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Berberine was neuroprotective against an *in vitro* model of brain ischemia: Survival and apoptosis pathways involved

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ABSTRACT

Berberine is an alkaloid derived from herb the *Berberis* sp. and has long-term use in Oriental medicine. Studies along the years have demonstrated its beneficial effect in various neurodegenerative and neuropsychiatric disorders. The subject of this study was to evaluate whether berberine protects against delayed neuronal cell death in organotypic hippocampal culture (OHC) exposed to oxygen and glucose deprivation (OGD) and the cell signaling mechanism related to its effect. Hippocampal slices were obtained from 6 to 8-days-old male Wistar rat and cultured for 14 days. Following, the cultures were exposed for 1 h to OGD and then treated with Berberine (10 and 20 μM). After 24 h recovery, propidium iodide (PI) uptake was analyzed and a decrease was observed in PI uptake on OGD Ber-treated culture, which means a decrease in cellular death. Western blot analysis showed that proteins Akt, GSK3β, ERK and JNK appear to play a role in berberine-mediated neuroprotection. Furthermore, capase-3 activity of OGD Ber-treated culture was diminished by control level in a fluorimetry assay. These findings suggest that berberine-mediated neuroprotection after ischemia involves Akt/GSK3β/ERK 1/2 survival/apoptotic signaling pathway as well as JNK and caspase-3 activity inhibition.

1. Introduction

Cerebral ischemia is one of the leading causes of death worldwide. The injured neurons undergo through biochemical cascade that leads to cell death. Despite increasing studies focused on mechanisms of ischemia have been reported, the only effective treatment is thrombolysis. However, due to its numerous limitations, only about 5% of the patients can be selected for this treatment (Hall et al., 2009).

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Berberine (Ber) is a major alkaloid isolated from medicinal herbs such as *Berberis* sp., *Coptidis* sp. and *Phellodendri* sp. and has a long history in Chinese and Indian medicines (Kulkarni and Dhir, 2010). Studies demonstrated its multiple pharmacological actions, including anti-inflammatory effects (Wu et al., 2012), diarrhea-treating action (Birdsall and Kelly, 1997) and glucose-lowering potential (Di Pierro et al., 2012). Furthermore, it was reported that berberine has potent neuroprotective effects against brain ischemia (Wang et al., 2004; Yoo et al., 2006), Alzheimer's disease (Zhu and Qian, 2006; Ji and Shen, 2011) and brain tumor (Eom et al., 2008).

There appears to be at least two major modes of cell death involved in ischemic neuron death, apoptosis and necrosis (Lipton, 1999). Increasing evidence shows that apoptosis is caused by an unbalance in signaling events, including phosphatidylinositide 3-kinases pathway (PI3K). The serine–threonine kinase, Akt (also known protein kinase B), is a downstream kinase of PI3K and becomes activated by phosphorylation of residues Thr-308 and Ser-473. This kinase can be phosphorylated by PDK1 and PDK2 or by PI3K directly and plays an important role for death/survival processes (Horn et al., 2005). Akt mediates its anti-apoptotic effects by phosphorylating, among others, GSK3β, Bad, NFkB and CREB (Mehta et al., 2007). Serine–threonine kinase glycogen synthase kinase 3-β (GSK3β) is particularly abundant in the central nervous system and is neuron-specific and is a key regulator in several physiological processes, such as cell cycle and apoptosis in neuronal cells during hypoxia. GSK3β induces caspase3 activation and activates the pro-apoptotic tumor suppressor gene, p53 (Loberg et al., 2002). Akt phosphorylation of GSK3β on Ser9 and its inactivation may mediate some anti-apoptotic stimuli (Zhang et al., 2012). Extracellular signal-Regulated Kinases (ERK 1/2) and stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) are members of the mitogen-activated protein kinases (MAPK) family and are activated by a variety of environmental stresses, inflammatory cytokines and growth factors. Those signals are delivered to this cascade by small GTPases of the Rho family (Rac, Rho, cdc42). SAPK/JNK translocates to the nucleus where it can regulate the activity of multiple transcription factors resulting in cell growth, differentiation, survival and apoptosis (Moulin and Widmann, 2004).

Despite global cerebral ischemia suppress the continuous blood flux during the outcome; the hippocampus is one of the most sensitive regions to the effects of the insult. Distinct subfields show differential vulnerability to cell death: the CA1 pyramidal neurons are vulnerable, whereas the granular cells of the dentate gyrus (DG) are resistant. This differential pattern of damage of CA1 cells *versus* preservation of DG cells creates an ideal model for comparing cell death vulnerability in areas within the same region of the brain (Lipton, 1999; Horn et al., 2005).

The aim of this study was to verify berberine neuroprotective effect in organotypic hippocampal culture exposed to oxygen and glucose deprivation, an *in vitro* model of cerebral global ischemia, focusing on PI3K/Akt pathway and its consequent signaling. For this purpose, cultures were treated with different concentrations of berberine, based on the literature, and then exposed to oxygen and glucose deprivation (OGD). We analyzed the cell death/survival by propidium iodide (PI) uptake, phosphorylation of signaling proteins and caspase activation.

2. Results

2.1. *Effect of berberine treatment in organotypic hippocampal cultures after OGD*

Exposure of cultures to 10–20 μM of berberine caused a marked decrease in cell death, as shown by the intense fluorescence due to incorporation of PI in hippocampus after 24 h of recovery (Fig. 1A). The neuroprotection of berberine against *in vitro* ischemia-induced hippocampal cell damage appeared to be more effective at the concentration of 10 μM, which was chosen for the following analysis. Quantification of PI fluorescence showed that the exposure of cultures to OGD caused around 30% of damage in hippocampus after 24 h of recovery, a significant increase when compared to control cultures (Fig. 1B). The damage induced by OGD was mainly observed in CA1, CA2 and CA3 areas. Additionally, a small damage was sometimes observed in the inner blade of the DG area, as observed in Fig. 1A. This data confirms our previous work using the same experimental model of ischemic cell death (Valentim et al., 2003). When cultures were exposed to berberine, 10 μM and 20 μM, around 15–10% of decrease in PI staining was observed, respectively, showing a statistically significant neuroprotective effect when compared to OGD culture (Fig. 1B).

2.2. *Phosphorylation of Akt/GSK3β signaling pathway involved with berberine neuroprotective effect*

As well established, kinase protein Akt plays a critical role to promote neuronal survival after global brain ischemia. And, as a downstream target, GSK3β is also involved in this type of neuronal injury, as we previously reported (Mehta et al., 2007). Therefore, we hypothesized that Akt pathway may be important for Ber-mediated neuroprotective effect. To detect the role of these signaling proteins, we performed western blots (Fig. 2A and C). The expression of pAkt and pGSK3β from OGD culture was significantly decreased when compared to control culture (Fig. 2B and D, correspondingly). As shown in Fig. 2B and D, OGD berberine-treated cultures indicate a significant increase in protein Akt and GSK3β phosphorylation when compared with OGD culture. Also, OGD berberine-treated cultures demonstrated a 2.0 fold increase in pAkt level when compared to control culture (Fig. 2B).

2.3. *ERK 1/2 activation and inhibition of JNK signaling pathway plays a role in berberine-mediated neuroprotection*

Apoptosis is involved in the ischemic cell death pathway and can be mediated by several signaling molecules, such as pro-apoptotic proteins BAX and Bcl-Xs (Cui et al., 2009), caspase family (Ouyang et al., 1999; Horn et al., 2005) and cytokines liberation (Yasuda et al., 2011; Simão et al., 2012). MAPK family proteins such as ERK and JNK are common upstream pathway proteins that can lead to those apoptotic responses signaling, s study that has been demonstrated (Wang et al., 2007). To confirm the role of ERK/JNK as protein-related to

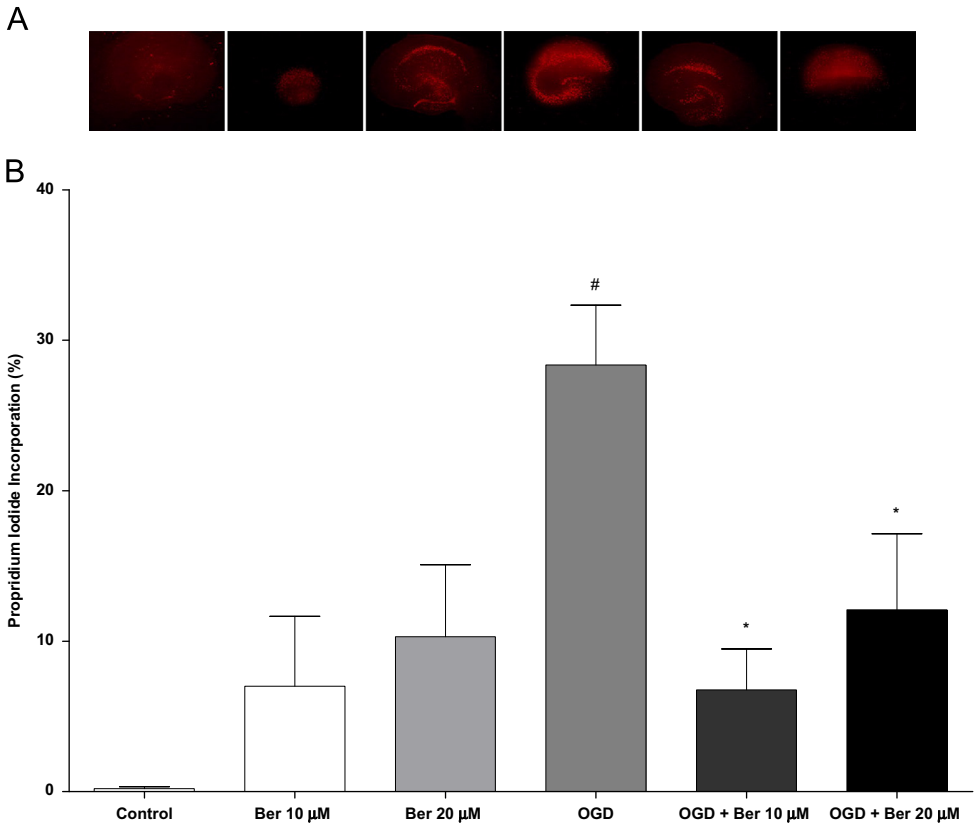

Fig. 1 – Administration of Berberine after OGD in OHC decrease PI uptake. (A) Representative pictures of OHC slices stained with propidium iodide after treatment with 10 and 20 µM of Ber and after 24 h recovery (magnification: 40×). (B) PI incorporation quantification from pictures shown in (A). Bars represent the mean ± S.E.M., $n=12$. # Significantly different from control cultures and * significantly different from cultures exposed to OGD alone; *$p<0.05$ (one-way ANOVA followed by Tukey's test).

ischemic death and if they are involved with the berberine neuroprotective effect, we analyzed the phosphorylation content in the cell culture.

The ERK MAP kinase pathway was also triggered by berberine, with clear increase (3-fold) in phospho-ERK levels in OGD-treated slice when compared to OGD group (Fig. 3A and B). As shown in Fig. 3C, the exposure to OGD induced an increase of 3.29 fold on JNK phosphorylation when compared to control culture. However when cultures were treated with berberine, the level of JNK phosphorylation was similar to control cultures (Fig. 3C and D).

2.4. *Berberine neuroprotective effect decreases caspase 3 activity*

There are further chemical changes that are suggestive of apoptosis and are expressed in vulnerable CA1 area. Induction of classical apoptosis by caspase 3 occurs in cells after global ischemia before and during the time of maximal cell death (Lipton, 1999). Based on that information we carried out a fluorimetry assay for the effector caspase 3. A higher caspase activity was detected in OGD culture (Fig. 4), whereas in cultures treated with berberine a lower caspase 3 activity was observed, which was similar to that observed in control cultures (Fig. 4).

3. Discussion

There are two major types of cerebral ischemia, focal and global. The brain injury caused by transient global cerebral ischemia is characterized by a delayed selective death of CA1 pyramidal neurons in the hippocampus (Kirino, 1982; Lipton, 1999). This region is critically involved in spatial learning and memory, and the degeneration of pyramidal neurons results in impairment of these functions (Bendel et al., 2005, Frozza et al., 2013). One of the principal features of OHC is to keep the tissue organization as *in vivo*: the slice presents all types and organized cells as in the *in vivo* form. This experimental model of brain ischemia has a great advantage over others cultures since they retain several global brain ischemia *in vivo* aspects, such as delayed neuronal death and selective vulnerability (Strasser and Fischer, 1995; Noraberg et al., 1999, 2005; Zamin et al., 2006).

The present study provides evidence that berberine addition to the culture medium of hippocampal organotypic

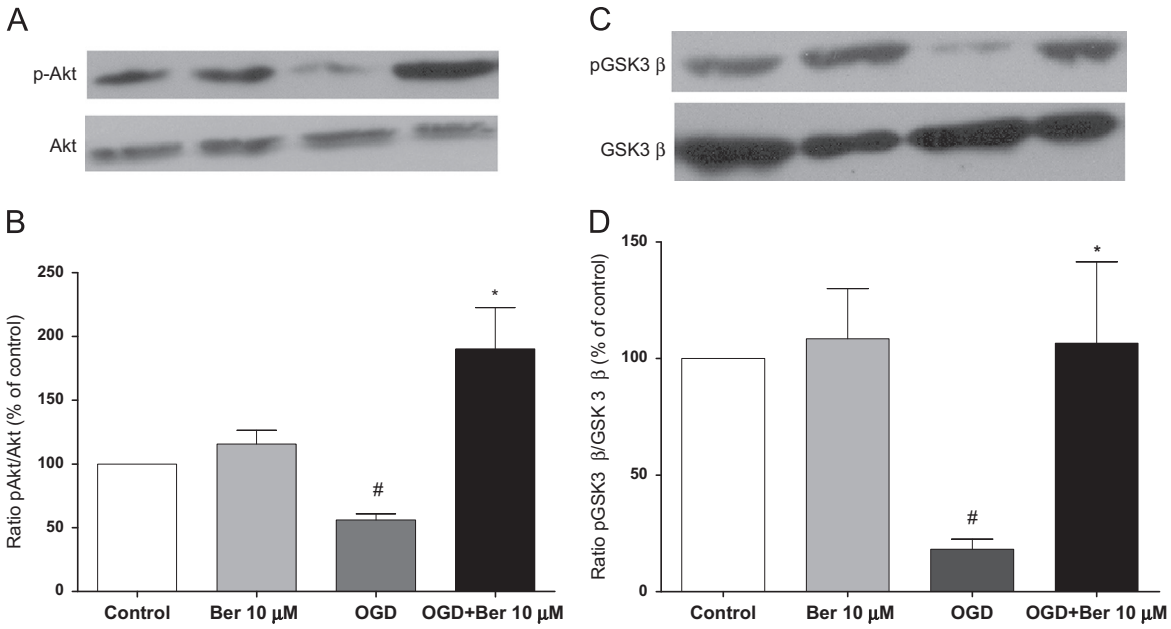

Fig. 2 – Berberine enhances Akt and GSK3β phosphorylation. Representative western blots showing levels of pAkt, Akt (A) and pGSK3β, GSK3β (C) in OHC culture. The average levels of pAkt (B) and pGSK3β (D) were increased in OGD Berberine-treated culture after 24 h recovery. Bars represent the mean ± S.E.M., $n=7/6$. # Significantly different from control cultures and * significantly different from cultures exposed to OGD alone; $p<0.05$ (one-way ANOVA followed by Tukey's test).

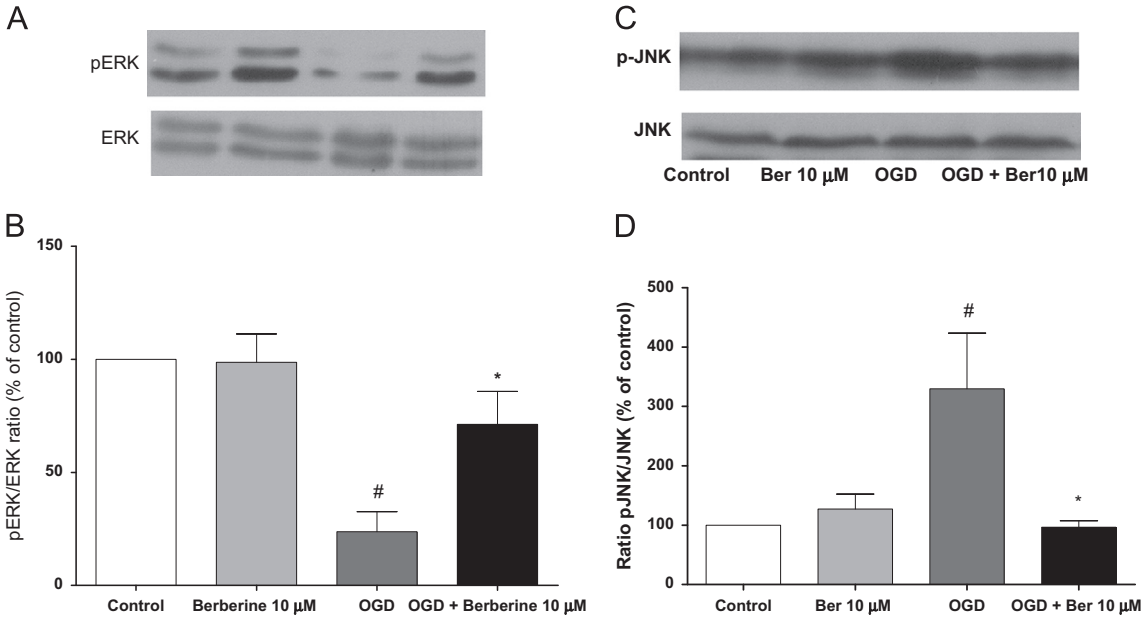

Fig. 3 – Berberine induces regulation on MAP kinase pathway as indicated by elevated pERK 1/2 and decrease in pJNK levels in OGD-treated culture. (A–C) Representative western blots showing levels of pERK 1/2, ERK, pJNK and JNK in OHC culture. The average levels of pERK 1/2 were increased (B) and decreased (D) in OGD Berberine-treated culture after 24 h recovery. Bars represent the mean ± S.E.M., $n=6$. # Significantly different from control cultures and * significantly different from cultures exposed to OGD alone; $p<0.05$ (one-way ANOVA followed by Tukey's test).

culture, after OGD exposition, an *in vitro* model of brain ischemia, has neuroprotective effect as seen by a decrease in incorporation of dye propidium iodide; and this effect appears to be mediated via modulation of cellular signaling PI3K/Akt and JNK, leading to decreased activation of caspase 3, involved in mediation of apoptotic cell death.

Studies have demonstrated potential cell protective mechanisms of berberine on cerebral ischemia such as

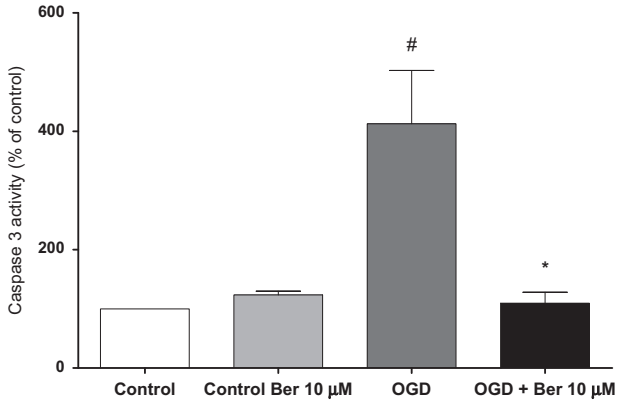

Fig. 4 – Lower caspase-3 activity following global ischemia after Ber treatment.Caspase-3 activity in OHC with Ber administration after OGD and 24 h recovery. Bars represent the mean ± S.E.M., $n=6$. # Significantly different from control cultures and * significantly different from cultures exposed to OGD alone; $p<0.05$ (one-way ANOVA followed by Tukey's test).

involvement of Akt, GSK3β, CREB on cerebral cortex in an *in vivo* rat model of permanent middle cerebral artery occlusion (pMCAO) (Zhang et al., 2012) and Bcl-2 (Cui et al., 2009), cytochrome C release (Zhou et al., 2008) in mouse OHC and PC12 cell OGD model. Akt is well known to be the primary basis of neuronal survival and prevents the progression of apoptotic stimuli after brain ischemia outcome. These finding is confirmed with the use of Akt inhibitor LY294002on brain ischemic model (Horn et al., 2005). In the present work, our results suggest that the Akt phosphorylation acts on the berberine-mediated neuroprotective effect and contributes to hippocampal cell survival and thus suppresses apoptosis.

The targets of the protein Akt are many and varied, but all of them are related to cell proliferation and/or survival and result in antiapoptotic effect. These physiological roles of Akt include involvement in metabolism, protein synthesis, apoptosis pathways, transcription factor regulation and the cell cycle. Akt exerts its effects in the cell by phosphorylating a variety of downstream substrates, including GSK3β, Bcl-2 – family member Bad and CREB (Downward, 1998). Our data demonstrated an enhanced phosphorylation of GSK3β at Ser9 in OGD-berberine treated culture which blocked the protein activation and consequently negatively regulates its pro-apoptotic signaling. A role for GSK3β in ischemia demonstrates the use of a GSK3β inhibitor to decrease infarct size in rodents (Kelly et al., 2004). As our data suggest, Akt/ GSK3β pathway is likely to be important for Ber-neuroprotective effect, given the abundance of GSK3β on the central nervous system and its role on ischemia.

In this study, we defined the relationship between Ber and Akt/GSK signaling, as well as ERK 1/2 and JNK in ischemic hippocampal tissue. Members of the MAP kinase family play crucial roles in regulating responses to various stresses, and in neural development, inflammation, and apoptosis (Simão et al., 2012). Besides stress, those signaling proteins can also be activated via G-Protein Coupled Receptors (GPCRs), Receptor Tyrosine Kinases (RTKs) and Cytokine Receptors. Growth Factors also activate ERK/JNKs. Although the signaling cascade from Growth Factor Receptors to ERKs is relatively well understood, the pathway leading to JNK activation is more unclear (Mehta et al., 2007). Growth Factors and Growth Factor Receptors stimulate Ras and PI3K; an Akt upstream protein also activates Ras (Downward, 1998). Our results demonstrated the MAP kinase pathway was for instance shown to be involved in Berberine-mediated neuroprotection in OGD culture. The causes of cell death in stroke are multifactorial and are influenced by the ischemic environment lacking in nutrients and oxygen, coupled with the loss of survival signals towards apoptosis and inflammation, for example. We have started investigation on cytokine secretion *in vitro* after OGD to combine with these data and have a more detailed knowledge of JNK activation by Berberine treatment. Thereby combined results and use of inhibitors of the PI3K/Akt and MAPK pathways could affect more significantly the Berberine-mediated neuroprotection.

Apoptosis is proved to play a role in neuronal death in human stroke and its animal models several hours after obtaining evidence based on studies of ischemia. The activation of caspases, particularly caspase-3, and the inhibition of cell death by inhibition of these proteases are the strongest criteria. Caspase do not appear to be activated in necrotic death (Lipton, 1999). The caspase cascade is activated by two distinct routes: one from cell surface and the other from mitochondria. The pathway leading to caspase activation varies according to the apoptotic stimulus. Another evidence for caspase involvement is the upregulation of the enzyme. This occurs either by cleaving the existing procaspases or by synthesizing a new enzyme that is then cleaved (Sugawara et al., 2004). In this study, we showed increase of caspase-3 activation in OGD culture. After Ber treatment, the protein activity was in the same control level. Other studies continue in the same direction as ours: caspase-3-like mRNA is upregulated between 8 and 24 h after 15–30-min ischemia in rat, predominantly in CA1 pyramidal cells, and total caspase-3 protein is increased by 8 h. And also, caspase-3-like activity measured in artificial substrate was elevated about 10-fold in hippocampus between 8 and 24 h after ischemia (Chen et al., 1998).

In conclusion, we demonstrated the phosphorylation of Akt/GSK3β pathway and the involvement of ERK 1/2, JNK and caspase-3 inhibition after Berberine treatment of organotypic hippocampal culture following an *in vitro* global brain ischemia model. Our study provides beneficial evidences for early administration of Berberine and its underlying mechanisms and interrelated pathways that may rescue neuronal cells after global brain ischemia.

4. Experimental procedures

4.1. *Organotypic hippocampal cultures*

Organotypic hippocampal cultures were prepared according to the method of Stoppini et al. (1991) with some modifications (Valentim et al., 2003). The local Animal Care Committee approved all animal procedures used. Hippocampal slices

were obtained from 6 to 8-day-old male Wistar rats by removing the brain, dissecting hippocampi and making transverse slices (400 μm), using a Mcllwain tissue chopper. Slices were separated in a Hank's balanced salt solution (HBSS) (Gibco) supplemented with 25 mM HEPES, 1% fungizone and 36 μl/100 ml gentamicin, pH 7.2. Six slices were placed on one Millicell culture insert (Millicell-CM, 0.4 μm) and the inserts were transferred to a six-well culture plate (Cell Culture Cluster, TPP) with 1 ml of culture medium consisting of 50% minimum essential medium (MEM) (Gibco), 25% heat inactivated horse serum (Gibco) and 25% HBSS (Gibco), supplemented with glucose 36 mM, glutamine 2 mM, HEPES 25 mM and $NaHCO_3$ 4 mM (final concentrations). Fungizone 1% and gentamicin 36 μl/100 ml were added to the medium. The pH was adjusted to 7.3 and immediately the solution was filtered (Millex-GS, Millipore). The plates were then placed in an incubator at 37 °C in an atmosphere of 5% CO_2. The medium was changed twice a week and experiments were carried out after 14 days *in vitro*.

4.2. *Oxygen and glucose deprivation (OGD)*

The induction of OGD was based on the method described by Strasser and Fischer (1995) with some modifications (Cimarosti et al., 2001). Cultures were carefully rinsed three times with OGD medium composed of $CaCl_2$ 1.26 mM, KCl 5.36 mM, NaCl 136.9 mM, H_2PO_4 0.34 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.44 mM, HEPES 25 mM, pH 7.2. Slices were left in 1 ml of this medium for 15 min, and then the medium was exchanged by one with the same composition previously bubbled with nitrogen for 30 min. The cultures were transferred to an anaerobic chamber at 37 °C in which the oxygen was replaced by nitrogen, and left in these conditions for 60 min. Slices were washed three times with HBSS and treated with 10 and 20 μM of berberine chloride (Sigma) diluted in water (37 °C) added to the culture medium. Afterwards, the plates were brought back to the usual culture conditions for 24 h recovery time with the culture medium treated with berberine.

4.3. *Quantification of cellular death*

Cellular damage was assessed by fluorescent image analysis of propidium iodide (PI) uptake (Valentim et al., 2003; Horn et al., 2005). PI 5 μM was added 1 h before the end of the recovery period and cultures were observed with an inverted microscope (Nikon Eclipse TE 300) using a standard rhodamine filter. Images were captured and analyzed using Scion Image software (http://www.scioncorp.com). The area where PI fluorescence was detectable above background levels was determined using the "density slice" option of Scion Image software and compared to the total slice area to obtain the percentage of damage.

4.4. *Western blot analysis*

After obtaining fluorescent images, slices were homogenized in lyses buffer, aliquots were taken for protein determination by Lowry assay and β-mercaptoethanol was added to a final concentration of 5%. Proteins were resolved (50 μg per lane) on SDS-PAGE. After electrophoresis, proteins were electro transferred to nitrocellulose membranes using a semi-dry transfer apparatus. Membranes were incubated for 60 min at 4 °C in blocking solution (Tris-buffered saline containing 5% powdered milk and 0.1% Tween-20, pH 7.4) and further incubated with the appropriate primary antibody dissolved in the blocking solution overnight at 4 °C. Primary antibodies anti-pAkt/Ser473 (1:1000), anti-Akt (1:1000), anti-pGSK3β/Ser9 (1:1000), anti- GSK3β (1:1000), anti-pERK 1/2/Thr202-Tyr204 (1:1000), anti-ERK 1/2 (1:1000), anti-pJNK/Thr183-Tyr185 (1:1000), anti-JNK (1:1000) and anti-β-actin (1:1000) were used. The membranes were then incubated with horseradish peroxidase–conjugated anti-rabbit antibodies (1:1000) for 2 h. The chemioluminescence was detected using X-ray films that were scanned and analyzed using the Optiquant software (Packard Instruments). All antibodies were purchased from Cell Signaling Technology, USA. Data are expressed as percentage of control cultures.

4.5. *Caspase activity measurement*

Caspase 3 activity was measured by the cleavage of the fluorescent substrate Ac-DEVD-AFC (Sigma). The procedure with OHC, OGD and treatment (Ber 10 μM) were the same described above. Twenty four hours after recovery, the slices were taken out from culture membranes and lysed in ice-cold solution of PBS and Triton x-100 0.2%. The extract was centrifuged at 10,000*g* for 5 min and the supernatant was collected. For each experiment, 30 μg of protein was incubated with a reaction buffer containing (g/ml): sucrose 0.1, CHAPS 0.001, BSA 0.0001 and HEPES–NaOH 0.024, pH 7.5 in a 96 wells black plate. The caspase 3 substrate was present at a final concentration of 20 μM. The plate was incubated at 37 °C for 10 min under agitation. Samples were assayed using SoftMax Pro fluorescence plate reader (Molecular Devices®, USA). Data are expressed as percentage of control cultures.

4.6. *Statistical analysis*

Data are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was applied to the means to determine statistical differences between experimental groups. Post hoc comparisons were performed by Tukey's test (GraphPad Prism software 5.0). Differences between mean values were considered significant when $p < 0.05$.

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