the Bcl2 protein expression was not significantly changed, whereas the apoptosis marker proteins, cleaved caspase-3, Bax, Bad, and cytochrome-C expression levels were increased. It is possibly due to the activation of ER resident protein caspase-12, which activates the cytoplasmic caspase-3 via TNFα during the ER stress (Junichi et al., 2004). Increased caspase-3 activation in hyperglycemia is due to the derepression of CHOP through interaction with mitochondrial protein Bax. Activated caspase-3 alters mitochondrial membrane depolarization leading to mitochondrial dysfunction (Edaena et al., 2020). The flow cytometry results also revealed that hyperglycemia causes 43% of apoptosis in HMC3 cells. The previous study also revealed the ER-resident protein caspase-12-dependent caspase-3 activation induces apoptosis in ER stress (Jin et al., 2015). The increased expression of CHOP in hyperglycemia suggests that ER and mitochondrial membrane

crosstalk pathway-mediated apoptosis is induced in HMC3, implying that inhibition of the ER stress response reduces apoptosis and protects HMC3 cells.

A recent study has also shown that apoptotic cell death occurs through ER and mitochondrial membrane crosstalk pathway-mediated apoptosis in ER stress (Sun et al., 2016; Sophonnithiprasert et al., 2017; Burton et al., 2017). Constitutive production of ROS is a primary inducer of apoptosis. Cells can produce endogenous antioxidants, such as glutathione and superoxide dismutase, to prevent ROS production and protect cells from stress and apoptosis (Hiebert et al., 2015). Nrf2 is the transcription factor which regulates the transcription of genes involved in antioxidant synthesis (Farina et al., 2012). Therefore, we evaluated whether the Nrf2-dependent redox homeostatic balance could protect the microglia in hyperglycemic conditions. In hyperglycemia, the Nrf2 expression was increased in the nuclear fraction, and no significant change was detected in total cell lysate (S1). However, the ROS levels were increased and apoptosis was not inhibited possibly due to prolonged chronic ER stress and the ratio of ER stress greater than the redox balance.

The BIP protein expression was upregulated and ER stress was reduced through the repression of p-eIF2α and CHOP in BIX-treated hyperglycemic cells. Subsequently, the proteins involved in the apoptosis, Bax, Bad, cleaved caspase-3, caspase-1, and cytochrome-C levels were significantly reduced in BIX-treated hyperglycemic cells. This further led to the inhibition of apoptosis in HMC3 cells during hyperglycemic conditions. The LC3-BII expression was not changed in BIX-treated cells compared to untreated hyperglycemia control, suggesting that BIX is protecting the microglia cells, but not through autophagy. A recent study has shown that BIX protects neuronal cells from apoptosis induced by ER stress (Kudo et al., 2008; Oidaa et al., 2008). But there was no evidence of whether microglia-mediated neuronal protection was observed in an *in-vivo* stroke mouse model. Understanding microglia function is complex; a decade of studies shows that activated microglia plays a crucial role in eliminating damaged neuron cells and secreting neurotrophic factors required for neurogenesis during the pathological condition. Microglial dysfunction results in behavioral deficits, indicating that microglia are essential for proper brain function. This defines a new role for microglia beyond being a mere pathologic sensor. For the first time, we described the BIP signaling mediated microglial cell protection from the ER stress in hyperglycemia, thus opening new therapeutic strategies for neurological disease.

The present study has some limitations that need to be addressed. First, we demonstrated the BIP expression was downregulated in the striatum of STZ-injected mice brains. To further verify these results additional systematic experiments are required to show BIP expression in primary microglia. Second, we reported that BIP repression-mediated ER stress is restored in HMC3 cells after BIX treatment, but this needs to be proved in the *in-vivo* mice model. Overexpression of the BiP/HSPA5 gene in HMC3 cells in hyperglycemic conditions is required for further confirmation. Thus, future studies are needed to acquire more thorough information on using inhibitors for microglia proliferation in mouse models to evaluate microglia function in diabetes conditions.

In conclusion, the results suggest that BIX, a selective inducer of BIP, inhibits the apoptosis induced by ER stress in hyperglycemia. BIX selectively induces BIP and may activate defense mechanisms against ER stress, resulting in a protective effect. BIX may therefore have potential as a new therapeutic intervention against neurological diseases and microglia dysfunctions mediated complications in diabetes conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Availability of Data and Materials

The datasets used in the current study are available with the corresponding author and will be shared with any investigator upon reasonable request.

Abbreviations

BIP	Binding of Immunoglobulin heavy chain protein
CNS	Central nervous system
ROS	Reactive Oxygen species
ER	Endoplasmic reticulum
STZ	Streptozotocin
BIX	BiP inducer-X
XBP1	Binding of X-box protein
ERSE	Endoplasmic reticulum stress responsive element
eIF2α	Eukaryotic translation initiation factor-2 alpha
ATF4	The active transcription factor-4
CHOP	C/EBP homologous protein
C-Caspase-3	Cleaved caspase-3
UPR	Unfolded protein response
BBB	Blood-brain barrier

AD	Alzheimer's disease
PERK	Protein kinase R-like ER kinase
Annexin V-FITC	Fluorescein isothiocyanate annexin V
DAPI	4, 6-diamidino-2-phenylindole
DCFDA	2'−7'-Dichlorodihydrofluorescein diacetate

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- The ER chaperone, BIP, is downregulated in hyperglycemia and streptozotocin-injected mice brains.
- Repression of BIP increases ROS production and causes apoptosis through ER stress
- BIP inducer-X reduces the p-eIF2α and CHOP and inhibits the ER stress in microglia
- BIP inducer-X protects microglia from apoptosis by de-repression of BIP in hyperglycemia

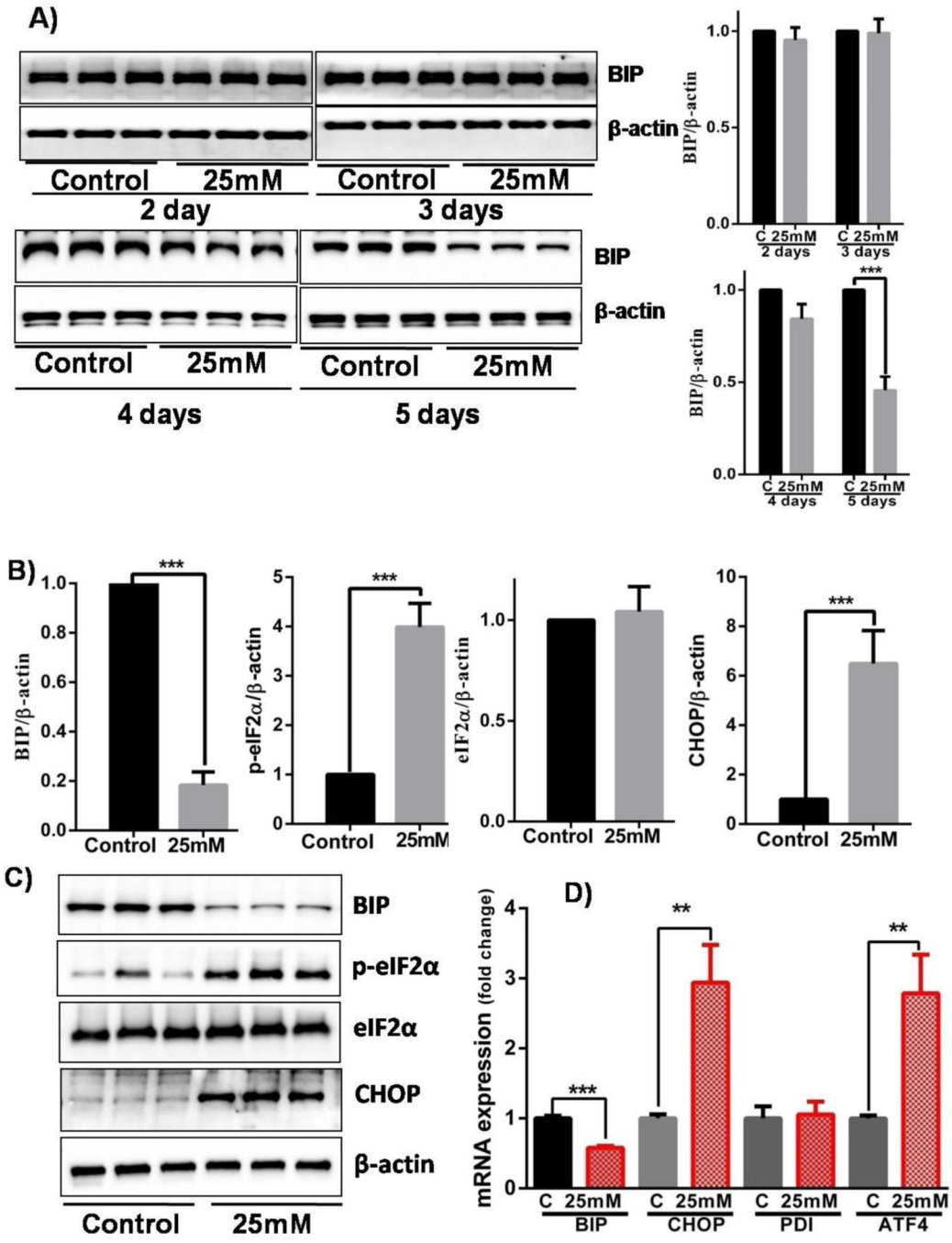

Figure.1.

BIP protein expression was downregulated and ER stress increased in hyperglycemic HMC3 cells, (A) Time-dependent expression of BIP in hyperglycemia, (B) relative quantification of ER stress pathway proteins, (C) The relative expression of BIP, eIF2α, p- eIF2α, CHOP, (D) the mRNA expression of genes involved in ER stress. The protein expression bands were quantified with Image-J software and normalized with loading control, β-actin. The data represent the means ± standard deviations (SD): n=3. Statistical significance was determined

by unpaired t-test and significant differences $P < 0.05$, $P < 0.01$, and $P < 0.001$ are symbolized as *, **, and ***, respectively.

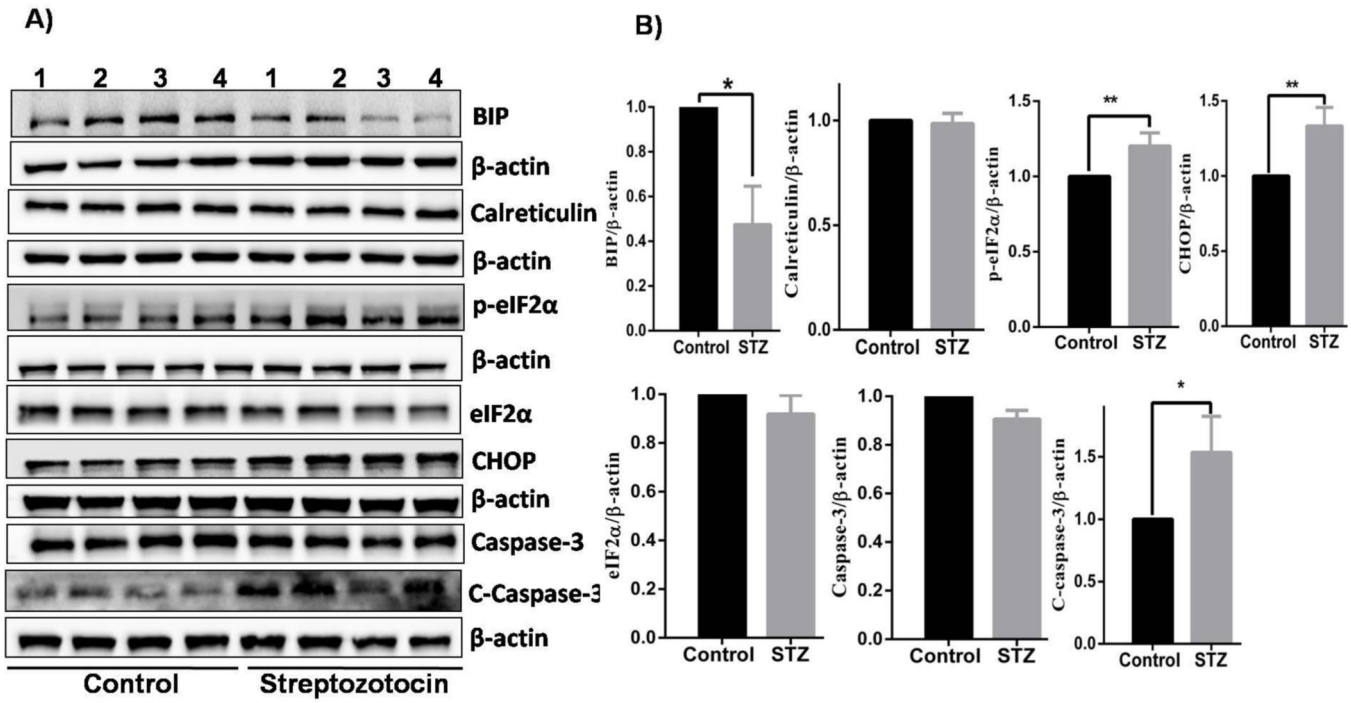

Figure. 2.

STZ-induced hyperglycemia inhibits BIP expression and induces ER stress in mice brain striatum, (A) The relative expression of BIP, calreticulin, eIF2α, p-eIF2α, CHOP, caspase-3, cleaved caspase-3, (B) relative quantification of ER stress and apoptosis pathway proteins. The protein expressions were quantified by Image-J software and normalized with β-actin. The data represent the means SD: n=5. Statistical significance was determined by unpaired t-test followed by comparisons and significant differences $P< 0.05$, $P< 0.01$, and $P< 0.001$ are symbolized as *and **, respectively.

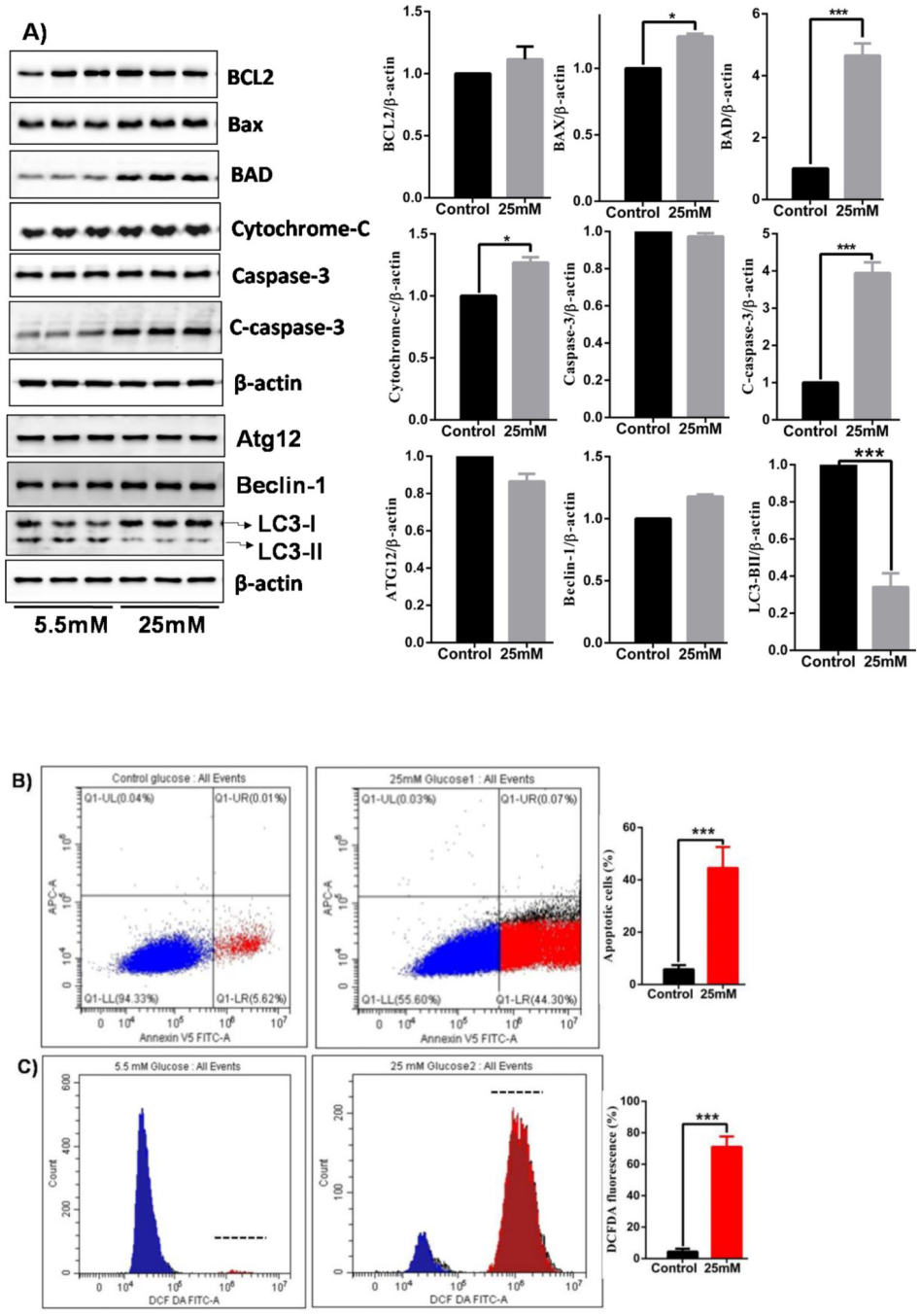

Figure. 3.

Hyperglycemia-induced apoptosis in HMC3 cells, A) Western blot analysis of proteins involved in apoptosis and autophagy and its quantifications presented in graphs. B) Flow cytometry analysis for apoptosis measured by Annexin-V5 staining and quantified and presented in percentage, (C) DCFDA staining-ROS levels in control and hyperglycemia. The fluorescence intensity of apoptosis and DCFDA for ROS level were measured and normalized from three (n=3) independent experiments, The statistical significance was

determined by unpaired t-test and significant differences $P< 0.05$, $P< 0.01$, and $P< 0.001$ are symbolized as *, ** and** * respectively.

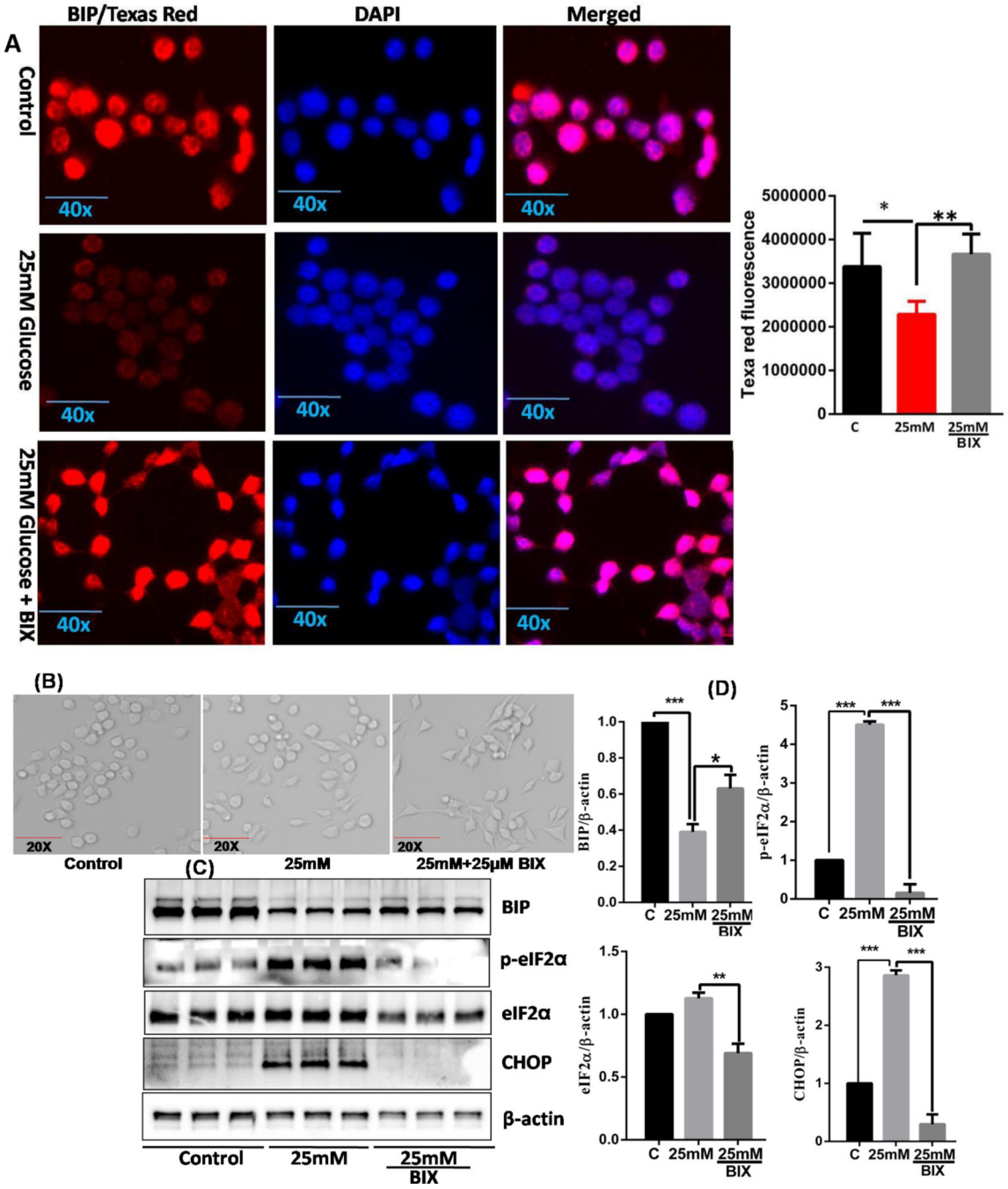

Figure. 4.

BIX derepresses the BIP expression and reduces the ER stress, A) Immunocytochemistry analysis of BIP, Texas Red fluorescence conjugated secondary antibody was used to detect BIP and DAPI for nuclear staining. The relative fluorescence intensity of BIP protein expression was measured from three biological triplicates. (B) Representative bright field image of BIX treated cells, (C) Expression of BIP, p-eIF2α, eIF2α, and CHOP (D) relative quantification of ER stress pathway proteins, and quantified with Image-J software and

normalized with β-actin. The statistical significance was determined by one-way ANOVA and significant differences $P < 0.05$ and $P < 0.01$ are symbolized as * and **, respectively.

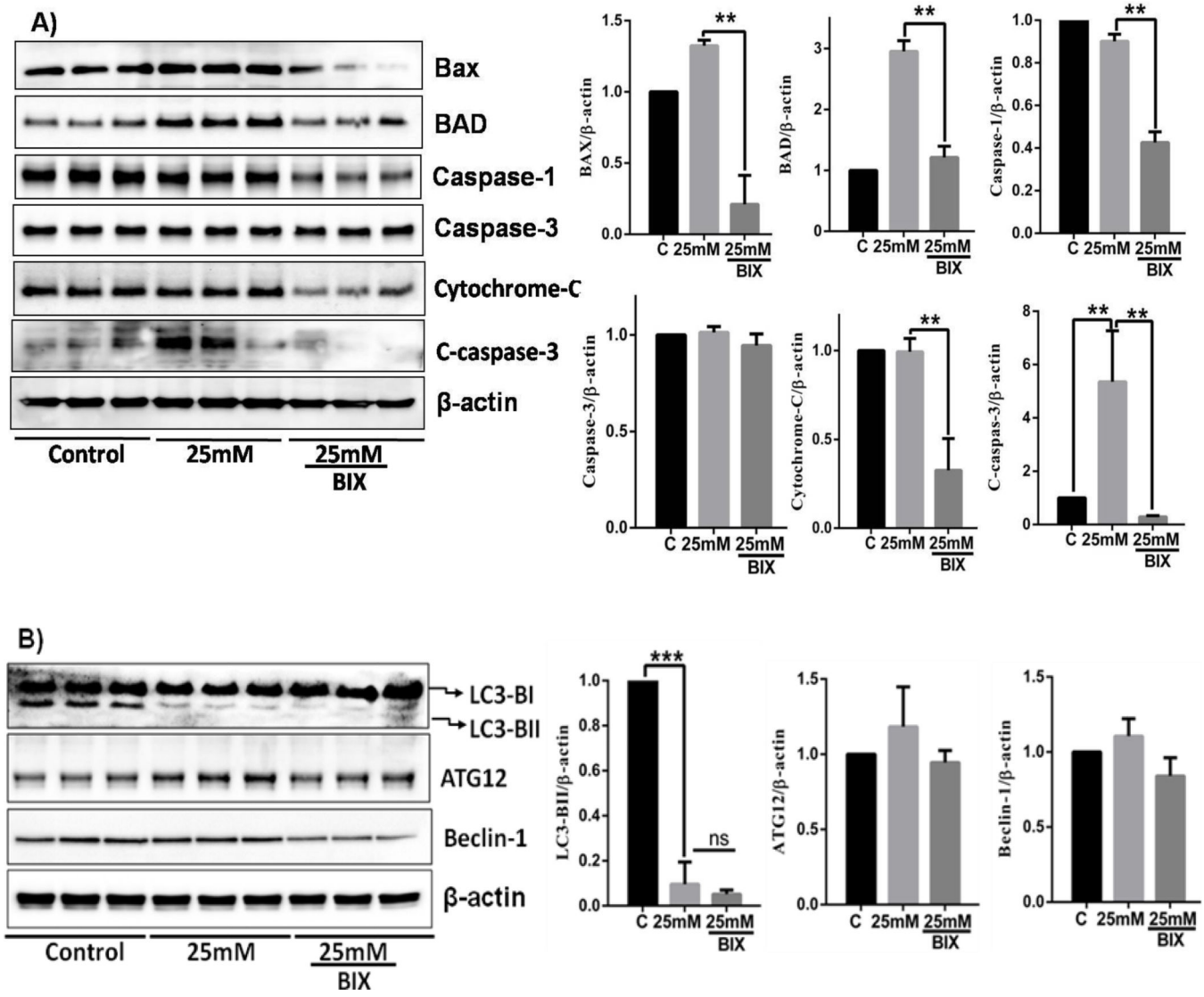

Figure. 5.

BIX treatment inhibits the apoptosis in HMC3 cells, (A) Expression of Bax, Bad, cytochrome-C, caspase-1, caspase-3, and cleaved caspase-3 and their relative quantification, (B) Expression of proteins involved in autophagy. The protein expression was quantified with Image-J software and normalized with β-actin. The data represent the means ± SD of three independent experiments. The statistical significance was determined by one-way ANOVA and significant differences $P< 0.05$, $P< 0.01$, and $P< 0.001$ are symbolized as *, and **, respectively.

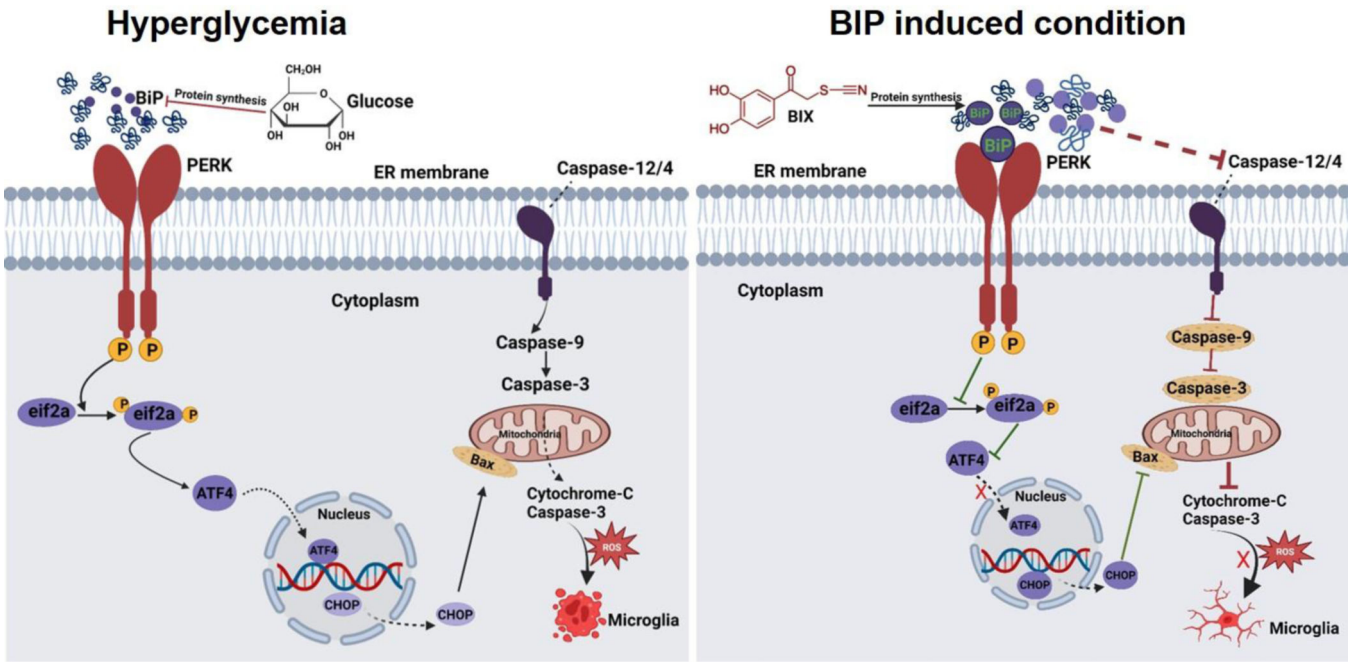

Fig. 6.

The proposed protective role of BIP in microglia during hyperglycemic conditions. In hyperglycemic conditions, the BIP protein expression was reduced, and misfolded proteins were accumulated in the ER lumen, which disassociated the BIP from the PERK and induced the phosphorylation of eIF2α, leading to translocation of ATF4 into the nucleus activating the transcription of CHOP, simultaneously caspase −3 was activated through ER resident caspase-12, and CHOP and Bax interaction, which caused the cytochrome-C release and increased ROS production leading to apoptosis. The BIX derepress the BIP expression and possibly associates with PERK and inhibits the phosphorylation of eIF2α leading to repression of CHOP transcription by ATF4. The microglia was protected from apoptosis through the increased expression of BIP, which might have increased the refolding capacity of accumulated misfolded proteins in the ER and inhibited the caspase-3 activation, cytochrome-C release, and pro-apoptotic markers.