Ischemic stroke remains a leading cause of death and disability around the world, with limited successful therapeutic strategies. In the past decade, preventing neuronal damage to improve recovery has always been a focus in stroke research, which is still a promising target. With regard to the major mechanisms of ischemic neuronal damage, early events such as oxidative stress, calcium overload, as well as excitotoxicity, may rapidly result in neuronal necrosis in the infarct core; however, later events such as inflammation are relevant to programmed cell death in the penumbra.

Apoptosis is a classic type I programmed cell death. Accompanied by the activation of caspases, the induction of apoptotic neuronal death is associated with proapoptotic factors such as Bad or Bax, and suppressed by anti-apoptotic factors such as Bcl-xL. Although apoptosis has been intensively
studied in the field of ischemic stroke, few anti-apoptotic strategies have proven to be useful, indicating that other molecular pathways might be involved in post-ischemic neuronal death.

Autophagy is a well-conserved mechanism activated in response to nutrient deprivation, which is a highly regulated process for degradation of macromolecules via the lysosomal system. Theoretically, autophagy may help promote cell survival, either by purging the cell of damaged organelles or toxic pathogens, or by regenerating metabolites for energy and growth during nutrient-limiting conditions. However, excess or prolonged autophagy may also promote cell death through excessive self-digestion and degradation of essential cellular constituents. Autophagic cell death is also known as type II programmed cell death. Despite the presence of underlying crosstalk, autophagic cell death is different from apoptotic cell death not only in morphology but also in gene regulation. Although some previous studies have indicated autophagy activation following cerebral ischemia, the underlying mechanism and its contribution to neuronal death after ischemic injury remain to be determined.

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcriptional factor composed of a constitutively expressed HIF-1β subunit and an inducibly expressed HIF-1α subunit. Under hypoxic conditions, because of inhibited ubiquitination, HIF-1α may accumulate and dimerize with HIF-1β; then, activating transcription of a broad range of hypoxia-responsive genes, including vascular endothelial growth factor (VEGF), erythropoietin (EPO), glucose transporter 1, and glycolytic enzymes. However, some important evidence recently suggested that HIF-1 might also contribute to the execution of programmed cell death. For example, HIF-1 may interact with Bcl-2 family members such as Bcl-2 adenovirus EIB 19 kDa interacting protein 3 (BNIP3); the later, an essential regulator in the autophagic pathway.

2-methoxyestradiol (2ME2) is a natural metabolite of estradiol, which has been widely used as an HIF-1α inhibitor in other studies. The present investigation was designed to clarify the possible relationship between HIF-1 up-regulation and autophagy activation following global cerebral ischemia in vivo. 2-methoxyestradiol was used to down-regulate HIF-1α expression. We tested the hypothesis that 2ME2 might attenuate post-ischemic autophagic cell damage and minimize neuronal injury. We also compared the effects of 2ME2 with those of 3-methyl-adenine (3-MA), which is an autophagy inhibitor, on post-ischemic neuronal injury.

Materials and Methods

The animal protocol of the present study was evaluated and approved by Shanghai Experimental Animal Center, Chinese Academy of Science.

Induction of Global Ischemia

The experiments were performed on adult male Sprague-Dawley rats weighing 250-280g (Shanghai Experimental Animal Center, Chinese Academy of Science). Global cerebral ischemia was induced by four-vessel occlusion (4-VO) as described before. In brief, rats were anesthetized with 3% pentobarbital sodium (1.5ml/kg, intraperitoneally (i.p)); then, bilateral vertebral arteries were electrocauterized and common carotid arteries were exposed. Rats were allowed to recover for 24 hour (h) and fasted overnight. Thereafter, ischemia was induced by occluding bilateral common carotid arteries with aneurysm clips for 15 minutes (min). Rats which lost their righting reflex within 30 seconds (s) and whose pupils were dilated and unresponsive to light during ischemia were selected for the experiments. Rats with seizures were excluded. After 15 min of ischemia, aneurysm clips were removed for reperfusion. Throughout the operation, rectal temperature was maintained at 36.5-37.5°C. Sham-operated animals underwent the same exposure procedures without clamping carotid arteries.

Drug Administration

2ME2 or 3-MA (Sigma Aldrich, USA) was administered half an hour before global ischemia. In 2ME2 group, the rats received 5 mg/kg 2ME2 intraperitoneally (i.p.), which was dissolved in 1% dimethyl sulfoxide (DMSO) to a final volume of 2 ml. In vehicle control group for 2ME2, the same volume of DMSO was injected (i.p.). For rats in 3-MA group, 5 μl 0.9% saline (NS) containing 500 nmol 3-MA was administrated through intracerebralventricular injection (i.c.v.). During i.c.v. injection, the rats were placed on ear bars of a stereotaxic instrument under anesthesia. Drug infusion into the left cerebral ventricle (from the bregma: anteroposterior: -0.8mm; lateral: 1.5mm; depth: 3.5mm) was performed using a stepper-motorized microsyringe at the rate of 1 μl/min. The same volume of vehicle was used as control.

Protein Sample Preparation

Rats were sacrificed under anaesthesia at different time points (0, 6, 12, 24 and 72 h after reperfusion). The hippocampal regions were microdissected at 0°C and immediately frozen in liquid nitrogen. For Beclin-1 and LC3 detection, tissues were homogenized in ice-cold homogenization buffer which consists of 50 mM HEPES (pH 7.4), 150 mM NaCl, 12 mM β-glycerophosphate, 3 mM dithiothreitol (DTT), 2 mM sodium orthovanadate (Na3VO4), 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, and 5 μg/ml each of leupeptin, pepstatin A and aprotinin. The homogenates were centrifuged at 15,000 g for 30 min at 4°C. Supernatants were collected and aliquots were stored at -80°C. For HIF-1α analysis, nuclear proteins were extracted and examined. In brief, tissues were homogenized in 10 mM HEPES (pH 7.9), 0.5 mM MgCl2, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 5 mM DTT, 10 mM β-phosphoglyceric acid, 1 mM Na3VO4, 1% NP-40, 1 mM benzamidene, 1 mM PMSF and enzyme inhibitors. The homogenates were centrifuged at 800g for 10 min at 4°C; then, the nuclear pellets were extracted with 20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 0.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and enzyme inhibitors for 30 min at 4°C with constant agitation. After centrifugation at 15,000g for 30 min at 4°C, the supernatants were removed and stored for use. The protein concentrations were determined by the method of Lowry with bovine serum albumin (BSA) as standard.
Western blot assay

Equal amounts of protein per lane (50 μg) were loaded onto an 8% or 12% polyacrylamide gel and separated by electrophoresis. Then, proteins were electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat dry milk/0.1% Tween-20 in Tris-buffered saline for 2 h at room temperature. Thereafter, the membrane was incubated with different primary antibodies overnight at 4°C, such as mouse anti-HIF-1α (1:500, Novus Biologicals), rabbit anti-beclin-1 (1:500, Sigma Aldrich, USA), rabbit anti-LC3 (1:800, Sigma Aldrich, USA), mouse anti-P53 (1:500, Santa Cruz), and goat anti-BNIP3 (1:500, Santa Cruz). Subsequently, the membrane was treated with appropriate horseradish peroxidase-labeled secondary antibodies for 2 h at room temperature. Immunoblots were probed using enhanced ECL chemiluminescence reagent (Amersham Bioscience). Band intensities were quantified by densitometric analyses using an AxioCam digital camera (Carl Zeiss GmbH, Jena, Germany) and a KS400 photo analysis system (version 3.0). Tata binding protein (TBP) or β-actin was used as internal control.

Quantitative real-time polymerase chain reaction (PCR)

Quantification of BNIP3 mRNA expression relative to β-actin was detected using real-time PCR. Total cellular RNA from the hippocampal region was extracted through Trizol reagent and reverse transcription was performed with a cDNA synthesis kit according to manufacturer’s instruction (Takara). For quantitative SYBR Green real-time PCR (2×SYBR Green Real-time PCR Master Mix, TOYOBO), the following primers were used: BNIP3 primer, sense: 5'-GCGCAGCAGCTACTCTCAGCA-3'; antisense: 5'-GTCAAGCCTCTCAGTA-3'; β-actin primer, sense: 5'-GATGACCTGTTGCTATC CAGGC-3'; antisense: 5'-CTCCCTTAATGTCACGCAGAT-3'. ABI 7900HT Sequence Detection System (Applied Biosystems) was applied and data were analyzed using ΔΔCT method.

Histological analysis

Rats were anaesthetized five days after ischemia and transcardially perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS). Brains were removed and post-fixed with the same fixation solution overnight at 4°C. After embedded in paraffin, coronal sections (6 μm) were prepared. Cresyl violet (0.1%) was used for Nissle staining. Under light microscope, the sections were examined and neuronal density was expressed as the number of cells per 1 mm linear length of hippocampal CA1 pyramidal layer. To detect cell apoptosis, sections were stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The TUNEL-positive cells were expressed by POD with 3,3'-diaminobenzidine (DAB) according to the manufacturer’s protocol (Roche).

Statistics

Five independent animals were sampled at each time point for Western blot assay and histology examination. Semiquantitative analysis of the bands was performed with the Image J analysis software (Version 1.30v; Wayne Rasband, NIH, USA). Semiquantitative data were expressed as mean ± S.E.M. Differences between groups were compared by one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test or Newman-Keuls test. A probability value of p < 0.05 was considered statistically significant.

RESULTS

HIF-1α up-regulation and autophagy activation following global cerebral ischemia

Since hippocampus is the most vulnerable region to transient global ischemia, we first examined HIF-1α protein expression in this particular region at different time points. As shown in Figure 1 (A and D), the protein level of HIF-1α increased rapidly after ischemia. As early as 6 h of reperfusion, significant up-regulation of HIF-1α was identified, which peaked at 12 h and did not decline until 24 h after ischemia.

Then, to explore the activation of autophagy after ischemia/reperfusion, we further detected beclin-1 and LC3 expression in the hippocampal regions. Beclin-1 is the mammalian ortholog of yeast Atg8/Vps30, which plays an important role in autophagosome formation. As shown in Figure 1B and E, the expression of beclin-1 elevated gradually after ischemia/reperfusion. Although no remarkable up-regulation was identified at the time point of 6 h, the expression of beclin-1 increased significantly at 12 h and peaked at 24 h of reperfusion. LC3 is the ortholog of Atg8 in mammals, which exists in two forms. When autophagy is activated, more LC3-I is converted to LC3-II; then the ratio of LC3-II/LC3-I increased. In our study, the LC3-II/LC3-I ratio elevated significantly at 12 and 24 h after ischemia (Figure 1C and 1F). The time course of LC3-II/LC3-I ratio was similar to that of beclin-1 expression, which provided reliable evidence of post-ischemic autophagy activation.

2ME2 inhibited post-ischemic autophagy activation

In order to investigate the effect of HIF-1α up-regulation in post-ischemic autophagy activation, we administrated 2ME2 to animal models half an hour before ischemia. Compared with sham group, the expression of HIF-1α increased significantly in DMSO group at 12 and 24 h of reperfusion as expected. While 2ME2 could remarkably inhibit the up-regulation of HIF-1α at the same time points (Figure 2A and 2D).

Moreover, we examined beclin-1 and LC3 expression in each group. In the DMSO group, the expression of beclin-1 and the ratio of LC3-II/LC3-I increased significantly at the time points of 12 and 24 h of reperfusion. However, when post-ischemic up-regulation of HIF-1α was inhibited by 2ME2, the expression of beclin-1 and the ratio of LC3-II/LC3-I declined remarkably in the 2ME2 group at the time points of 12 and 24 h, compared with the DMSO group (Figure 2B, 2C, 2E, 2F).

To elucidate the possible mechanism between HIF-1α down-regulation and autophagy inhibition after 2ME2 administration, we further examined P53 and BNIP3 expression in the 2ME2 and the DMSO group. Through Western blot, we demonstrated that P53 expression increased significantly after ischemia/reperfusion. While 2ME2 remarkably inhibited P53 high-expression, compared with DMSO group (Figure 3A and 3C). In ischemic models, BNIP3 expression was up-regulated at both transcription and protein level. After 2ME2 administration, high-expression of BNIP3 was inhibited significantly (Figure 3B, 3D and 3E).
2ME2 and 3-MA attenuated post-ischemic neuronal death

To further elucidate the possible relationship between autophagy activation and post-ischemic neuronal injury, histology analysis was performed after 15 min ischemia/5 days reperfusion in animal models. The surviving cells of CA1 pyramidal neurons were calculated through cresyl violet staining. As shown in Figure 4, normal cells showed round and pale stained nuclei. The shrunken cells with pyknotic nuclei after reperfusion were counted as dead cells. Compared with sham group (Figure 4A and 4F), severe cell death was identified in both vehicle groups, including DMSO group (Figure 4B and 4G) and saline group (Figure 4D and 4I). However, 2ME2 administration could obviously minimize ischemia/reperfusion-induced neuronal degeneration (Figure 4C and 4H), as well as those identified in 3-MA group (Figure 4E and 4J). The numbers of surviving pyramidal cells in the CA1 region of the sham group, DMSO group, 2ME2 group, saline group, and 3-MA group were 188.6±15.6, 41.3±20.4, 122.1±16.2, 45.2±22.1 and 138.1±19.3, respectively (Figure 4K). Results of TUNEL staining indicated that 2ME2 did not increase the number of TUNEL positive cells compared with DMSO group (Figure 5).

DISCUSSION

In the present study, we mainly examined the effects of 2ME2, a natural HIF-1α inhibitor, on post-ischemic autophagy activation. Our results demonstrated that 1) global cerebral
ischemia could induce autophagy activation in the hippocampal region; 2) HIF-1α inhibitor 2ME2 might decrease post-ischemic autophagy activities; 3) inhibiting autophagy helped to reduce neuronal death after ischemia/reperfusion. Our study for the first time explored the possible relationship between HIF-1α up-regulation and autophagy activation in vivo.

The 4-VO model described by Pulsinelli is now widely used to create global ischemia, which selectively impairs hippocampus and its surrounding structures. As demonstrated in previous studies, we also identified rapid and sustained HIF-1α up-regulation in vulnerable region after cerebral ischemia. However, HIF-1α induction has different temporal profiles depending on the type of experimental models. After transient focal ischemia, a biphasic activation of HIF-1α was identified, which peaked at 6 h and 6 days respectively; while in cardiac arrest/resuscitation rats, the high expression of HIF-1α in the cortex lasted from 1 h after ischemic injury until 7 days later. Another previous investigation also suggested that the age of experimental animals might influence the pattern of HIF-1α expression after ischemic insults in brain. In our study using the 4-VO model in adult rats, HIF-1α protein expression increased as early as 6 h after ischemia and peaked at the time point of 12 h. Our results were basically in accordance with previous study using a similar model. We proposed that the temporal profiles of HIF-1α expression related not only to the duration and severity of ischemic damage, but also to the nature of impaired tissues.

HIF-1α is a master regulator of oxygen homeostasis. Meanwhile, HIF-1α may also serve as an important pro-death factor through interacting with tumor suppressor gene p53. In our study, we demonstrated that HIF-1α up-regulation might induce p53 stabilization; in contrast, when the expression of HIF-1α was inhibited by 2ME2, p53 was down-regulated. Several previous reports have provided evidence that p53 participated in autophagy activation. For example, the inhibition of p53 could reduce tumor necrosis factor α (TNF-α)-induced autophagy activation in vitro; in excitotoxic rat models, induction of p53 contributed to the increase of autophagy activities. Therefore, in the present study, we supposed that HIF-1α induced p53 stabilization was an important reason contributing to the post-ischemic autophagy activation.

BNIP3 is an important member of HIF-1α target genes. Our results confirmed that HIF-1α up-regulation after global ischemia might increase both transcription and protein expression of BNIP3. Our results were in accordance with previous findings. In 2ME2 group, BNIP3 expression was correspondingly down-regulated when HIF-1α was inhibited. As revealed before, BNIP3 might competes with beclin-1 for binding to bcl-2, thus freeing beclin-1 to trigger autophagy. We supposed that BNIP3 high-expression as a result of post-ischemic HIF-1α up-regulation was also an important mechanism for autophagy activation after cerebral ischemia. Other strong evidence for BNIP3 participating in autophagy activities included that knockdown of BNIP3 might reduce autophagic cell death under conditions of nutrient deprivation, as demonstrated previously.

As a known HIF-1α inhibitor, 2ME2 has been already evaluated in several clinical trials for cancer patients. According to previous experimental studies performing in adult rats, 5mg/kg 2ME2 was administrated half an hour before ischemia. Our results supported that this dosage of 2ME2 could remarkably inhibit post-ischemic up-regulation of HIF-1α. Moreover, we also identified that 2ME2 significantly decreased autophagy activities following cerebral ischemia.

**Figure 3:** Effects of 2ME2 on p53 and BNIP3 expression in the hippocampal region after global ischemia/reperfusion. Protein levels were detected through Western blot analysis. BNIP3 mRNA levels were evaluated by real-time quantitative RT-PCR. A) p53 protein expression. B) BNIP3 protein expression. C) Bar graph of p53 protein expression. D) Bar graph of BNIP3 protein expression. E) Bar graph of BNIP3 mRNA levels. Bands were scanned and the optical density (O.D.) was represented as fold versus sham group (C, D). Values are expressed as means ± S.E.M. from five independent animals (n = 5). **p < 0.01 versus sham group, ##p < 0.01 versus DMSO group.
Figure 4: 2ME2 and 3-MA reduced hippocampal neuronal death after 15 min ischemia/5 days reperfusion. Compared with sham group (A, F), neuronal density decreased significantly in DMSO group (B, G) and saline group (D, I). While 2ME2 (C, H) and 3-MA (E, J) remarkably rescued post-ischemic neuronal death compared to the corresponding vehicle controls. Cell density was expressed as the number of cells per 1 mm length of the CA1 pyramidal cells counted under a light microscope (×400) (K). Values are presented as mean ± SD (n = 5). Scale bars = 400 μm (A-E); 10μm (F-J). White arrow indicated normal cell; black arrow indicated pyknotic cell. **p < 0.01 versus sham group, #p < 0.05 versus corresponding vehicle controls.

Figure 5: 2ME2 reduced hippocampal TUNEL-positive cells after 15 min ischemia/5 days reperfusion. Compared with sham group (A, D), TUNEL-positive cell density increased significantly in DMSO group (B, E). While 2ME2 (C, F) remarkably reduced post-ischemic TUNEL-positive cell density in the hippocampal CA1 region compared to DMSO group. Cell density was expressed as the number of cells per 1 mm length of the CA1 pyramidal cells counted under a light microscope (×400) (G). Values are presented as mean ± SD (n = 5). Scale bars = 400 μm (A-C); 10μm (D-F). Black arrow indicated TUNEL-positive cell. **p < 0.01 versus sham group, #p < 0.05 versus DMSO group.
HIF-1α might contribute to autophagy activation through stabilizing p53 and promoting BNIP3 transcription, we supposed that 2ME2 probably down-regulated autophagy activities through inhibiting post-ischemic HIF-1α up-regulation. By means of TUNEL staining, our study suggested that 2ME2 did not increase apoptotic cell death when inhibiting autophagy in global ischemia models. These findings provided forceful evidence that 2ME2 actually rescued neurons rather than pushing them from type-II to type-I cell death. Our investigation, combined with previous studies, indicated that not only early administration of 2ME2 after cerebral ischemia but also prophylactic administration of 2ME2 before cerebral ischemia could protect neurons from post-ischemic damage.\(^{16,31}\) Since 2ME2 might induce cell death in cancer therapy, discrepancy of 2ME2 effects in cancer and cerebral ischemia exists. An important reason responsible for the discrepancy might be the complex role that HIF-1 and its down-stream molecules play in different diseases. To date, over 60 genes have been identified as HIF-1 target genes, which participate in a broad range of cellular physiological or pathophysiological activities. In cancer, chronic hypoxic conditions induce sustained expression of HIF-1, the main role of which is to promote cancer cell proliferation, survival and invasion.\(^{32}\) Therefore, 2ME2 has been proved to be a promising anti-cancer compound by inhibiting HIF-1. In cerebral ischemia, acute severe hypoxic insult induces the expression of cell death-related HIF-1 target genes soon after ischemia.\(^{16,31}\) Thus, prophylactic or post-ischemic early administration of 2ME2 might protect neurons from ischemia-induced neuronal injury.

As those identified in focal cerebral ischemia models,\(^{10}\) our study confirmed that autophagy participated in post-ischemic neuronal injury. Beclin-1 expression and LC3-II/LC3-I ratio increased significantly as early as 12 h after ischemia, peaked at 24 h, and continued to be high until 48 h of reperfusion. It should be noted that the peak of autophagy activation was a little later than that of HIF-1α expression. These findings were in accordance with our speculation that HIF-1α might be correlative to autophagy activation through activating p53 and BNIP3. As revealed in our results, inhibiting autophagy, either by direct inhibitor 3-MA or by indirect inhibitor 2ME2, might prevent pyramidal neuron death after ischemia. Therefore, these findings provided powerful evidence that autophagy could be a potential target for post-ischemic neuronal protection.

**CONCLUSIONS**

In summary, our study suggested that 2ME2 inhibited autophagy activation following cerebral ischemia, probably through the HIF-1 pathway; therefore, 2ME2 could attenuate post-ischemic neuronal damage through autophagy inhibition. Further studies focusing on the accurate mechanisms of HIF-1 influencing autophagy activities after cerebral ischemia are still needed.

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