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A new antioxidant compound H-290/51 attenuates nitric oxide synthase and heme oxygenase expression following hyperthermic brain injury

An experimental study using immunohistochemistry in the rat

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Summary. Influence of a new anti-oxidant compound H-290/51 on expression of nitric oxide synthase (NOS) and heme oxygenase (HO) enzymes responsible for nitric oxide (NO) and carbon monoxide (CO) production, respectively was examined in the CNS following heat stress in relation to cell injury. Exposure of rats 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 constitutive isoforms of neuronal NOS (nNOS) and HO-2 revealed marked upregulation in damaged and distorted neurons located within the edematous brain regions. Pretreatment with H-290/51 (50 mg/kg, p.o., 30 min before heat stress) significantly reduced the edematous swelling and cell injury and resulted in a marked attenuation of nNOS and HO-2 expression. These observations suggest that upregulation of NOS and HO is associated with cell injury, and the antioxidant compound H-290/51 is neuroprotective in heat stress.

Keywords: Amino acids – Hyperthermia – Heat stress – Brain edema – Nitric oxide synthase – Heme oxygenase – Oxidative stress – H-290/51 – Cell injury

Introduction

Heat stress induced hyperthermia is associated with profound brain damage (Malamud et al., 1946; Sharma, 1982; Sharma and Cervós-Navarro, 1990; Sharma et al., 1997a–d, 1998a–c; Sharma and Westman, 1998; Sharma, 1999).

The probable mechanisms of brain injury caused by hyperthermia include several endogenous factors such as, release of neurochemicals, oxidative stress, generation of free radicals, and alterations in cellular metabolism (Sharma, 1982, 1999; Sminia et al., 1994; Alm et al., 1998; Sharma and Westman, 1998, 1999). Generation of free radicals and lipid peroxidation will induce direct membrane damage of the nerve cells, glial cells and the endothelial cells (Chiueh et al., 1994). Breakdown of the blood-brain barrier (BBB) will result in extravasation of serum proteins leading to the formation of vasogenic edema (Cervós-Navarro and Ferstz, 1980; Wahl et al., 1988; Bradbury, 1992; Black, 1995). All these factors together will induce profound cell injury in the CNS.

Although, oxidative stress significantly contribute to cell damage, its role in hyperthermia induced brain injury is not known in details. One way to study the contribution of oxidative stress in hyperthermia is to use a pharmacological approach using anti-oxidant drugs. Our laboratory is engaged in this direction using the potent antioxidant compound H-290/51 to understand the influence of oxidative stress in the pathophysiology of hyperthermic brain injuries (Alm et al., 1998; Sharma, 1998, 1999; Sharma et al., 1998a,b). This compound is known to attenuate BBB disturbances and edema formation in hyperthermia (Sharma et al., 1997c; Alm et al., 1998) indicating that oxidative stress significantly contribute to the pathophysiology of hyperthermic brain injury.

The problem of cell injury in hyperthermia is extremely complex (Sminia et al., 1994; Sharma and Westman, 1998, 1999; Sharma, 1999). Recently, the newly discovered free radicals nitric oxide (NO) and carbon monoxide (CO) have been suggested to be involved in the mechanisms of hyperthermic brain injury like in many other experimental or clinical conditions (Chiueh et al., 1994; Dawson and Snyder 1994; Dawson and Dawson 1996; Sharma et al., 1997c,d, 1998b; Sharma, 1999). NO and CO are biological gases capable of diffusing from one cell to the other in an extremely short duration in order to influence cell to cell communication (Ewing and Maines, 1991; Bredt and Snyder, 1992; Ceccatelli et al., 1993; Dawson and Snyder, 1994; Abraham et al., 1996; Dawson and Dawson, 1996). These gaseous molecules in biological system act as free radicals and generation of NO or CO in the CNS will thus contribute to the cell injury (Chiueh et al., 1994). However, until now experimental evidences either supporting or rejecting this hypothesis are controversial.

NO is synthesised from the enzyme nitric oxide synthase (NOS) which is normally present in the CNS (Bredt and Snyder, 1992). CO is synthesised from the heme oxygenase (HO) enzyme which is also found in the normal CNS (Verma et al., 1993). Upregulation of NOS and HO occur in many experimental conditions involving ischemia, hypoxia, trauma or in several other neurodegenerative diseases (for review see Sharma et al., 1998b). This indicates that upregulation of NOS and HO seems to be harmful and invokes cell injury. This idea is supported by the fact that upregulation of NOS and HO is associated with increased production of NO or CO (Chiueh et al., 1994;

Abraham et al., 1996; Sharma et al., 1998b). Increased production of NO or CO may induce breakdown of the BBB, either by a direct membrane disruption, or indirectly via increased production of cGMP synthesis in the endothelial cells (Kubes and Granger, 1992). Thus, available line of evidences indicate that upregulation of NOS and HO synergistically contribute to the brain pathology. However, further studies are needed to confirm this hypothesis.

Previously, our laboratory demonstrated a powerful neuroprotective effect of the antioxidant compound H-290/51 in spinal cord trauma induced cell changes (Sharma et al., 1997c, 1998b; Alm et al., 1998). One possible mechanism of neuroprotection with H-290/51 is its capacity to inhibit the formation of free radicals probably by a chain-braking reaction (Alm et al., 1998). Thus, pretreatment with this compound will result in an inhibition of free radicals formation thereby reducing the consequences of cell injury.

There are reasons to believe that generation of NO or CO in hyperthermia is somehow associated with the oxidative stress (Chiueh et al., 1994; Dawson and Dawson, 1996). Thus, a possibility exist that pretreatment with antioxidant compound significantly inhibits the production of these gaseous molecules in hyperthermia and induce neuroprotection. Production of NO and CO can be easily examined by immunohistochemical detection of their synthesising enzymes NOS and HO, respectively (Ewing and Maines, 1991; Bredt and Snyder, 1992; Verma et al., 1993). The present investigation was carried out to examine whether pretreatment with H-290/51 is associated with a reduction in NOS and HO-2 expression in the CNS following hyperthermia. In addition, the effect of H-290/51 was also examined on the brain edema formation, BBB permeability and cell injury.

Materials and methods

Animals

Experiments were carried out on 30 male Sprague Dawley rats housed at controlled ambient temperature (21 ± 1°C) with 12 h light and 12 h dark schedule. The rat food and water were supplied ad libitum.

Exposure to heat stress

Animals were exposed to 4 h heat stress at 38°C in a biological oxygen demand (BOD) incubator (relative humidity 45–47%; wind velocity 20–25 cm/sec). This experimental condition is approved by the Ethical committee of Lund University, Lund; Uppsala University, Uppsala, Sweden and Banaras Hindu University, Varanasi, India.

Control group

Normal rats kept at room temperature (21 ± 1°C) served as controls.

H-290/51 treatment

The compound H-290/51 (AstraZeneca, Mölndal, Sweden) was administered per os (50 mg/kg) as a suspension in distilled water 30 min before the onset of heat stress (Sharma et al., 1997c, 1998b). One group of animals treated with H-290/51 was used as drug-treated control (Alm et al., 1998).

Immunohistochemistry of NOS and HO

Immunohistochemistry of NOS and HO was performed on free floating vibratome sections obtained from several brain regions from the control, untreated heat stressed rats and the drug-treated control or heat stressed rats in parallel (Sharma et al., 1998b).

In brief, at the end of the heat stress experiments, animals were anaesthetised with Equithesin (3 ml/kg, i.p.) and the chest was rapidly opened. The animals were perfused via the heart with 0.1 M sodium-potassium phosphate buffer (PBS, pH 7.4) solution to washout the blood inside the microvessels, followed by 4% paraformaldehyde in 0.1 M PBS (about 150 ml). At the end of the perfusion, the animals were wrapped in an aluminium foil and kept at 4°C in a refrigerator overnight. On the next day, the brain and spinal cord of the animals were dissected out and small pieces from different regions were placed in the same fixative and stored at 4°C for one week (Sharma et al., 1997; Sharma, 1999).

Immunostaining was performed on 40μm vibratome sections using a monoclonal NOS antiserum (Alm et al., 1998) and HO-2 antiserum (StressGene, Canada) as described earlier (Sharma et al., 1997c,d, 1998b). The reaction product was developed using peroxidase-antiperoxidase techniques and visualised at light microscope. To understand the ultratstructural localisation of NOS and HO immunostaining, some vibratome sections were post-embedded in osmium tetraoxide and embedded in epon. About 1 micrometer thick semithin sections were cut from these immunostained vibratome sections to identify labelling and then ultrathin sections were made by ultramicrotome using a diamond knife. The ultrathin sections were mainly passing through the immunostained nerve cells and dendrites (Sharma et al., 1998b).

Blood-brain barrier permeability

The BBB permeability was examined using Evans blue albumin and radioactive iodine labelled to sodium ($^{[131]}$I-sodium)as described earlier (Sharma, 1987; Sharma and Dey, 1987).

Brain edema formation

The brain edema formation was examined using measurement of the brain water content (Sharma and Cervós-Navarro 1990; Sharma et al., 1997a,b).

Cell changes

The cell changes were examined using light and electron microscopy according to the standard procedures as described earlier (Sharma et al., 1997a–c; Sharma 1999).

Statistical significance

The Student's unpaired t-test was used to evaluate the statistical significance of the semi-quantitative or quantitative data obtained. A p-value less than 0.05 was considered as significant.

Results

Effect of H-290/51 on NOS upregulation

Subjection of rats to 4 h heat stress resulted in a marked upregulation of nNOS activity in many nerve cells located within the cerebral cortex, hippocampus, cerebellum, brain stem and the spinal cord. Upregulation of nNOS was mainly seen in the distorted neurones located within the edematous regions. In control animals, few nNOS labelled neurons could be seen sparsely distributed within the cortex, hippocampus, cerebellum and brain stem. Spinal cord usually did not exhibit nNOS stained neurones. Thus, hyperthermia induced expression of nNOS in many nerve cells that do not normally show NOS immunoreactivity.

Pretreatment with H-290/51 did not influence the normal distribution of nNOS in the CNS. However, a significant reduction in upregulation of nNOS activity was seen in hyperthermic animals which received H-290/51 as a pretreatment (Fig. 1). The nNOS positive cells were mainly seen in the regions of their normal localisation and only in a few areas nNOS upregulation was seen in regions not normally showing this activity. A representative example of nNOS upregulation in the cortex of one untreated and one H-290/51 treated rat is shown in Fig. 2.

As apparent from the Fig. 2, NOS upregulation is mainly seen in the cytoplasm and in most cases the nerve cell nucleus is also stained. Few dendrites also exhibit NOS immunostaining. Pretreatment with H-290/51 resulted in significant reduction in NOS staining and the intensity of immunostaining is significantly less in the nerve cells or dendrites.

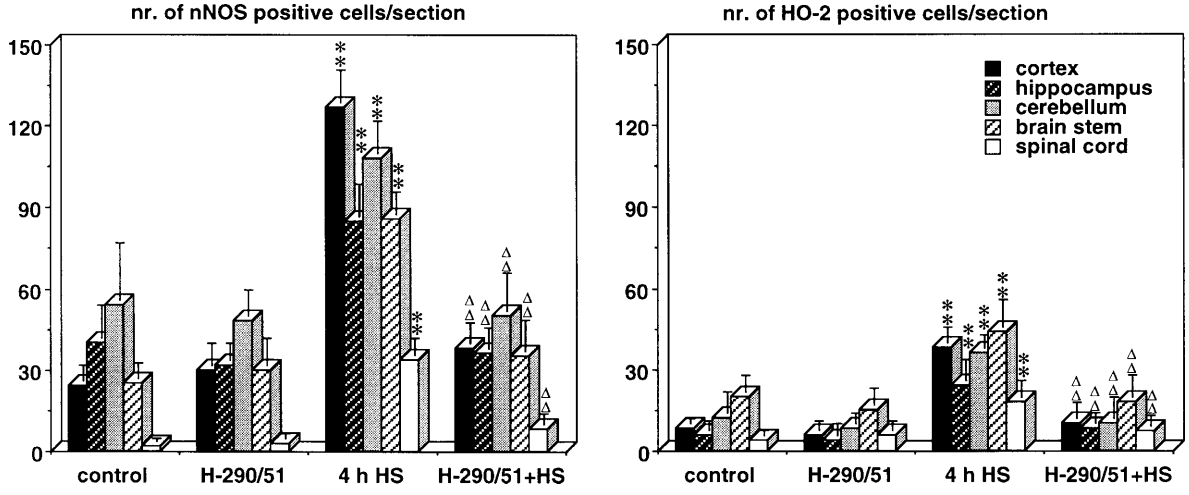

Fig. 1. Semiquantitative analysis of NOS and HO positive cells in the control or 4 h heat stressed rats and their modification with H-290/51 pretreatment. **P < 0.01, compared from control group; ΔΔ = P < 0.01, compared from 4 h heat stressed (*HS*) group; Student's unpaired t-test

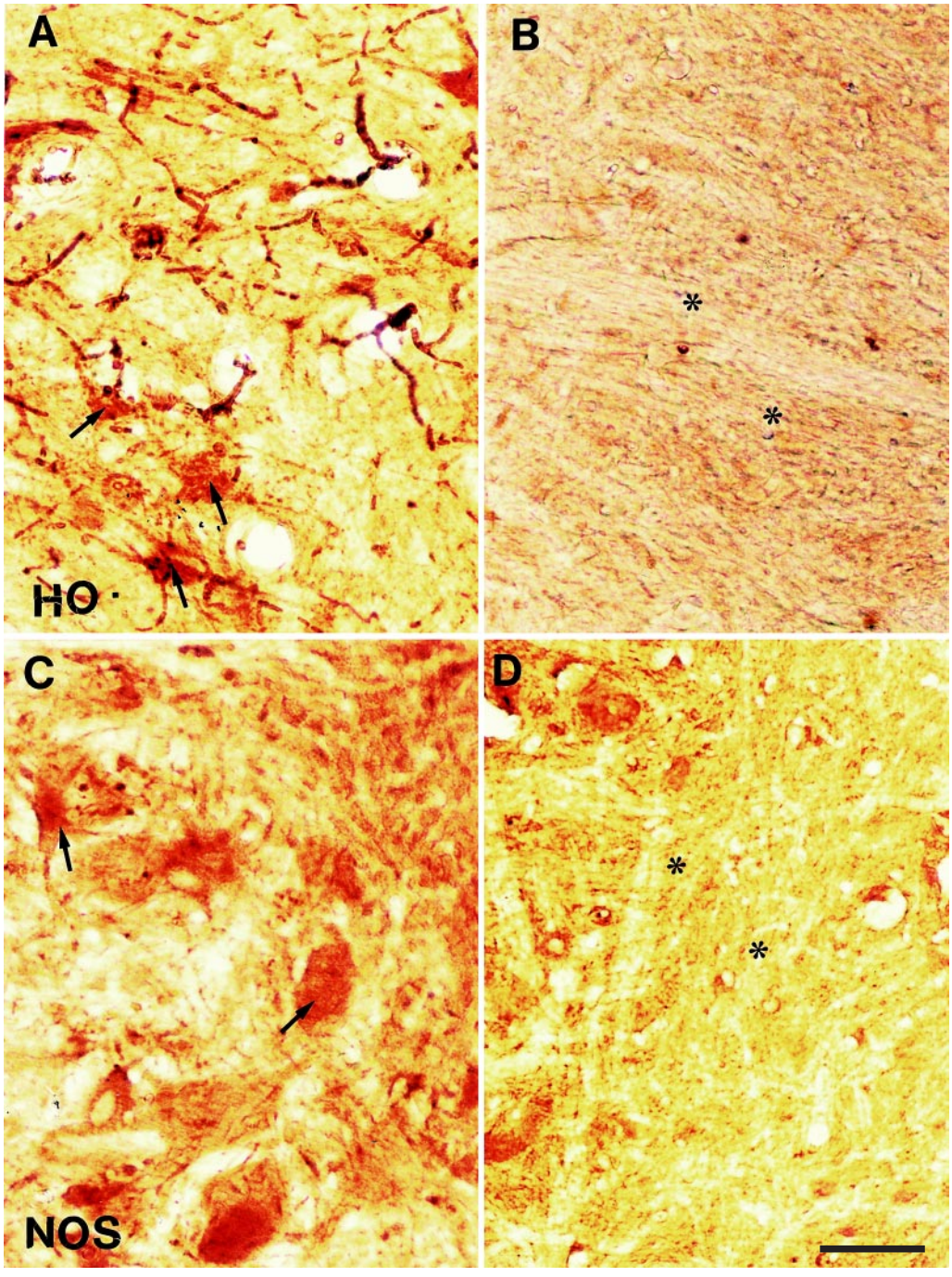

Fig. 2. A representative example of HO-2 (*HO*) immunolabelling (arrows) in the thalamus of one untreated 4 h heat stressed rat (**A**) and its modification with H-290/51 pretreatment (**B**). Immunolabelling of cNOS (*NOS*) positive cells in the thalamus of another heat stressed rat (**C**) is markedly attenuated by pretreatment with H-290/51 (**D**) (bar A,B = 40 μm; C,D = 60 μm)

Effect of H-290/51 on HO-2 upregulation

Normal rats exhibit very few HO-2 positive nerve cells. However, subjection of rats to 4 h heat stress significantly increased the number of HO-2 positive cells in the regions not normally showing HO-2 positive activity (Fig. 2). Thus many HO-2 positive cells could be seen in heat stressed rats in the cortex, cerebellum, hippocampus, brain stem and the spinal cord (Fig. 1). The upregulation of HO-2 positive cells were mainly located within the damaged nerve cells in the edematous brain regions.

Pretreatment with H-290/51 in normal rats did not influence the normal distribution of HO-2 positive pattern in the CNS. However, this compound significantly attenuated the upregulation of HO-2 expression in rats subjected to hyperthermia. One representative example of HO-2 expression in the cerebral cortex of one untreated heat stressed rat and one H-290/51 treated rat is shown in Fig. 2. As apparent from the figure that HO-2 immunostaining is mainly localised in the nerve cell cytoplasm, Occasionally, few nerve cell nucleus is also stained. Pretreatment with H-290/51 significantly attenuated the nerve cell staining and the intensity of immunostaining is markedly decreased in the drug-treated stressed rat.

Effect of H-290/51 on the BBB permeability

Pretreatment with H-290/51 significantly attenuated the extravasation of Evans blue and radioactive iodine in several brain regions following hyperthermia. The drug treatment alone in normal rats however, did not influence the tracer distribution in the brain (Table 1).

Table 1. Pathophysiology of hyperthermic brain injury and its modification with H-290/51 pretreatment

Type of Exp.	BBB permeability		Brain Water %	CNS damage/distortion		
	EBA mg %	[131]I-Na %		Nerve cell	glial cell	myelin
Control (n = 5)	0.24 ± 0.05	0.34 ± 0.06	76.38 ± 0.38	nil/+	nil/+	nil/+
H-290/51 (n = 5)	0.32 ± 0.10	0.42 ± 0.09	75.47 ± 0.67	nil	nil	nil/+
4 h Heat Stress (n = 6)	1.54 ± 0.23***	2.31 ± 0.33***	80.54 ± 0.89***	++++	++++	++++
H-290/51+ 4 h HS (n = 6)	0.45 ± 0.08[a]	0.48 ± 0.11[a]	77.38 ± 0.45[a]	++	++	++

Values are mean ± SD, *** = $P < 0.001$, compared from control, [a] $P < 0.001$, compared from 4 h heat stress, Student's unpaired t-test; *nil* absent, + occasional, ++ mild, ++++ severe.

Effect of H-290/51 on brain edema formation

Pretreatment with H-290/51 markedly reduced the brain edema formation in several brain regions of heat stressed rats. The distribution of brain water was not significantly altered in normal animals with H-290/51 pretreatment (Table 1).

Effect of H-290/51 on cell injury

Marked cell changes were apparent in untreated heat stressed animals. Thus, many nerve cells showed distortion, swelling of glial cells and myelin vesiculation. Perivascular edema and damage of intra- and extracellular membrane was quite common in many brain regions of the heat stressed rats. Pretreatment with H-290/51 significantly reduced the occurrence of cell injury in hyperthermia. Thus, in drug treated stressed rats, the neuropil was quite compact and the signs of membrane disruption, perivascular edema and damage to nerve cells, glial cells and myelin vesiculation were considerably reduced (Table 1).

Discussion

The salient new findings of the present investigation show that the compound H-290/51 has the capacity to attenuate NOS and HO upregulation in heat stress. This indicates that oxidative stress plays important role in NO and CO upregulation in hyperthermia, not reported earlier. Our study further shows that breakdown of the BBB permeability, brain edema formation and cell injury were considerably reduced in heat stressed animals pretreated with H-290/51. These observations show that the antioxidant compound H-290/51 induces significant neuroprotection in heat stress.

Our observations further suggest that upregulation of NOS and HO seems to be injurious to the cell. This is evident from the fact that in H-290/51 treated heat stressed rats upregulation of NOS and HO were considerably reduced and the cell changes were not apparent in these animals. These results strongly advocate the idea that NO and CO probably work in synergy to induce cell damage in hyperthermic brain injury.

NO and CO are biological gases which influence neural communication and thereby exert important influence on brain function (Ewing and Maines, 1991; Bredt and Snyder, 1992; Verma et al., 1993; Chiueh et al., 1994). NO is synthesised from the amino acid L-arginine by the enzyme NOS which is found in normal cells located within the CNS (Dawson and Dawson, 1996). On the other hand, CO is synthesised by the enzyme hemeoxygenase (HO) from the heme during conversion into biliverdin and iron (Abraham et al., 1996). Recent observations suggest that both NO and CO exert powerful influence on neuronal function and are involved in several disease processes of the CNS (Verma et al., 1993; Chiueh et al., 1994; Sharma et al., 1998b). In many neurodegenerative conditions, upregulation of NOS and HO

is reported (Abraham et al., 1996; Dawson and Dawson, 1996; Fukuda et al., 1996). There are evidences that several neurochemicals, hormones and growth factors influence upregulation of these enzymes (Chiueh et al., 1994; Abraham et al., 1996). Identification of HO-1 as heat shock protein-27 suggest that induction of HO may occur following induction of cellular stress response or upregulation of HO represents the cellular stress reaction (Verma et al., 1993; Sharma et al., 1998b). However, further studies are needed to clarify this point.

Generation of NO and CO in the biological system is greatly influenced by oxidative stress commonly seen during trauma, ischemia or hypoxia (Chiueh et al., 1994). The present results show that hyperthermia is also associated with the upregulation of NOS and HO. This indicates that oxidative stress associated with hyperthermia is capable to induce an increased production of NO and CO as evidenced with the increase of their synthesising enzymes NOS and HO, respectively.

There are reports that activation of NOS and HO is associated with increased production of the NO and CO (Abraham et al., 1996; Dawson and Dawson, 1996; Sharma et al., 1997c,d, 1998b; Sharma, 1999). Upregulation of NOS and HO-2, the constitutive isoform of the enzymes, produces small quantities of NO and CO respectively which can act locally to induce signal transduction mechanisms leading to an increase in cGMP levels in the both neural and non neural cells (Bredt and Snyder, 1992; Dawson and Snyder, 1994). Increased cGMP levels are associated with increase in the breakdown of the BBB permeability (Kubes and Ganger, 1992; Verma et al., 1993). This idea is further supported by the fact that NO is associated with breakdown of the BBB permeability in many experimental conditions (Sharma et al., 1997c, 1998b). An increased permeability of the BBB in the present investigation together with upregulation of NOS is in line with the above idea. Our observations further show that HO-2 expression was also increased in regions associated with BBB leakage. This indicates that CO production is also associated with breakdown of the BBB permeability, not reported earlier.

That CO and NO are associated with breakdown of the BBB permeability in hyperthermia is further confirmed by the results obtained with H-290/51 pretreatment. Pretreatment with H-290/51 significantly attenuated the BBB permeability in hyperthermia in the present investigation together with nNOS and HO-2 expression. Obviously, a less production of NO or CO in H-290/51 treated group in hyperthermia is associated with a significant reduction in the BBB permeability.

Brain edema formation following hyperthermia is significantly attenuated in heat stressed rats pretreated with H-290/51. This indicates that H-290/51 has the capacity to reduce brain edema formation. Brain edema formation is directly related with breakdown of the BBB permeability (Sharma and Dey, 1978, 1984, 1986, 1987; Sharma, 1982; Sharma et al., 1986). Thus, an increased extravasation of serum proteins following disruption of the BBB will allow water to enter into the cerebral compartment simply because of increased osmotic pressure across the cerebral vascular wall due to shift in protein osmolarity from the vascular to the brain compartment (Cervós-Navarro and

Frestz, 1980; Sharma et al., 1998a). A direct damage of the cell membrane is also responsible for brain edema formation and intracellular accumulation of brain water. The methods to determine dry and wet weight for brain edema formation does not allow us to discriminate between extracellular or intracellular water accumulation in the brain following hyperthermia. Ultrastructural studies suggest that hyperthermia induced brain swelling is due to both extracellular and intracellular accumulation of the brain water (Sharma et al., 1998a; Sharma, 1999).

Direct membrane damage by hyperthermia induce free radical formations as well as NO and CO production also contribute to brain edema formation. A considerable reduction in NOS and HO-2 upregulation following hyperthermia in H-290/51 treated rats supports this idea.

Our morphological observations show that H-290/51 pretreatment is associated with a significant reduction in cell injury in hyperthermic animals compared to the untreated rats. Thus, in the drug treated animals, cell changes were considerably reduced. A significant reduction in cell injury by this drug treatment suggests that BBB breakdown and edema formation play important roles in cell injury. Since, H-290/51 pretreatment is associated with a reduction in NOS and HO expression, a direct effect of NO and CO on cell injury cannot be ruled out in hyperthermia.

In conclusion, our results strongly suggest that hyperthermia is associated with upregulation of NOS and HO which is associated with NO and CO production. Pretreatment with H-290/51 attenuated NOS and HO expression indicating that oxidative stress is important in production of NO and CO in hypothermia which seems to be a key factor in inducing breakdown of the BBB permeability, brain edema formation and cell injury. Taken together, our results show that H-290/51 offers significant neuroprotection in hyperthermia induced brain damage, and the breakdown of the BBB appears to be instrumental in cell injury.

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