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Nitric oxide in the pathophysiology of hyperthermic brain injury. Influence of a new anti-oxidant compound H-290/51

A pharmacological study using immunohistochemistry in the rat

P. Alm[1], **H. S. Sharma**[2], **S. Hedlund**[2], **P.-O. Sjöquist**[3], and **J. Westman**[2]

[1] Department of Pathology, University Hospital, University of Lund, Lund, [2] Laboratory of Neuroanatomy, Department of Anatomy, Biomedical Centre, Uppsala University, Uppsala, and [3] Pharmacology CV, Astra Hässle AB, Mölndal, Sweden

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Summary. The possibility that nitric oxide (NO) is involved in the pathophysiology of brain injury caused by heat stress (HS) was examined using neuronal nitric oxide synthase (NOS) immunohistochemistry in a rat model. In addition, to find out a role of oxidative stress in NOS upregulation and cell injury, the effect of a new antioxidant compound H-290/51 (Astra Hässle, Mölndal, Sweden) was examined in this model. Subjection of conscious young rats to 4 h HS in a biological oxygen demand (BOD) incubator at 38°C resulted in a marked upregulation of NOS in many brain regions compared to control rats kept at room temperature (21 ± 1°C). This NOS immunoreactivity was found mainly in distorted neurons located in the edematous regions not normally showing NOS activity. Breakdown of the blood-brain barrier (BBB) permeability, increase in brain water content and marked neuronal, glial and myelin reaction were common findings in several brain regions exhibiting upregulation of NOS activity. Pretreatment with H-290/51 significantly attenuated the upregulation of NOS in rats subjected to HS. In these animals breakdown of the BBB permeability, edema and cell changes were considerably reduced. Our results suggest that hyperthermic brain injury is associated with a marked upregulation of NOS activity in the CNS and this upregulation of NOS and concomitant cell injury can be reduced by prior treatment with an antioxidant compound H 290/51. These observations indicate that oxidative stress seems to be an important endogenous signals for NOS upregulation and cell reaction in hyperthermic brain injury.

Keywords: Heat stress – Nitric oxide – Cell injury – Antioxidant – H-290/51 – Blood-brain barrier – Brain edema

Introduction

Heat stress is a serious clinical problem in many parts of the world. The clinical symptoms include hyperthermia, which if exceeds beyond 41° C can lead to the

development of various neurological symptoms and mental abnormalities (Sterner 1990). The death rate of heat stress victims is often more than 50% (Malamud et al., 1946). Sporadic case reports and post mortem studies of heat stress victims show marked cell changes in several regions of the brain and spinal cord (Malamud et al., 1946). However, the probable mechanisms underlying brain dysfunction following heat stress is not known in all details.

Experiments carried out by Sharma and his group on rats in the past has shown that brain pathology in heat stress appears to be closely related with breakdown of the blood-brain barrier (BBB) permeability to proteins (Sharma, 1982; Sharma et al., 1991, 1992). A breakdown of the BBB can induce leakage of plasma proteins into the brain extracellular compartment and induce vasogenic edema formation (Sharma et al., 1997b). Leakage of various neurochemicals, ions, hormones, immunoactive compounds can induce a wide variety of immunological, chemical and metabolic reactions within the CNS compartment (Bradbury, 1990). Influence of several vasoactive substances on the cerebral microcirculation will lead to disturbances in the cerebral blood flow and may cause ischemia (Sharma and Dey 1986, 1987). All these factors alone, or in combination, may influence adverse cell reaction leading to brain damage.

Recently, nitric oxide (NO) has emerged as one of the new class of gaseous molecules which can influence the neurotransmission and signal transduction mechanisms (Kimura and Steinbusch, 1996). NO is a free radical gas with a very short half life (less than 5 sec) (for review see Dawson and Dawson, 1996). Thus, most studies are focussed on its synthesising enzyme, nitric oxide synthase (NOS) which is found in the normal CNS. Recent reports suggest that NO is involved in neurotoxicity caused by ischemia, infarction, stroke and trauma (Dawson and Dawson 1996). It seems quite likely that, the basic mechanisms of cell injury under a wide variety of experimental or clinical situations appear to be similar in nature. Thus, it appears that NO may participate in the process of brain injury following heat stress as well.

We have initiated a series of investigations to explore the role of NO in hyperthermic brain injury and its involvement in cell changes in the CNS (Sharma et al., 1997a, 1998). In this investigation the constitutive isoform of neuronal NOS expression was examined in the brain and spinal cord of heat stressed rats as described earlier (Sharma et al., 1997a). Furthermore, in order to find out a role of cellular and oxidative stress in pathophysiology of heat stress, the effect a new antioxidant compound H-290/51 (Astra Hässle, Mölndal, Sweden) (Mustafa et al., 1995) on heat stress induced brain damage and NOS expression was also examined.

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 12 h light and 12 h dark schedule. The rat food and tap water were supplied *ad libitum*.

Exposure to heat stress

Rats were exposed to 4 h heat stress in a biological oxygen demand (BOD) incubator (relative humidity 45–50%, wind velocity 20–25 cm/sec) maintained at 38°C, as described by Sharma and Dey (1986). Rats kept at normal room temperature were used as controls. This experimental condition is approved by the Ethical Committee of Banaras Hindu University, Varanasi, India, Uppsala University, Uppsala, Sweden, and Lund University, Lund, Sweden.

Effect of H-290/51 pretreatment

In separate group of rats a new anti-oxidant compound H-290/51 (Astra Hässle, Mölndal, Sweden) was given as pretreatment. This compound is a potent antioxidant and has the capacity to inhibit lipid peroxidation (Svensson et al., 1993; Westerlund et al., 1996). Rats received this compound (50 mg/kg, per oss) 30 min before subjection to heat stress (Mustafa et al., 1995). This dose of the compound effectively inhibits the lipid peroxidation (Sharma et al., 1997a).

Parameters measured

The following parameters were measured routinely in both controls, heat stressed, and drug-treated heat stressed rats.

Stress symptoms and physiological variables

Changes in rectal temperature, occurrence of behavioural salivation and prostration as well as gastric ulceration in the stomach at autopsy were used as indices of stress following heat exposure (Sharma and Dey, 1987). In addition, mean arterial blood pressure, blood gases and arterial pH were recorded as other physiological variables as described earlier (Sharma and Dey, 1986).

Microvascular permeability and the water content

In separate group of rats, brain water content and the microvascular permeability disturbances were determined in several brain and spinal cord regions using standard protocol (Sharma and Cervós-Navarro, 1990).

NOS immunohistochemistry

NOS immunohistochemistry was done on free floating vibratome sections (about 40–60 μm thick) obtained from various regions of the brain and spinal cord according to the standard protocol (Sharma et al., 1997a). In brief, after *in situ* fixation with buffered cold 4% paraformaldehyde in 0.1 M phosphate buffer, the brain and spinal cord segments were dissected out and kept in the same fixative at 4°C. For NOS immunohistochemistry, selected tissue pieces were cut on a Vibratome section and immunolabelled with neuronal NOS antiserum (Alm et al., 1993). This antiserum is specific for the constitutive isoform of neuronal NOS (for details see Sharma et al., 1996, 1998).

Cell changes at light and electron microscopy

Some tissue pieces were embedded in epon for routine light and electron microscopy as described earlier (Sharma and Cervós-Navarro, 1990). In brief, about 1 μm thick sections were cut for high resolution light microscopy. These sections were stained with toluidine blue and examined under a light microscope. The desired area of the section was then selected, and ultrathin sections were cut on a LKB ultramicrotome using a diamond knife

and these sections were collected on a one hole copper grid. These sections were counterstained with uranyl citrate and lead acetate, and examined under a Hitachi Transmission Electron microscope.

Statistical evaluation

The quantitative data were analysed using Student's unpaired t-test. A p-value less than 0.05 was considered to be significant.

Results

Effect of heat stress on stress symptoms and physiological variables

Subjection of conscious young rats to a 4 h heat stress resulted in marked hyperthermia and behavioural symptoms. Measurement of blood pressure showed profound hypotension whereas the blood gases and the arterial pH did not alter significantly (results not shown). Post-mortem examination showed many gastric haemorrhages in the stomach (Sharma et al., 1997a, b).

Effect of heat stress on NOS immunohistochemistry

Subjection of rats to 4 h heat stress resulted in a marked upregulation of NOS in many brain and spinal cord regions compared to control rats. This NOS immunoreactivity appeared mainly in distorted neurons located in edematous regions not normally showing NOS immunolabelling. A representative example of NOS upregulation in the cervical spinal cord segment is shown in Fig. 1. It appears from the figure that neuronal cytoplasm is mostly stained and in some occasions, the cell nucleus also exhibited NOS immunoreactivity.

A semiquantitative analysis of NOS immunopositive neurons is shown in Fig. 2A. As apparent from the figure, NOS immunoreactivity was increased in the gray matter and this increase was most pronounced in the ventral gray matter compared to the dorsal horn (Fig. 2A).

Effect of heat stress on brain edema and microvascular permeability

Measurement of Evans blue extravasation and radioactive iodine showed significant increase in several brain and spinal cord regions associated with NOS upregulation (Fig. 2B). These regions also showed pronounced edematous swelling as evident from measurement of the brain water content (HS Sharma, unpublished observations).

Effect of heat stress on cell injury

Marked neuronal, glial and myelin reaction are common findings in several brain and spinal cord regions in heat stress (Sharma et al., 1997a,b). Most of these distorted neurons exhibit NOS positive reaction (Sharma et al., 1998).

Effect of H-290/51 in heat stress

Pretreatment with the antioxidant compound H-290/51 significantly attenuated NOS upregulation in the CNS of various regions following heat stress (Figs. 1, 2A). However, the stress symptoms and physiological variables were not significantly affected by this drug treatment (Sharma et al., 1997a). Measurement of BBB permeability (Fig. 2B) and brain edema showed significant reduction compared to untreated heat stressed rats (H. S. Sharma, unpublished observations). The compound was also very effective in reducing cell changes. Thus neuronal, glial and myelin changes were much less severe in this drug-treated heat stressed rats compared to the untreated group (H. S. Sharma et al., unpublished observations).

Discussion

Our results suggest that hyperthermic brain injury is associated with a marked upregulation of NOS immunoreactivity in thalamus, hypothalamus and the spinal cord. This upregulation of NOS immunoreactivity was found in neurons not normally showing NOS immunoreaction. It appears that neurons which exhibited NOS upregulation may be more liable to cell injury and cell death (Dawson and Dawson, 1996). This is evident from the findings that upregula-

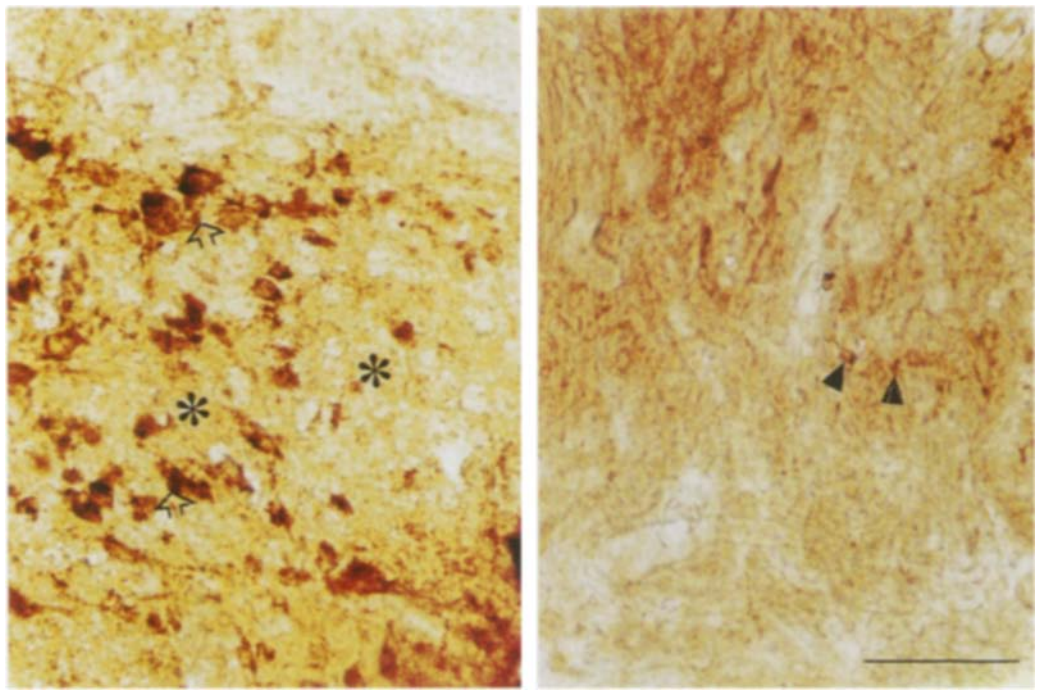

Fig. 1. NOS upregulation in the dorsal horn of the spinal cord from C-5 segment of one four h heat stressed rat at 38°C (left) and its modification with H-290/51 treatment (right). Upregulation of NOS is evident in heat stressed rat (arrow heads) and mainly located in the edematous area (*). On the other hand only a very few NOS positive cells (filled triangles) are visible in rats pretreated with the new anti-oxidant compound (bar = 50 μm)

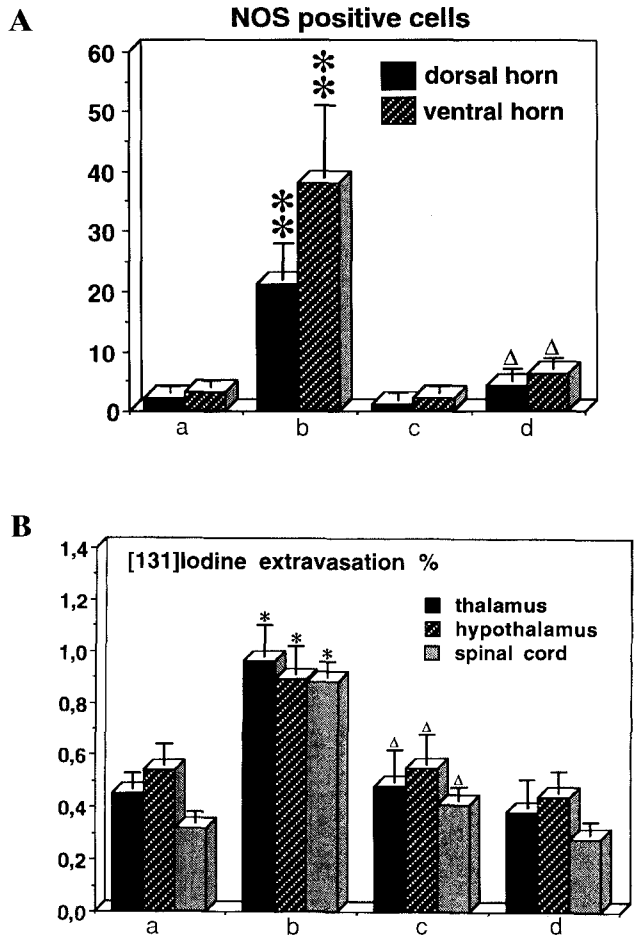

Fig. 2. **A** Semiquantitative data of NOS positive cells in the spinal cord C-5 segment of heat stressed rats and their modification with the compound H-290/51 (**p < 0.01 from control, Δ p < 0.05 from 4 h heat stressed group). **B** Extravasation of radioactive iodine in heat stressed rats and its modification with H-290/51 (*p < 0.001 from control, Δ p < 0.05 from 4 h heat stressed group, Student's unpaired t-test). *a* control; *b* 4 h HS; *c* H-290/51 + control; *d* H-290/51 + 4 h HS

tion of NOS is seen in distorted nerve cells found in the edematous areas of the CNS. This observation is in line with our previous investigation in which an upregulation of NOS can be seen in the parietal cerebral cortex and hippocampus of heat stressed rats which is most pronounced in the edematous brain regions (Sharma et al., 1997a). The present observation confirms our previous findings and further extends the idea that upregulation of NOS is somehow associated with cell injury and cell death. This idea is in line with the findings in ischemic brain injury in which upregulation of NOS in neurons not normally showing NOS immunoreactivity are more susceptible to cell death.

Recently involvement of NO in cell injury has been demonstrated in various brain diseases involving ischemia, infarction, stroke, AIDS, dementia, Parkinson's and Alzheimer's diseases (Dawson and Dawson, 1996). These

observations are based on the fact that in many disease conditions upregulation of NOS occurs and in some experimental situations, neuronal NOS null mice are quite resistant to cell injury following ischemic insults to the CNS (for review see Dawson and Dawson, 1996; Sharma et al., 1997a, 1998). Another approach to examine this issue came from pharmacological studies. Thus, use of NOS inhibitors significantly attenuated cell injury in most cases. However in some cases same NOS inhibitors appear to aggravate the cell injury indicating that pharmacological blockade of NOS is not always associated with cell protection (Kimura and Steinbusch, 1996). However, most of the studies using a pharmacological approach did not examine NOS immunohistochemistry. Thus, it is not certain whether the compound used by various investigators were sufficient enough to inhibit NOS upregulation in that experimental model.

There are some reports that blockade of endothelial NOS (eNOS) is harmful in ischemia because this isoform is involved in the regulation of cerebral blood flow (Dawson and Dawson, 1996). Some reports suggest the specific blockade of neuronal NOS or inducible isoforms of NOS with specific inhibitors are neuroprotective in stroke and ischemia. Thus, the pharmacological blockade of NOS and neuroprotection is still a matter of controversy and further research is needed to clarify this point.

We have approached this issue in a different way. We used an antioxidant to induce neuroprotection in heat stress and examined NOS upregulation in this drug treated stressed animals. NO is a free radical gas and thus lipid peroxidation and membrane damage following heat stress can increase NO production. Pretreatment with H-290/51 which is an inhibitor of lipid peroxidation (Mustafa et al., 1995) may have some inhibitory effect on NO production and consequently on cell injury.

Our results show that a new anti-oxidant compound H-290/51 significantly attenuated the cell injury, edema and microvascular permeability indicating that this compound has the capacity to induce neuroprotection in heat stress which is very similar following trauma to the spinal cord (Mustafa et al., 1995). Interestingly, our results show that this anti-oxidant compound was also associated with downregulation of NOS in brain and spinal cord regions after heat stress. This observation support our previous findings in the parietal cortex and hippocampus (Sharma et al., 1997b) and further extends the idea that antioxidant can induce a widespread neuroprotection in the CNS following heat stress.

This investigation, however, does not shed any light on the possible mechanisms of action of antioxidants in heat stress induced cell injury. One possibility that this compound may prevent free radical induced lipid peroxidation of cell membranes following hyperthermic brain injury (Svensson et al., 1993). Free radical production is associated with cell damage and secondary injury cascade. Direct damage of membranes may influence Ca^{++} uptake and intracellular accumulation of calcium will induce activation of NOS (Dawson and Dawson, 1996). The other possibility seems to be related with the activity of these compounds in inhibiting the general mechanisms of stress response, a feature which require additional investigation using expression of heat shock

protein (HSP) response (Sharma et al., 1995). From this observation it appears that a direct effect of anti-oxidants on NOS upregulation is unlikely. This is because of the fact that pretreatment with H-290/51 in normal rats did not significantly attenuate the normal NOS activity.

In conclusion, our results suggest that NOS upregulation, which probably causes an activation of NO production, is associated with cell injury and cellular and oxidative stress are important endogenous signals for NOS upregulation in thermal brain injury.

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Authors' address: H. S. Sharma, Ph.D., Laboratory of Neuroanatomy, Department of Anatomy, Box 571, Biomedical Centre, Uppsala University, S-751 23 Uppsala, Sweden; phone and fax +46-18-243899, E-mail: Sharma@anatomi.uu.se

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