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Taurine reduces caspase-8 and caspase-9 expression induced by ischemia in the mouse hypothalamic nuclei

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Summary. Taurine is a sulphur-containing amino acid abundant in the nervous system. It protects cells from ischemia-induced apoptosis, but the mechanism underlying this is not well established. The aim of our study was to explore the effects of taurine on two main pathways of apoptosis induced by ischemia: receptor-mediated and mitochondrial cell death. Brain slices containing the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus were incubated in vitro under control and simulated ischemic (oxygen-glucose deprivation for 30 min) conditions in the absence and presence of 20 mM taurine. Brain slices were harvested after the 180-min "postischemic" period and fixed in 4% paraformaldehyde. To estimate apoptosis, immunostaining was done for caspase-8 and caspase-9 in paraffin-embedded sections. Immunoreactive caspase-8 and caspase-9 cells were observed in SON and PVN in all experimental groups, but in the "ischemic" group the expression of caspase-8 and caspase-9 and the number of immunoreactive cells was significantly increased in both hypothalamic nuclei. Addition of taurine (20 mM) to the incubation medium induced a marked decrease in caspase-8 and caspase-9 immunoreactivity after ischemia in SON and PVN when compared with the taurine-untreated "ischemic" group. Taurine reduces ischemia-induced caspase-8 and caspase-9 expression, the key inductors of apoptosis in SON and PVN.

Keywords: Taurine – Ischemia – Caspase-8 – Caspase-9 – Hypothalamus – Brain slices – Mice

Abbreviations: ACSF, Artificial cerebral spinal fluid; DAB, diaminobenzidine; GL, grey level; IR, immunoreactive; PBS, sodium phosphate buffer; PVN, paraventricular nucleus; SON, supraoptic nucleus

Introduction

Ischemic injury causes severe neurodegeneration and consequently a loss of normal brain functions (Schwartz et al., 1998; Block, 1999; Lipton, 1999). The mechanisms underlying neuronal degeneration and the potential neuroprotective effects of some pharmacological treatments have been actively studied. In different animal models cells dying after ischemic injury exhibit both necrotic and apoptotic features (Lipton, 1999; Liou et al., 2003). In the ischemic core, cell death is due to necrosis (Garcia et al., 1995) and it is difficult, even impossible, to preserve the necrotic cells. In the penumbra, however, dying cells display morphological manifestations of apoptosis (Linnik et al., 1993; MacManus et al., 1993) and with the right treatment they may be saved (Goto et al., 1990; Linnik et al., 1995). Caspases are a family of aspartate-specific cysteine proteases involved in various pathways of programmed cell death and play a crucial role as initiators or effectors of apoptosis (Earnshaw et al., 1999; Budihardjo et al., 1999). Evidence is mounting to indicate a major role of caspases in ischemia-mediated cell death (Thornberry and Lazebnik, 1998; Love, 2003). Caspases begin to be expressed at high levels and become activated under post-ischemic conditions. For this reason, genetic knockout or pharmacological block of caspases seems to be effective in reducing cell damage in both in vivo and in vitro models of ischemia (Le et al., 2002).

There are two main pathways of caspase activation: death receptor-mediated and mitochondrial. Death

receptor-mediated apoptosis is initiated by activation of caspase-8 from cell surface receptors. Caspase-8 subsequently causes direct activation of caspase-3 (Scaffidi et al., 1998) or/and activates downstream caspases, which destroy cells (Budihardjo et al., 1999). The mitochondrial pathway of programmed cell death involves the release of cytochrome C, procaspase-9, and Apaf-1 from the mitochondrial intermembrane space and a series of subsequent biochemical interactions, including the activation of caspase-9 and thereafter activation of caspase-3 (Earnshaw et al., 1999; Budihardjo et al., 1999). Both apoptotic pathways are involved in brain ischemia (Velier et al., 1999; Love, 2003).

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid abundant in the nervous system. It plays an important role in the modulation of neurotransmitter release, calcium homeostasis, osmoregulation and neuroprotection (Oja and Saransaari, 1992; Birdsall, 1998; Saransaari and Oja, 2000). Taurine has been reported to protect the myocardium (Milei et al., 1992) and cardiomyocytes (Takahashi et al., 2003) from ischemia-induced damage, but the mechanism of this protection remains unclear. We now investigated how taurine affects the expression of caspase-8 and caspase-9 in the supraoptic (SON) and paravetricular (PVN) nuclei, chosen as a model of ischemic neurodegeneration because they have high levels of taurine (Palkovits et al., 1986), which may underlie the resistance of these neurons to damage.

Materials and methods

Preparation and incubation of slices

Young adult (3-month-old) male NMRI mice were decapitated and their brains rapidly excised and placed in ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebral spinal fluid (ACSF) containing (in mM) 126 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, 2.4 $CaCl_2$, 10 D-glucose, and 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (Hepes), pH 7.4. Slices containing SON and PVN (400–500 µm thick) were manually prepared and preincubated (two slices in each chamber) in 30 ml ACSF bubbled with 95% O_2 and 5% CO_2 in a shaking water bath at 37 °C for 30 min. After the preincubation period, the slices were divided into 3 experimental groups. Those in the first (“control”) group were incubated in the above-mentioned conditions until the end of experiments. The slices in the second (“ischemic”) group were incubated for 30 min in ACSF without glucose and bubbled with N_2 to mimic ischemic conditions. The third group (“ischemia + taurine”) were incubated for 30 min in ACSF with taurine (20 mM) without glucose and bubbled with N_2. We used this high taurine concentration because it has been shown that 20 mM of taurine most effectively protects tissue against apoptosis and cell loss (Eppler and Dawson, 2002; Takahashi et al., 2003; Takatani et al., 2004).

After the 30-min “ischemic” period, the medium was replaced every 30 min with fresh ACSF bubbled with 95% O_2 and 5% CO_2 to simulate a “reperfusion” period. In the “ischemia + taurine” group taurine was present during all periods of incubation. Slices were removed at 180 min after the end of the “ischemic” period and fixed in 4% paraformaldehyde in sodium phosphate buffer (PBS) (0.1 M, pH 7.4) overnight at 4 °C.

Conventional histology

Paraformaldehyde-fixed slices were embedded in paraffin and cut with a microtome into 5 µm-thick sections. The sections were mounted on glycerin-albumin-coated slides and dried overnight at 37 °C. Hematoxylin/eosin staining was used to demonstrate the general histoarchitecture of the slices after incubation in vitro. Deparaffinized sections were hydrated over the xylene and ethanol series to distilled water and stained with hematoxylin, washed, stained with eosin, washed again and dehydrated using ethanol and xylene for embedding.

Immunohistochemistry

The sections were deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. After antigen retrieval by microwave [20 min at 1000 W in 10 mM citric acid buffer (pH 6.0)], washing in PBS and blocking with 0.5% hydrogen peroxide in PBS for 20 min, specimens were preincubated for 30 min in serum-blocking solution (1% bovine serum albumin and 0.3% Triton X-100 in PBS), thereafter specimens were incubated with polyclonal caspase-8 (Caspase-8 (FLICE) Ab-4, Lab Vision Corp., diluted 1:200 in serum-blocking solution) or polyclonal caspase-9 antibody (sc-8355, Santa Cruz Biotechnology Inc., diluted 1:200 in serum-blocking solution) in moist chambers overnight at 4 °C. After incubation with primary antibody, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit 1:500 in blocking solution) and ABC complex (Vectastain Elite ABC Kit, Vector Laboratories, Inc.) each for 30 min. Diaminobenzidine (DAB) was used as a chromogen to visualize the sites expressing caspase-8 and caspase-9 immunoreactivity. The control sections were incubated without the primary antibodies to rule out nonspecific staining. Finally, the sections (without additional counter-staining) were dehydrated and mounted.

Semi-quantitative analysis of caspases

The sections were processed under standardized conditions in every experiment, which allowed semi-quantitative analysis of the protein amount in the histological slices (Smolen, 1990). An image analysis system comprising IBM PC, Nikon Microphot-FXA microscope, SensiCam digital camera (PCO Computer Optics GmbH), Image-Pro Plus (Media Cybernetics) program was used for a semi-quantitative analysis of caspase-8 and caspase-9 expression in the histological sections of SON and PVN of the hypothalamus. Five sections cut at the same level of each hypothalamic nucleus from every animal were analyzed. The sections were reviewed at 250-fold magnification under light microscope. Optical density was evaluated by two parameters reflecting the expression level of this protein in the selected hypothalamic nuclei. As the first parameter, the number of caspase-immunoreactive (IR) cells was calculated in every slice and the average number of caspase-8-IR or caspase-9-IR cells per slice of hypothalamic nucleus counted. As the second parameter, the relative optical density of DAB precipitates in the perikaryons of individual cells was estimated in every section and the average optical density with its SEM calculated. Optical density was analyzed by the software as a “grey level” (GL). Optical density reflecting the content of the proteins studied in neurons was calculated as the GL of an immunoreactive field of cell by subtracting the background GL. The optical density of the background was estimated in the same slice in the field of non-immunoreactive brain tissue.

Statistical analysis

Statistical significance was determined by ANOVA and Student’s *t*-test. Each value was expressed as mean ± SEM. Differences were considered significant when the calculated p value was <0.05.

Results

Hematoxylin-eosin stained paraffin sections displayed normal parenchymal architecture and cell morphology of SON and PVN. In no experimental group were necrosis and apoptotic bodies morphologically discernible.

Effect of ischemia on the caspase-8 and caspase-9 expression in SON and PVN

Immunoreactive (IR) caspase-8 and caspase-9 cells were observed in SON and PVN in all experimental groups, but in the "ischemic" group their numbers were significantly increased ($p<0.05$) in both hypothalamic nuclei (Fig. 1A, B). Moreover, ischemia led to upregulation of caspase-8 expression in individual neurons of both SON and PVN (Fig. 2A, B). All these changes indicate that ischemia led to the initiation of two main apoptotic pathways in SON and PVN of the hypothalamus.

Effect of taurine on caspase-8 and caspase-9 expression induced by ischemia

After incubation with taurine, the number of caspase-8-IR cells in PVN was significantly decreased ($p<0.05$) when compared to PVN cells in the ischemic group (Fig. 1B).

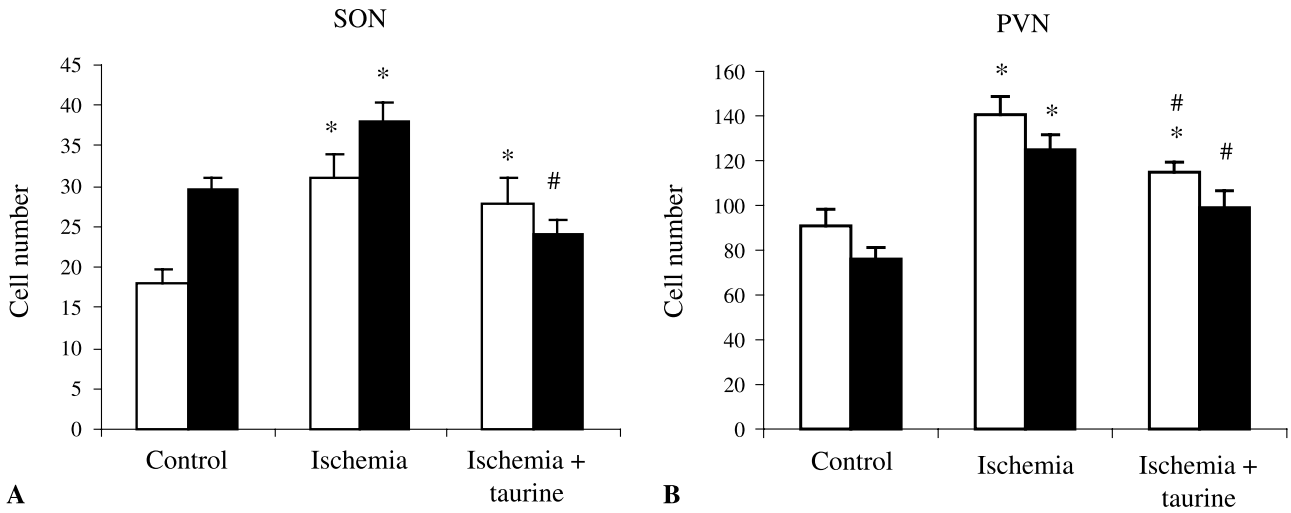

Fig. 1. Effect of taurine on the number of caspase-8- and caspase-9-immunoreactive cells in the supraoptic (SON) (**A**) and paraventricular (PVN) (**B**) nuclei after ischemia. Open bars show caspase-8 and filled bars caspase-9. Data are mean values ± SEM. $^{*}p<0.05$ compared with the control group, $^{\#}p<0.05$ compared with the "ischemia" group

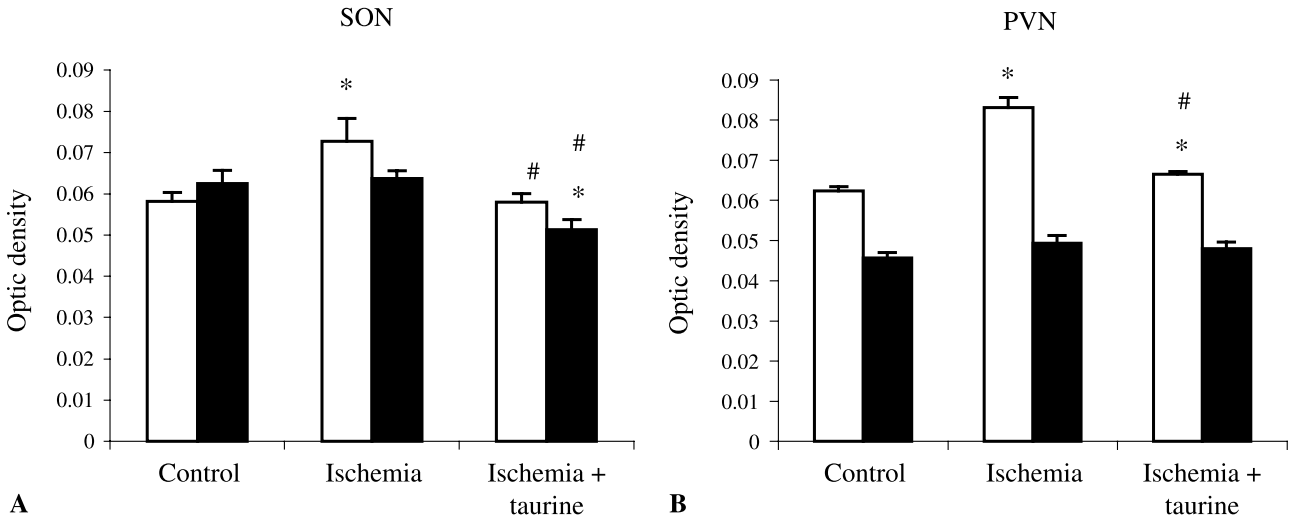

Fig. 2. Effect of taurine on the expression of caspase-8 and caspase-9 in neurons of the supraoptic (SON) (**A**) and paraventricular (PVN) (**B**) nuclei after ischemia. Open bars indicate caspase-8 and filled bars caspase-9. Data are mean values ± SEM (bars). $^{*}p<0.05$ compared with the control group, $^{\#}p<0.05$ compared with the "ischemia" group

Taurine also reduced caspase-8 immunoreactivity in neurons of SON and PVN under ischemic conditions (Fig. 2A, B). The presence of taurine likewise affected the expression of caspase-9. It reduced the number of caspase-9-IR cells after ischemia in SON and PVN (Fig. 1A, B), and the expression of caspase-9 in neurons of SON (Fig. 2A) in comparison with the taurine-untreated "ischemic" group.

Discussion

It has been demonstrated that oxygen-glucose deprivation-exposed slices is a useful in vitro model to study many physiological processes (Joshi and Andrew, 2001; Saransaari and Oja, 2002, 2004, 2005) and in particular apoptosis during ischemic brain injury in adult mice (Gottron et al., 1997; Martin-Villalba et al., 2001; Plesnila et al., 2001; Le et al., 2002). Caspase-mediated apoptosis can be induced through two major upstream pathways. The first is via the release of cytocrome C from the mitochondria into the cytoplasm, where it consequently binds to procaspase-9 and Apaf-1, forming an apoptosome. This cleaves caspase-9 which further cleaves caspase-3 (Ferrer and Planas, 2003). The second pathway is via the binding of the Fas to the Fas-associated death domain, which activates procaspase-8. Procaspase-8 cleaves caspase-8 which activates caspase-3 (Boatright and Salvesen, 2003). To determine which pathway of apoptosis induction is utilized by ischemia in SON and PVN of the hypothalamus, we measured caspase-8 and caspase-9 expression. The results showed that caspase-8 and caspase-9 expressions significantly increased in neurons of both hypothalamic nuclei. Our data thus suggest that ischemia induces apoptosis in SON and PVN through both caspase-8 and caspase-9 pathways.

Excessive accumulation of Ca^{2+} occurs during ischemia-mediated mitochondrial injury (Mattson et al., 2000; Fiskum, 2004). Reactive oxygen species generated during ischemia damage the mitochondrial membrane (Mattson et al., 2000; Fiskum, 2004; Warner et al., 2004) lead to release of apoptotic proteins which activate apoptosis through the caspase-9-dependent pathway. Taurine can modulate the intracellular calcium homeostasis by reducing the intracellular calcium level in neurons (Foos and Wu, 2002) and in this manner promote its neuroprotective action. Furthermore, taurine acts as an antioxidant and preserves cells from apoptosis induced by reactive oxygen species (Biasetti and Dawson, 2002; Eppler and Dawson, 2002).

In the first demonstration of the molecular mechanisms of the antiapoptotic effects of taurine it suppressed the formation of the Apaf-1/caspase-9 apoptosome in cardiomyocytes and thereby prevented caspase-9 activation and apoptosis (Takatani et al., 2004). In our experiments taurine reduced caspase-9 expression in neurons of SON and PVN, which is in good accord with the results of Takatani et al. (2004).

There are no previous data on the effects of taurine or taurine derivatives on caspase-8 and caspase-8-dependent apoptosis in neurons. Experiments carried out on a primary culture of rat hepatocytes demonstrated that taurine derivatives exert controversial effects on caspase-8 and apoptosis. For example, tauroursodeoxycholic acid reduced the caspase-8 activity induced by glycochenodeoxycholic acid to one half (Schoemaker et al., 2004). Taurocholate also evinced a protective effect on vagotomy-induced cholangiocyte apoptosis, which was associated with the downregulation of caspase-3, -8, and -9 (Marzioni et al., 2003). On the other hand, taurolithocholate 3-sulfate has induced caspase-8 activation and led to apoptosis in a primary culture of rat hepatocytes (Graf et al., 2002, 2003). In our experiments, taurine in the incubation medium reduced caspase-8 expression induced by ischemia in neurons of SON and PVN in the hypothalamus. Taurine can thus protect these hypothalamic neurons from caspase-8-dependent apoptosis.

We have now shown that taurine can reduce the expression of caspase-8 and caspase-9 induced by ischemia. What are the possible mechanisms? Caspase-8 resides in the cytosol as an inactive 53-kDa precursor and like all other caspases is activated by proteolytic processes at what constitute caspase consensus cleavage sites. When activated, procaspase-8 zymogen is cleaved into large (~43-kDa) and small (~12-kDa) polypeptides, which assemble to form the heterotetrameric structure shared by all known active caspases (Earnshaw et al., 1999; Kruidering and Evan, 2000). In our work we used the caspase-8 antibody, which detects the inactive 53-kDa precursor and the processed forms (43-kDa, 12-kDa) of caspase-8. The increasing expression of caspase-8 induced by ischemia might thus indicate an increased level of precursor procaspase-8 expression as well as caspase-8 activation. Hence, the reduction of caspase-8 expression by taurine would indicate that taurine can influence the gene expression of caspase-8 or can suppress its activation. The data presented by Park and his associates (Park et al., 2006a, b) corroborate the gene-regulation hypothesis whereby taurine affects gene expression and downregulates the genes implicated in signal transduction, cell proliferation and apoptosis. Theirs are the first studies on

the effects of taurine on gene expression but the detailed mechanisms of these effects are still unknown.

Recently, Matsumori et al. (2006) have shown that under ischemic conditions overexpression of heat shock protein 70 increases the expression of cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (FLIP) and decreases caspase-8 cleavage. Taurine enhances the expression of heat shock protein 70 (Kurz et al., 1998) and in such a manner may prevent the activation of caspase-8, though this assumption is in need of confirmation.

Caspase-9 is synthesized as a 50-kDa precursor protein. Like caspase-8 and other caspases, it consists of three domains: an N-terminal pro-domain, a large subunit (∼37-kDa) and a small subunit (∼12-kDa). Procaspase-9 is converted to the active form of caspase-9 by cleavage at specific sites between the different subunits. The active caspase-9 enzyme is a heterotetramer containing two small and two large subunits (Earnshaw et al., 1999). The caspase-9 antibody used in our experiments reacts with the large subunit (∼37-kDa) and the precursor of caspase-9. We cannot therefore recognize which kind of caspase-9, active form or zymogen, was detected. In any case, the increased caspase-9 level in the cells indicates that its synthesis is enhanced. Consequently, the reduction of the caspase-9 expression in ischemic neurons by taurine suggests that it affects caspase-9 synthesis via down-regulation of the corresponding genes (Park et al., 2006a, b).

Only relatively high concentrations of taurine have been found to be effective when other parameters have been studied with various brain preparations (del Olmo et al., 2000; Messina and Dawson, 2000; El Idrissi et al., 2003). The extracellular concentrations of taurine in the brain in vivo are many times higher those of, for instance, amino acid neurotransmitters (Lerma et al., 1986; Miele et al., 1996; Molchanova et al., 2004a). Ischemia itself evokes an approximately 30-fold increase in extracellular taurine (Molchanova et al., 2004b). Furthermore, there is unambiguous evidence that 20 mM of taurine is able to protect most effectively against apoptosis and cell loss (Eppler and Dawson, 2002; Takahashi et al., 2003; Takatani et al., 2004). In keeping with the investigations in question, a rather high concentration of taurine had now to be used to elicit significant reductions in caspase expression.

In conclusion, our findings show that taurine can suppress ischemia-induced apoptosis in hypothalamic neurons of SON and PVN by reducing caspase-8 and caspase-9 expression. Taurine participates in the regulation of the death receptor-mediated and mitochondrial pathways of apoptosis, but the exact mechanisms of its effects on caspase-mediated apoptosis are still unclear and subjected to further studies.

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