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Antioxidant compounds EGB-761 and BN-520 21 attenuate heat shock protein (HSP 72 kD) response, edema and cell changes following hyperthermic brain injury

An experimental study using immunohistochemistry in the rat

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Summary. Influence of the extract of Gingko biloba (EGB-761) and one of its constituent Gingkolide B (BN-52021) on hyperthermia induced cellular damage and heat shock protein (HSP 72 kD) response was examined in a rat model. Rats subjected to 4 h heat stress at 38°C in a biological oxygen demand (BOD) incubator (relative humidity 50–55%, wind velocity 20–25 cm/sec) resulted in profound edema and cell injury in many parts of the cerebral cortex, hippocampus, cerebellum, thalamus, hypothalamus and brain stem. Immunostaining of HSP 72 kD showed marked upregulation in the damaged and distorted neurons located within the edematous area. Pretreatment with EGB-761 (50 mg/kg/day, p.o.) and BN-520 21 (2 mg/kg, p.o.) per day for 5 days significantly reduced HSP expression and attenuated cell damage. Our results show that EGB-761 and its component Gingkolide B (BN-52021) has the capacity to reduce edema and cell injury following hyperthermia and this effect of the compound is somehow associated with a reduction in cellular stress response as evidenced with a reduction in HSP expression.

Keywords: Amino acids – Hyperthermia – Heat stress – Heat shock protein (HSP 72 kD) – Edema – Cell injury – Antioxidants – EGB-761-BN 52021

Introduction

Heat stress and associated hyperthermia induces profound cellular stress which seems to be primarily responsible for leakage of the blood-brain barrier (BBB) permeability, brain edema formation and cell injury in many parts of the brain (Sharma, 1982; Sharma and Dey, 1987; Sharma and Cervós-Navarro,

1990; Sharma et al., 1997a,b, 1998a; Sharma, 1999). However, the detailed information regarding hyperthermia induced cellular stress is not well known (Craig, 1985; Welch, 1992; Marcuccilli and Miller, 1994). Previously, increased expression of heat shock protein (HSP 72 kD) was first seen in cell cultures which were accidentally heated from 37°C to 45°C (see Craig, 1985; Welch, 1992). This abnormal expression of proteins following heat stress was termed as "heat shock proteins (HSP)" which is also sometimes referred to as "stress proteins". Since upregulation of this protein is now seen following ischemia, trauma, hypoxia, immobilization stress, chemical neurotoxicity or even hypothermia, many workers suggested to use the term "stress proteins" instead of "HSP" (Marcuccilli and Miller, 1994; Westman and Sharma, 1998). However, recent discovery of several other proteins like "ubiquitin" which are also expressed following stress, the term stress proteins does not refer to HSP only (Kato et al., 1993). Therefore, the term "heat shock proteins (HSP)" is still widely used by most workers.

The functional significance of upregulation of HSP is not known in all details. It seems quite likely that upregulation of HSP will result due to an increased threat to the cell survival under diverse conditions and depending on this kind of stimulus, the cells will start producing abnormal amount of the protein (Riabowol et al., 1988; Manzerra et al., 1993). However, induction of this protein is beneficial to the cell; or it simply represents the cellular stress or injury is still controversial.

Injection of HSP antibodies into the oocyte and subjection of cells to the light stress decreases the cell survival indicating a beneficial effect of these proteins for cell survival (Brown et al., 1989; Gonzalez et al., 1989; Li et al., 1992; Marcuccilli and Miller, 1994). This evidence is also supported in cells which were pretreated with mild heat stimulus to initiate HSP induction and when these cell were subjected to irradiation induced damage, no significant damage of these cells can be seen (see Westman and Sharma, 1998). However, these observations require further interpretation and detailed analysis. It seems possible that pretreating with mild heat has increased the cell adaptability and therefore, increased the threshold of the cell damage.

The probable mechanisms of brain pathology following hyperthermia is not well known. Our previous works demonstrate marked upregulation of HSP 72 kD response in several brain regions following hyperthermia which is significantly attenuated by prior treatment with neuroprotective drugs (Sharma et al., 1992; Westman and Sharma, 1998; Sharma, 1999). These observations suggest that HSP seems to be an indicator of cellular stress and could be used as a marker of cell injury.

Since hyperthermia is associated with oxidative stress, and antioxidant compounds are well known neuroprotective agents in this model (Sharma et al., 1997c, 1998b), a possibility exists that pretreatment with antioxidant compounds will also influence HSP expression. Thus, in order to further characterise the role of HSP in heat stress as a marker of cell injury or cellular stress, the present investigation was undertaken to find out if upregulation of HSP following hyperthermia is attenuated by pre-treatment with two potent

antioxidant compounds EGB-761 and BN-520 21 (DeFeudis, 1998). In addition, the neuroprotective effects of these compounds were investigated on edema formation and cell injury as well.

Materials and methods

Animals

Experiments were carried out on 30 male Sprague Dawley rats (body weight 90–110 g, age 8–9 weeks) housed at controlled room temperature (21 ± 1°C) with free access to food and tap water before experiments.

Heat exposure

Rats (8–9 weeks) were exposed to 4 h heat stress at 38°C in a biological oxygen demand (BOD) incubator (relative humidity 50–55%, wind velocity 20–25 cm/sec) (Sharma, 1982; Sharma and Dey, 1987). This experimental condition is approved by the Ethical Committee of Uppsala University, Uppsala, Sweden; Lund University, Lund, Sweden; and Banaras Hindu University, Varanasi, India. Rats kept at room temperature were served as controls.

Pharmacological treatments

We have used two potent antioxidant substances EGB-761 and BN-520 21. EGB-761 is a standardised extract from Ginkgo biloba leaves. It contains falvonoids (24% of flavonol hetrosides and about 7% proanthocyanidins), and 6% terpene trilactones (Ginkgolides A, B, C and bilobalide) (DeFeudis, 1998). In separate group of rats, EGB-761 (50 mg/kg, Beufour-IPSEN, France) or Ginkgolide B (BN-520 21) (2 mg/kg, Beufour-IPSEN, France) were administered per os in distilled water (0.3 ml/rat). This treatment was given daily for 5 days. One dose of the drugs was also administered 30 min before the onset of heat stress experiments (Janssens et al., 1995). Separate group of EGB-761 or BN-520 21 treated rats were used as drug treated controls and were not subjected to heat stress.

Perfusion and fixation

Immediately after heat stress, animals were anaesthetised with Equithesin (0.3 ml/100 g, i.p.) and the chest was rapidly opened. The right auricle was cut and a 21 gauge butterfly needle was inserted into the left ventricle of the heart which was connected to the perfusion apparatus. About 50 ml of phosphate buffer saline (0.1 M, pH 7.0) was perfused to washout the remaining blood in the blood vessels. This was followed by perfusion of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer saline (Sharma et al., 1995; Sharma and Westman, 1997). The perfusion pressure was effectively maintained at 90 torr throughout the process. After perfusion, the animals were wrapped in an aluminium foil and kept overnight in a refrigerator at 4°C. On the next day, brain and spinal cord tissues were dissected out and kept into the same fixative at 4°C for one week.

HSP immunohistochemistry

The HSP immunoreactivity was examined using monoclonal antibodies directed against HSP 72 kD (Sharma et al., 1992; 1995; Sharma and Westman, 1997; Westman and Sharma,

1998) (StressGene, Canada) in the several brain regions in the control, heat stressed rats and EGB-761 or BN-520 21 treated normal or heat stressed rats in parallel.

Immunohistochemistry of inducible isoforms of HSP was examined on 40μm thick vibratome sections obtained from the desired regions of the brain or spinal cord using standard procedures (Sharma et al., 1995). In brief, the antibodies of HSP were diluted 1:5000 and applied on free floating vibratome section for 48 h with continuous shaking at room temperature (Sharma and Westman, 1997). The immune reaction was developed using peroxidase-antiperoxidase technique. HSP immunoreactivity was examined on vibratome sections obtained from control, heat stress, EGB-761 or BN-520 21 treated normal and heat stressed rats in parallel. In few sections, the primary antibody step was omitted and the reaction product was developed as usual. The number of HSP positive immunostained cell in each group were counted in a blind fashion in all the animals (Westman and Sharma, 1998).

Cell injury

Light and electron microscopy was used to evaluate cell injury in the brain according to the standard protocol (Sharma et al., 1997a; 1998a).

Statistical analysis

Student's unpaired t-test was used to determine the statistical significance of the data obtained. A p-value less than 0.05 was considered to be significant.

Results

Effect of EGB-761 and Ginkgolide B on HSP immunoreactivity

Our results showed that rats exposed to heat stress at 38°C for 4 h exhibited a selective and specific upregulation of HSP 72 kD expression in the cerebral cortex, thalamus, hypothalamus, and spinal cord (Fig. 1). Normal rats did not show HSP expression but few HSP positive cells were seen in some areas in the brain. A representative example of HSP expression in the cerebral cortex in heat stress is shown in Fig. 2.

Pretreatment with EGB-761 (50 mg/kg/day, p.o. for 5 days) or BN-520 21 (2 mg/kg/day, p.o. for 5 days) significantly attenuated the HSP expression in the brain following heat exposure (Fig. 2). The effect of EGB-761 on HSP expression was far more superior than BN-520 21 pretreatment. However, pretreatment with EGB-761 or BN-520 21 alone did not influence HSP activity in normal rats (results not shown).

Effect of EGB-761 and BN-520 21 on cell injury

Pretreatment of rats with EGB-761 or BN 520 21 significantly attenuated brain edema formation and cell injury. Thus the neuropil appears more compact in EGB-761 treated animals and the signs of membrane damage, distorted nerve cells, glial cells and myelin vesiculation were considerably reduced (Fig. 3). Pretreatment with BN 520 21 also significantly attenuated

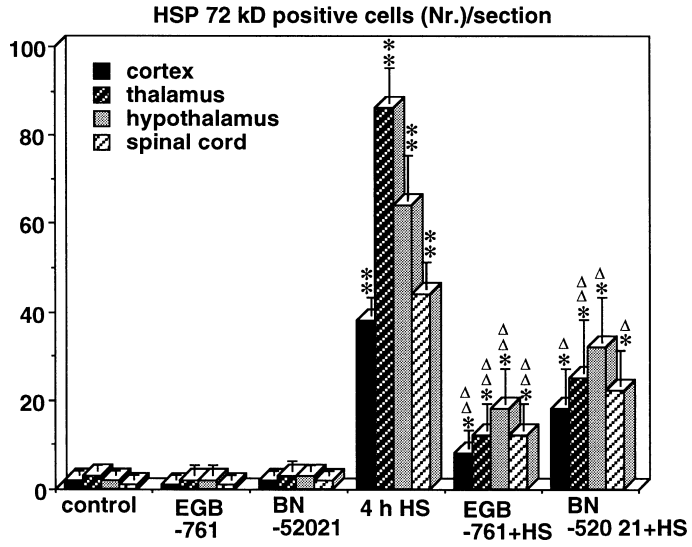

Fig. 1. Semiquantitative analysis of heat shock protein (HSP 72 kD) immunoreactivity in control or 4 h heat stressed (38°C) rats and its modification with EGB-761 or BN 520 21 pretreatment. * = $P < 0.05$; ** = $P < 0.01$, compared from control, △ = $P < 0.05$, △△ = $P < 0.01$, compared from 4 h HS, Student's unpaired t-test

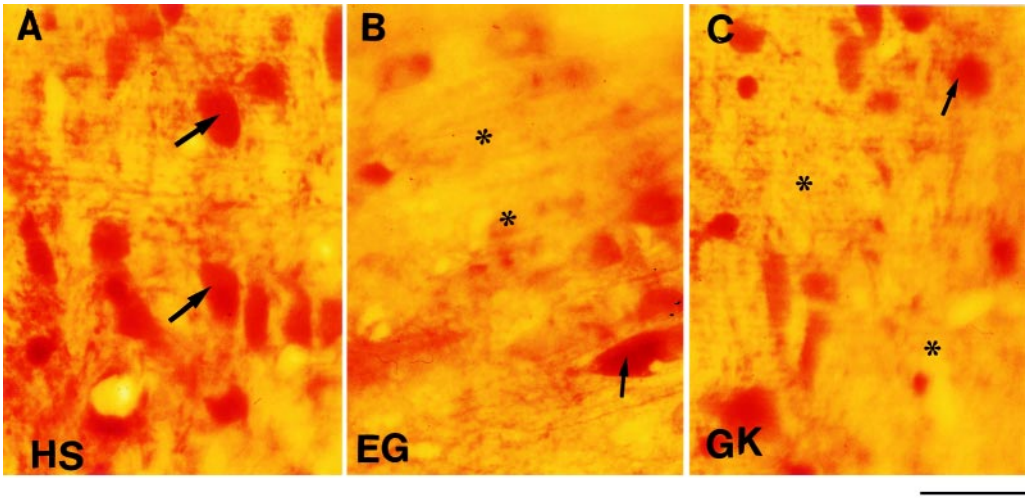

Fig. 2. A representative example of HSP 72 kD immunoreactivity (arrows) in the parietal cerebral cortex of a 4 h heat stressed (HS) rat (**A**) and its modification with EGB-761 (EG) (**B**) or Gingkolide B (GK) (BN 520 21, **C**) pretreatment. The number of HSP immunolabelled nerve cells are considerably reduced in the drug treated rats (bar = 50 μm)

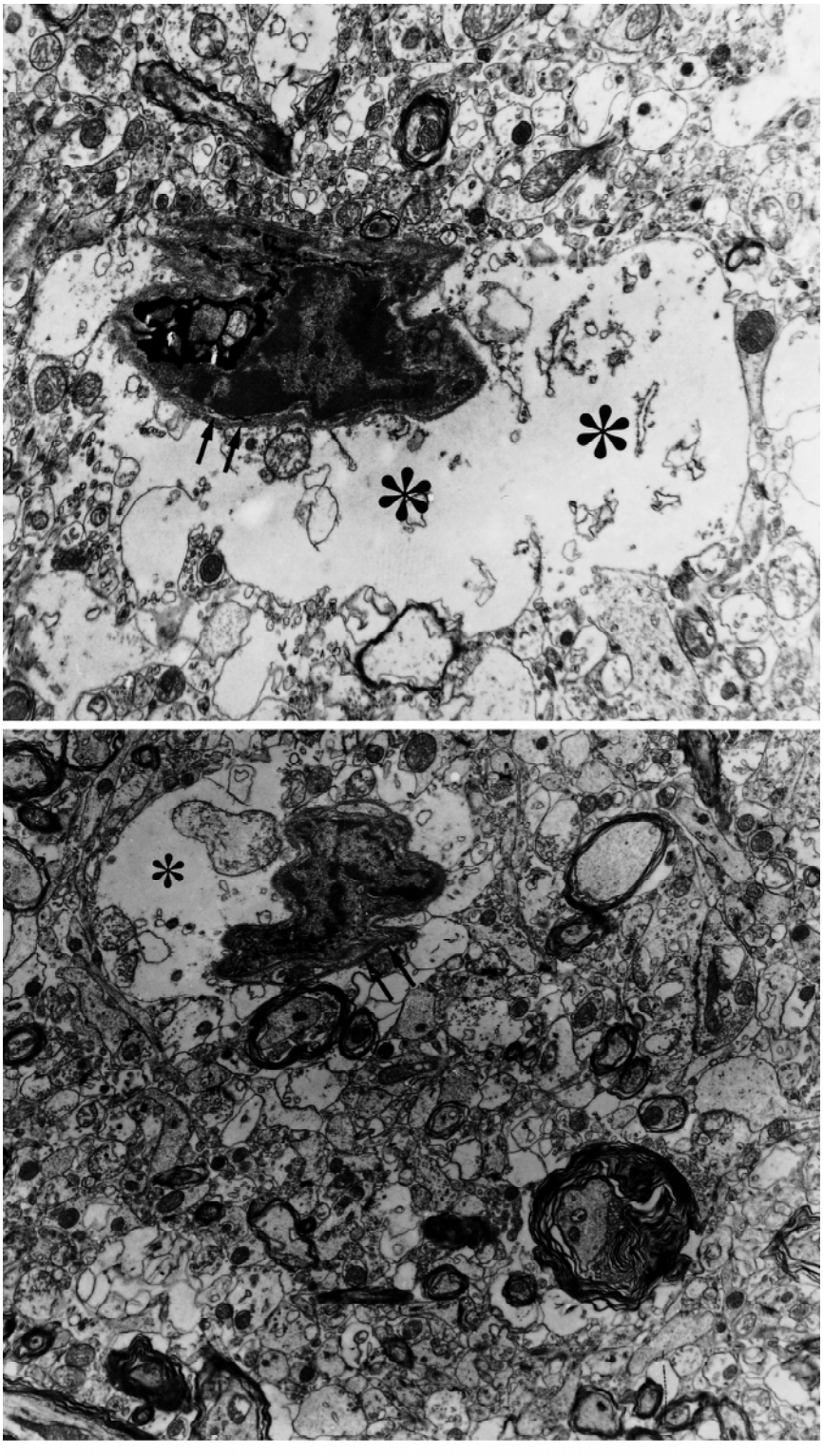

Fig. 3. Low power electron micrograph from the parietal cerebral cortex of one heat stressed rats (upper panel) and its modification with EGB-761 pretreatment (lower panel). In untreated rat, perivascular edema (arrows), membrane damage (*) and myelin vesiculation are quite prominent compared to the EGB-761 treated rat (bar = 1 μm)

cell injury (results not shown), however, the beneficial effects of EGB-761 is far superior on cell injury than BN 520 21 pretreatment.

Discussion

The present results suggest that heat stress has the capacity to induce HSP upregulation in several brain regions. This would indicate that HSP upregulation in heat stress is mainly associated with hyperthermia induced cellular stress or cell injury. The HSP expression can be upregulated in the CNS following ischemia, trauma, hypoxia or following several other kinds of emotional, chemical or metabolic stress (Sloviter and Lowenstein, 1992; Linsberg et al., 1996). It seems quite likely that emotional stress trigger a variety of chemical reaction within the CNS and in the periphery which may directly or indirectly influence HSP expression (Sharma, 1999; Sharma and Westman, 1999).

The molecular mechanisms behind expression of HSP in several stressful experimental or clinical conditions are not well known. There are reports that ACTH released following stress in the circulation will induce an upregulation of HSP expression (Marcuccilli and Miller, 1994). The other potential source of HSP expression are ischemic, oxidative and/or metabolic stress (Brown et al., 1989; Gonzalez et al., 1989; Rordorf et al., 1991; Kato et al., 1994). Previous reports from our laboratory suggest that neurochemicals involved in secondary injury cascade also influence upregulation of HSP in the CNS following spinal cord trauma (Sharma et al., 1995; Sharma and Westman, 1997). Thus drugs modifying serotonin, prostaglandin and opioids metabolism attenuate upregulation of HSP expression in trauma (Sharma et al., 1995; Westman and Sharma, 1998; Sharma and Westman, 1999). This indicate that release of neurochemicals following trauma will influence HSP expression.

Recently, oxidative stress occurring following trauma, ischemia or hypoxia is known to play an important role in cellular damage in the CNS and profound oxidative stress is associated with hyperthermia induces brain damage (Sharma et al., 1997c; Sharma, 1999). Thus hyperthermia exceeding 41°C for few hours will directly damage membranes and kill several cellular enzymes (Sharma et al., 1998a). In addition to a direct cell damage, hyperthermic stress will induce release of several secondary injury factors such as, neurochemicals, ions, enzymes which will altogether influence the pathophysiology of brain damage (Sharma et al., 1997a–c, 1998a,b; Sharma, 1999; Sharma and Westman, 1998, 1999). Release of prostaglandins, leukotrienes and other related neurochemicals lead to free radical generation and membrane damage (see Sharma and Westman, 1998). Increased lipid peroxidation and production of free oxygen radicals seems to be the key elements in inducing cell damage, edema formation and membrane disruption (see Sharma et al., 1998a).

This idea is in line with our morphological observations in heat stress. Thus, ultrastructural studies revealed widespread cell damage, disruption of

cell membranes, distortion or complete disintegration of nerve cell and cell nucleus (Sharma et al., 1997a, 1998a). Since these regions are associated with HSP immunoreaction, it seems quite likely that generation of free radicals and lipid peroxidation induce cellular or molecular signals responsible for abnormal production of HSP.

In addition, spread of edema fluid, local circulatory disturbances and/or breakdown of the BBB permeability could also contribute to an abnormal production of HSP expression (Sharma et al., 1995). Breakdown of the BBB seems to be instrumental in eliciting HSP induction in the brain (Sharma and Westman, 1997). This is well supported with the findings that the regions exhibiting BBB breakdown are associated with HSP expression (Westman and Sharma, 1998). A breakdown of the BBB is mainly responsible for edema formation (Sharma et al., 1997a, 1998a). This breakdown of the BBB however, may as well the results of a direct membrane disruption of the endothelial ells or indirectly due to the effect of several neurochemicals involving several signal transduction mechanisms at the molecular level (Sharma, 1999).

The idea that oxidative stress participate in HSP expression is clearly supported by the results obtained with drug-treatments. Thus pretreatment with EGB-761 significantly attenuated HSP expression in the CNS of heat stress. EGB-761 is a rather complex substance extracted from the dry green leaves of Gingko biloba (Boveris and Puntarulo, 1998; DeFeudis, 1998). This extract has been suggested to posses powerful anti-oxidant activity (Amri et al., 1996; Pietri et al., 1997; Kim et al., 1998). The detailed mechanisms of EGB-761 induced antioxidant effects are not clearly known. However, it is well known that flavonoids posses radical scavangering activity and Gingkolides have been shown to inhibit the formation of free radicals and also inhibit lipid peroxidation (Pietri et al., 1997; DeFeudis, 1998).

Inhibition of lipid peroxidation in heat stress seems to be one of the important factors in attenuating HSP reaction following hyperthermia. This is evident with the fact that pretreatment with EGB-761 is also significantly attenuated brain damage (Shen et al., 1998; Spinnewyn, 1992). The neuroprotective effects of EGB-761 in heat stress was obviously related with its antioxidant property. Previous studies using EGB-761 suggest that the compound is well known neuroprotective agent in other models of ischemia and trauma (see DeFeudis, 1998). EGB-761 is also neuroprotective in retinal cells following damage induced by light as well as following several neurochemical toxins. It seems thus clear that EGB-761 is neuroprotective and that this effect is mainly mediated via its antioxidant activity (for review see DeFeudis, 1998).

Our results for the first time show that EGB-761 has the capacity to inhibit HSP response in the CNS probably by attenuating cellular stress (Amri et al., 1996; Rapin et al., 1994). It seems that cellular stress is one of the most important signal to HSP induction in the CNS (DeFeudis, 1998). Our results clearly suggest that HSP induction is seen following membrane damage and that many factors are contributing to its induction. This supports the idea that HSP expression may be used as a marker of cell injury.

Our observations in heat stress however, does not contradict the suggestion that induction of HSP is essential for cell survival. Once HSP is induced in the cell, further induction of HSP is not possible with the same insult in a short time because of the desensitization of the protein or stress reaction (Welch, 1992; Marcuccilli and Miller, 1994; Sharma and Westman, 1999). Repeated similar insults will enhance the threshold for cell damage as well (Manzerra et al., 1993; Westman and Sharma, 1998). Thus, it seems quite likely that HSP induction is related with the magnitude and threshold of cell survival. An absence of HSP induction does not necessarily mean that the cells are going to die. In the absence of a sufficient cellular or molecular signal, the cell simply cannot respond or upregulate HSP expression.

Inhibition of HSP response in hyperthermia with EGB-761 and BN 520 21 did not aggravate the cell damage. These results clearly support the idea that induction of HSP itself has nothing to do with increased cell survival. The HSP induction may represent cellular stress which seems to be related with cellular integrity. An absence of HSP induction in drug-treated rats simply mean that the compound has effectively prevented cellular stress and/or the secondary injury factors that are responsible for induction of HSP (Chen et al., 1998; Shen et al., 1998). However, further studies are needed to clarify this point.

We also used Ginkgolide B (BN-520 21) to understand the effect of Ginkgolides on heat stress induced neuroprotection and HSP reaction. It is possible that its less efficiency in attenuating HSP response and cell injury is mainly due to a less bioavailability of the compound when given orally. Although, previous reports suggest that this pure compound has marked neuroprotective effects in ischemia, our results suggest that this compound neither effectively induce a major neuroprotection nor was able to completely reduce the HSP expression (DeFeudis, 1998). Taken together, these results for the first time suggest that HSP induction is somehow related to the magnitude and degree of cellular stress leading to cell damage.

In conclusion, our results suggest that HSP expression represents cellular stress which may be instrumental for inducing cell injury. Induction of HSP is absent in hyperthermia by pretreatment with EGB-761 or BN-520 21 which is related with their capacity to limit oxidative stress to achieve neuroprotection. Our results, thus opened a new aspect of HSP expression and function in the CNS, which, however require further investigation. Studies are in progress to find out whether HSP expression is related with an increased synthesis of HSP mRNA or other transcription factors like heat shock factor (HSF), and/or hemeoxygenase-1, to further understand the function of HSP in CNS injury.

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