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Nitric oxide synthase inhibitors influence dynorphin A (1–17) immunoreactivity in the rat brain following hyperthermia

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Summary. The possibility that nitric oxide synthase (NOS) inhibitors influence dynorphin immunoreactivity following hyperthermia was examined in a rat model using a pharmacological approach. Previous reports from our laboratory show that hyperthermia induces an upregulation of NOS in several brain regions that seems to be instrumental in causing cell injury. Recent reports suggest that nitric oxide (NO) can influence dynorphin neurotransmission in the normal brain as well as in several pathological states. Since dynorphin is neurotoxic in different animal models of brain or spinal cord injury, it may be that the peptide will contribute to the cell injury in hyperthermia. The present investigation was carried out to determine whether hyperthermia can influence dynorphin immunoreactivity in the brain, and if so, whether inhibition of NOS will influence the peptide distribution in the brain following heat stress. Rats subjected to hyperthermia at 38°C for 4 h in a biological oxygen demand incubator (BOD) resulted in a marked upregulation of dynorphin immunoreactivity in several brain regions e.g., cerebral cortex, hippocampus, cerebellum and brain stem. Pretreatment of rats with two potent NOS inhibitors, L-NAME (30 mg/kg/day, i.p. for 7 days) or L-NMMA (35 mg/kg/day, i.p. for 7 days) significantly attenuated the dynorphin immunoreactivity in the brain. These drugs were also able to reduce hyperthermia induced blood-brain barrier (BBB) permeability, brain edema formation and cell injury. Taken together, our results suggest that (i) hyperthermia has the capacity to upregulate dynorphin immunoreactivity in the brain, (ii) inhibition of NOS considerably attenuates the dynorphin immunoreaction following heat stress and (iii) upregulation of dynorphin is somehow contributing to hyperthermia induced brain damage, not reported earlier.

Keywords: Dynorphin – Hyperthermia – Brain injury – Immunoreactivity – Nitric oxide – Ultrastructure – L-NAME – L-NMMA – Nitric oxide synthase – Brain edema – Evans blue – [131]Iodine

Introduction

The central nervous system (CNS) is very rich in several opioid and non-opioid peptides (Björklund et al., 1990; Nyberg et al., 1995). These neuropeptides participate in several key functions within the CNS such as memory, sleep, stress, thermoregulation, behaviour and drug addiction. The neuropeptides are well-known neuromodulators in the CNS and usually found co-localized with many other classical neurotransmitters (Hökfelt et al., 1978, 1987). Thus, a possibility exists that modulation of neurotransmitters in the CNS will influence neuropeptide function or metabolism. However, this is a new feature that requires further investigation.

The dynorphins represent one of the three major groups of opioid peptides that have been found in the CNS. The dynorphin was first localised in the pituitary (Cox et al., 1975). Later on, its widespread localisation in the whole CNS as well as in the periphery was found (Goldstein and Ghazarhosian, 1980; for details see Fallon and Ciofi, 1990). Several forms of dynorphin exist in the CNS that are derived from the prodynorphin genes. Interestingly, all forms of prodynorphin derived peptides, i.e., α-neoendorphin, dynorphin B, dynorphin A (1–8) and dynorphin A (1–17) are extensively co-localised in the same neuronal system (Watson et al., 1983; Weber and Barchas, 1983). In some neurons, dynorphins are known to co-exist with enkephalins (Hökfelt et al., 1978). Recently, using antisera raised against different forms of dynorphins (Herrera-Marschitz et al., 1984), the concept of a specific distribution of the peptide as a separate system in the CNS has emerged (Guthrie and Basbaum, 1984; Sasek and Elde, 1986).

A widespread localisation in several brain regions of dynorphin suggests that the peptide participates in

many important functions in the CNS. However, surprisingly, the functions of dynorphins in the CNS are still not well understood. There are evidences that the physiological and behavioural characteristic of the peptide correlates well with their anatomical distribution (Smith and Lee, 1988). Thus, the peptide is involved in the central mechanisms of analgesia (Basbaum and Fields, 1984), self stimulation, reward and drug abuse (Herrera-Marschitz et al., 1984; Watson et al., 1989). The dynorphins are also implicated in stress response (Akil et al., 1984), cardiovascular depression at the levels of hypothalamus and medulla (Gautret and Schmitt, 1985; Punnen and Sapru, 1986), brain injury following stroke (Baskin et al., 1985) and spinal cord injury (Faden, 1993; Sharma et al., 1995). The dynorphins participate in the central mechanisms of thirst (Leander, 1982), feeding (Morely et al., 1984), respiratory depression (Woo et al., 1983), neuroendocrine functions at the hypothalamus and pituitary level (Przewlocki et al., 1987; Akil et al., 1984), motor function at the level of extrapyramidal motor system (Mulder et al., 1984) as well as the temperature regulation (Lee, 1984). However, the role of dynorphin in heat stress induced alteration in brain function is still unknown.

Heat stress is associated with profound hyperthermia that often exceeds 41°C. At this stage, sweating ceases to exist and the rise in body temperature continues further (Sharma and Westman, 1998). It is believed that failure of thermoregulatory mechanisms at the level of hypothalamus seems to be one important factor contributing to hyperthermia induced brain dysfunction (Sharma, 1999). If hyperthermia continues to persist, mental abnormalities, severe neurological symptoms and coma ensue that eventually leads to the death in more than 50% of the victims (Dematte et al., 1998). Although, the symptoms of heat stress are known since Biblical time, no suitable therapeutic strategy has yet been worked out (Malamud et al., 1946; cf Sharma, 1999). Thus, further studies are highly warranted to understand the mechanisms underlying heat stress induced brain pathology.

Our laboratory is engaged to map the distribution of several neurotransmitters and neuropeptides in the CNS following hyperthermia using immunohistochemistry (Alm et al., 2000; Sharma et al., 1998a,b; Sharma and Westman, 2000; Sharma et al., 2000a,b). Preliminary data from our laboratory using radioimmunoassay suggest that dynorphin A activity is altered profoundly in several brain regions following hyperthermia (Sharma et al., 1994). However, to our knowledge, alteration in dynorphin immunoreactivity in hyperthermia has so far not been examined. It seems quite likely that hyperthermia can induce alterations in dynorphin immunoreactivity in the CNS. Since the dynorphin is known to induced adverse cell reactions in animal models of brain and spinal cord injury (Faden, 1993; Sharma et al., 1995), it seems likely that the peptide may contribute to hyperthermia induced neurotoxicity.

Previously, we observed a positive role of nitric oxide (NO) in cell injury caused by heat stress induced hyperthermia in our rat model (Sharma et al., 1998b). Marked upregulation of nitric oxide synthase (NOS) occurred in several brain regions associated with blood-brain barrier (BBB) disruption, edema formation and cell damage (Sharma et al., 2000b). Drugs which directly or indirectly inhibited NOS upregulation also attenuated edema formation, BBB permeability and cell injury (Sharma et al., 1997, 1998b; Sharma, 1999). Recently, it has been suggested that NO can influence dynorphin neurotransmission in the CNS, either directly or through stimulating NMDA receptors (Hu et al., 1999, 2000). Likewise, dynorphins can also modulate the activity of NO in the CNS (Hu et al., 1999; Tang et al., 2000; Hauser et al., 2001). Thus, it would be interesting to see whether hyperthermia can alter dynorphin immunoreactivity in the CNS, and if so, whether inhibition of NOS can influence the peptide distribution in the brain following heat stress.

In present study, the dynorphin A (1–17) immunoreactivity was examined in the rat brain following hyperthermia. To understand the functional interaction between dynorphin and NO in heat stress, influence of two potent NOS inhibitors, L-NAME and L-NMMA (Sharma et al., 1998b) on the peptide immunoreactivity was examined. Furthermore, changes in the BBB permeability, brain edema and cell injury in the regions associated with dynorphin immunoreactivity were also investigated.

Materials and methods

Animals

Experiments were carried out on 24 male Sprague Dawley rats. The animals were kept at controlled room temperature (21 ± 1°C) with 12 h light and 12 h dark schedule. The rat feed and tap water were supplied ad libitum before the experiments.

Exposure to heat

Animals were exposed to heat stress in a biological oxygen demand (BOD) incubator at 38°C for 4 h. The relative humidity (45 to 47%) and wind velocity (21–25 cm/sec) were kept constant (Sharma and Dey, 1986, 1987). This experimental condition is approved by the Animal Ethics Committee of Lund University, Lund. Sweden.

Control group

Litter mates kept at normal room temperature (21 ± 1°C) were used as controls. Previously, rats kept in the BOD chamber at 21°C for 4 h did not exhibit any significant differences in their body temperature, behavioural parameters, arterial pH, mean arterial blood pressure (MABP) and blood gases compared to the animals kept at room temperature (Sharma and Cervós-Navarro, 1990a,b). Thus, all intact or drug-treated control groups in present investigation were kept at normal room temperature (Sharma et al., 1998a).

Treatment with NOS inhibitors

Separate groups of rats were treated with two potent NOS inhibitors. In these drugs treated rats, one group was maintained at normal room temperature while the other group was subjected to 4 h heat stress. The following drug treatments were carried out in this study.

(a) L-NAME (Sigma Chemical Co., USA). L-NAME hydrochloride ($N^{(G)}$-Nitro-L-arigine methyl ester) non-selective NOS inhibitor soluble in water was administered 30 mg/kg (i.p.) once daily for 7 days (Steinbush et al., 2000). On the 8th day, one group of rats treated with L-NAME were subjected to 4 h heat stress. The remaining animals were used as drug-treated controls. The drug in the doses used in the present study will inhibit the constitutive isoforms of neuronal and endothelial NOS (Sharma et al., 1998b; Steinbush et al., 2000).

(b) L-NMMA (Tocris, UK) L-NMMA (N^{G}-Monomethyl-L-arginine acetate) soluble in water, non-selective NOS inhibitor, competitive, irreversible inhibitor of all three NOS isoforms (Steinbush et al., 2000) was administered in a dose of 35 mg/kg intraperitoneally daily for one week. On the 8th day, one group of L-NMMA treated animals was exposed to 4 h heat stress at 38°C while the remaining rats were used as drug treated controls.

Parameters measured

The following parameters were measured in control, heat stressed, L-NAME or L-NMMA treated controls and heat exposed rats.

Stress symptoms

The changes in rectal temperature, the occurrence of behavioural salivation and prostration, and the gastric haemorrhages in the stomach were used as indices of stress symptoms in each animal (Sharma and Dey, 1987).

Physiological variables

In separate set of experiments, the MABP, arterial pH and blood gases were analysed according to the standard procedures as described earlier (Sharma and Dey, 1987).

Brain edema

Brain edema was measured using water content (Sharma and Cervós-Navarro, 1990a). For this purpose, rats were killed by decapitation and the brains were quickly removed and dissected into small pieces. The tissue pieces were weighed immediately on a preweighed filter paper and placed in an oven maintained at 90°C for 72 h to obtained dry weight of the samples. The dry weight was considered final if the three consecutive measurements of the dry weight samples were constant (Sharma et al., 1998a).

Blood-brain barrier permeability

The blood-brain barrier permeability was examined using Evans blue (2% in sterile saline solution, pH 7.4) and $^{[131]}$Iodine as protein tracers (Sharma and Dey, 1987). At the end of the experiment, both the Evans blue (0.3 ml/100 g) and $^{[131]}$Iodine (10 μCi/rat) were injected under Equithesin anaesthesia (3 ml/kg, i.p.) into the right femoral vein through a needle puncture. The tracers were allowed to circulate for 5 min. The intravascular tracers were washed-out with 0.9% saline perfusion through heart followed by perfusion with a formalin based fixative (see below). After perfusion, the brain was removed, examined and photographed immediately for Evans blue extravasation. Extravasation of the Evans blue was also examined into the internal parts of the brain following a mid-sagital section of the brain. After examination of the Evans blue staining into different parts of the brain, one half of the brain was cut into several pieces, weighed immediately and counted in a 3-in well type Gamma counter (Packard, USA; energy window 25–50 KeV). Immediately before perfusion, a sample of the whole blood was also withdrawn through cardiac puncture to measure the whole blood radioactivity. Extravasation of the radiotracer was expressed as percentage increase in the radioactivity in the desired tissue pieces over the blood radioactivity as described earlier (Sharma, 1987). In the remaining half, the Evans blue dye entered into the brain was extracted by homogenising of the brain tissues in a mixture of n-butanol (pure grade 1.4 ml) and sodium sulphate (0.5% 0.6 ml) (Sharma, 1987). The supernatant was extracted after centrifugation (×900 g) and the Evans blue dye extracted into the supernatant was measured colorimetrically in a spectrophotometer (620 nm). The standard curve of Evans blue was obtained using different concentration of the dye ranging from 0.01 mg to 1 mg % (Sharma and Cervós-Navarro, 1990a).

Dynorphin immunohistochemistry

Dynorphin A (1–17) immunoreactivity was examined on free floating Vibratome sections (40 μm thick) using a standard protocol (Fallon and Ciofi, 1990). In brief, Dynorphin A antiserum (Calbiochem, Polyclonal, USA, 1 : 2000) was used. This antiserum cross reacts with Dynorphin A 1–8, but not with Dynorphin B or Met-Enk (unpublished observations).

Perfusion and fixation

Immediately after heat stress, the animals were anaesthetised with Equithesin (3 ml/kg, i.p.) and the chest was opened. Intravascular blood was washed out with about 50 ml of cold 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde solution (150 ml) in PBS containing 2.5% picric acid (Sharma et al., 1998a,b). After perfusion, the animals were wrapped in an aluminium foil and kept overnight at 4°C. On the next day, the brain and spinal cord were dissected out and left in the same fixative at 4°C for one week.

Selected brain regions

The desired parts of the brain were then dissected out according to the atlas of Paxinos and Watson (1986). The brain regions examined includes cortex, hippocampus, thalamus and hypothalamus at the

same plane (−3.3 mm from the bregma). The coronal sections were divided into two halves and one half was mounted on the Vibratome. The other half was used to study ultrastructural changes using standard procedures (see below).

Immunostaining

Vibratome sections (40 μm thick) from the selected brain regions were collected in PBS and processed free-floating as described earlier (Sharma et al., 1998b). The sections were washed in PBS and incubated overnight (18 to 20 h) with a rabbit polyclonal dynorphin A (1–17) antiserum (1 : 2000 diluted in PBS containing 0.2% Triton-X) at room temperature under gentle agitation with goat anti-rabbit IgG linked to horseradish peroxidase diluted 1 : 100 in PBS-X (see Fallon and Ciofi, 1990). The sections were washed in PBS-X and then rapidly rinsed in distilled water (30 to 45 sec). The peroxidase activity was revealed by incubation of the sections for 1–3 min in 0.1 M Tris buffer (pH 7.6) containing 0.015% H_2O_2 and 0.03% 3–3′ diaminobenzidine tetra hydrochloride (Sigma Chemical Co., USA). The reaction was stopped by a 1 min wash in distilled water. The sections were then dehydrated, mounted in DPX (Flukachemie AG, Switzerland) and observed under bright field Leica light microscope.

Quality control

To examine the specificity of immunohistochemical methods, we used two different types of controls. In one group of sections, the primary antiserum was omitted and rest of the processing was done as described above. These negative controls do not show immunostaining of nerve cells or nerve fibres (Fig. 3). In second group of controls, the primary antiserum was first pre-absorbed with 5 or 10 μM of Dynorphin A (1–17) synthetic peptide and then used for immunostaining. No immunostaining using this pre-absorbed antisera was detected in these vibratome sections (Fig. 3). These observations indicated an excellent specificity of the antisera used in the present investigation.

Image processing

Microphotographs were taken on Kodak Supra 100 ASA negative films and digital images (size 52 cm × 32 cm, 80 pixels/inch) were processed by Kodak Colour laboratories (Stockholm, Sweden). The digital images (size 8 × 13 cm, 300 pixels/inch) were modified using Adobe Photoshop 3.5 programme on a G-4 Macintosh computer. Identical colour filter or colour balance was used on the images obtained from the control, heat stressed or drug-treated controls or heat exposed animals (Sharma, 1999).

Transmission electron microscopy

Tissues from the remaining half of the brain (see above) were dissected into small pieces containing parietal cortex, hippocampus, dorsal thalamus and anterior hypothalamus. These small pieces were embedded in epon according to the standard protocol and processed for routine transmission electron microscopy (Sharma et al., 1998a),

Statistical analysis

The quantitative and the semiquantitative data were analysed using commercial software StatView II (Abacus Concepts Inc, USA). For multiple group comparison ANOVA followed by Dunnett test was used. A P-value less that 0.05 was considered significant.

Results

Stress symptoms

Rats subjected to 4 h heat stress at 38°C exhibited marked hyperthermia (41.4 ± 0.4°C, $P < 0.01$) compared to the control group (36.6 ± 0.43°C) kept at the room temperature (21 ± 1°C). These rats exhibited profound salivation and behavioural prostration during the heat exposure session. At post mortem, many microhaemorrhages were observed in the mucosal wall of the stomach (Table 1). Pretreatment with either L-NAME or L-NMMA did not significantly reduce the salivation or behavioural prostration. However, there was a significantly less hyperthermia in rats treated with L-NAME (40.1 ± 0.23°C, $P < 0.05$) or L-NMMA (39.8 ± 0.32°C, $P < 0.01$) following heat exposure. On the other hand, pretreatment with these drugs in control rats did not alter the body temperature significantly (Table 1).

Physiological variables

Untreated rats subjected to heat stress showed profound hypotension at 4 h (78 ± 8 torr, $P < 0.01$) compared to the control group (120 ± 10 torr). There was a significant increase in PaO2 (82.54 ± 0.23 torr, $P < 0.05$) in heat stressed rats compared to the control group (80.56 ± 0.18). On the other hand, the PaCO2 showed a mild decrease in stressed rats (33.15 ± 0.23 torr; controls 34.67 ± 0.18, n.s.). However, the arterial pH in control group (7.38 ± 0.04) was not significantly different from the heat stressed rats (7.37 ± 0.06). Pretreatment with L-NAME or L-NMMA significantly attenuated the magnitude of hypotension seen in heat stressed rats compared to the untreated stressed rats (L-NAME, 86 ± 4, $P < 0.05$; L-NMMA, 88 ± 8, $P < 0.05$). There was no significant difference in arterial pH and blood gases in the drug-treated or untreated rats after heat exposure. The drug treatment alone however, did not influence these physiological variables significantly (Table 1).

Blood-brain barrier permeability

Evans blue extravasation

Normal rats did not show extravasation of Evans blue in the brain except at the non-barrier regions, such as pineal gland, infundibulum, and choroid plexuses in the cerebral ventricles. In normal rats, the staining of

Table 1. Stress symptoms and physiological variables in 4 h heat stressed rats and their modification with nitric oxide synthase (NOS) inhibitors

Rats were subjected to 4 h heat stress in a biological oxygen demand incubator (BOD) at 38°C (relative humidity 45–47%, wind velocity 20–25 cm/sec). L-NAME or L-NMMA were administered in separate group of rats (30 or 35 mg/kg, i.p.) daily for 7 days. On the 8th day, animals were subjected to heat stress. For details see text

Expt type	Control 4 h HS		Treatment with NOS inhibitors			
	n = 6	n = 6	L-NAME + Control n = 5	L-NAME + HS n = 6	L-NMMA + Control n = 5	L-NMMA + HS n = 6
Stress symptoms						
Rect. T°C	36.60 ± 0.43	41.41 ± 0.40**	37.22 ± 0.51	40.12 ± 0.23[a]	37.41 ± 0.67	39.81 ± 0.32[aa]
Salivation	–	++++	–	+++	–	++
Prostration	–	++++	–	++	–	++
Gastric haemorrhages	–	40 ± 8[b]	–	20 ± 6[a]	–	28 ± 8[a]
Physiological variables						
MABP torr	120 ± 10	78 ± 8**	118 ± 6	86 ± 4*[a]	124 ± 7	88 ± 8*[a]
Arterial pH	7.38 ± 0.04	7.37 ± 0.06	7.36 ± 0.03	7.37 ± 0.06	7.36 ± 0.04	7.36 ± 0.03
PaO_2	80.56 ± 0.18	82.54 ± 0.23*	81.34 ± 0.22	81.67 ± 0.54	81.43 ± 0.54	81.54 ± 0.44
$PaCO_2$	34.67 ± 0.18	33.15 ± 0.23	34.32 ± 0.22	33.87 ± 0.23	34.08 ± 0.43	33.67 ± 0.32

Values are mean ± SD; * $P < 0.05$; ** $P < 0.01$, compared to controls; [a] $P < 0.05$; [aa] $P < 0.01$, compared to 4 h heat stress (HS), ANOVA followed by Dunnet's test for multiple group comparison, [b] many microhaemorrhages in the stomach wall; *MABP*, mean arterial blood pressure

Fig. 1. Shows extravasation of Evans blue in one untreated rat subjected to 4 h heat stress at 38°C (**a**) and its modification by pretreatment with L-NMMA (**b**). The dorsal surface of the cerebral cortex and spinal cord shows mild blue staining in the untreated rat, a feature not seen in the drug-treated animal. In this untreated heat stressed rat (**a**), the dorsal surface of cerebellar cortex took only faint staining (bar = 12 mm)

either the ventricular walls or the dorsal surfaces of the internal brain structures were not evident. Gross examination of the rat brain from the 4 h heat stressed animals showed extravasation of the Evans blue dye on the dorsal surface of the cerebral cortex and in some rats on the cerebellum as well as on the brain stem and the spinal cord (Fig. 1). On the ventral surface of the brain, the pyriform cortex, hypothalamus, medulla, pons and brain stem were stained with the Evans blue dye. However, there were some minor variations in the staining pattern of the Evans blue dye on the dorsal and ventral surfaces of the brain in individual rats. Interestingly, these regional variations were most marked in the cerebellum. A mid sagital section of the brain was done to examine Evans blue staining in the inside of the brain structures. These observations show that the walls of the lateral ventricle were stained in 4 h heat stressed animals. The dorsal surface of hippocampus took mild blue staining. Extravasation of Evans blue was also evident in the massa intermedia and the dorsal thalamus. The anterior hypothalamus was mildly stained with the Evans blue dye.

Pretreatment with L-NAME or L-NMMA alone did not alter the pattern of extravasation of Evans blue

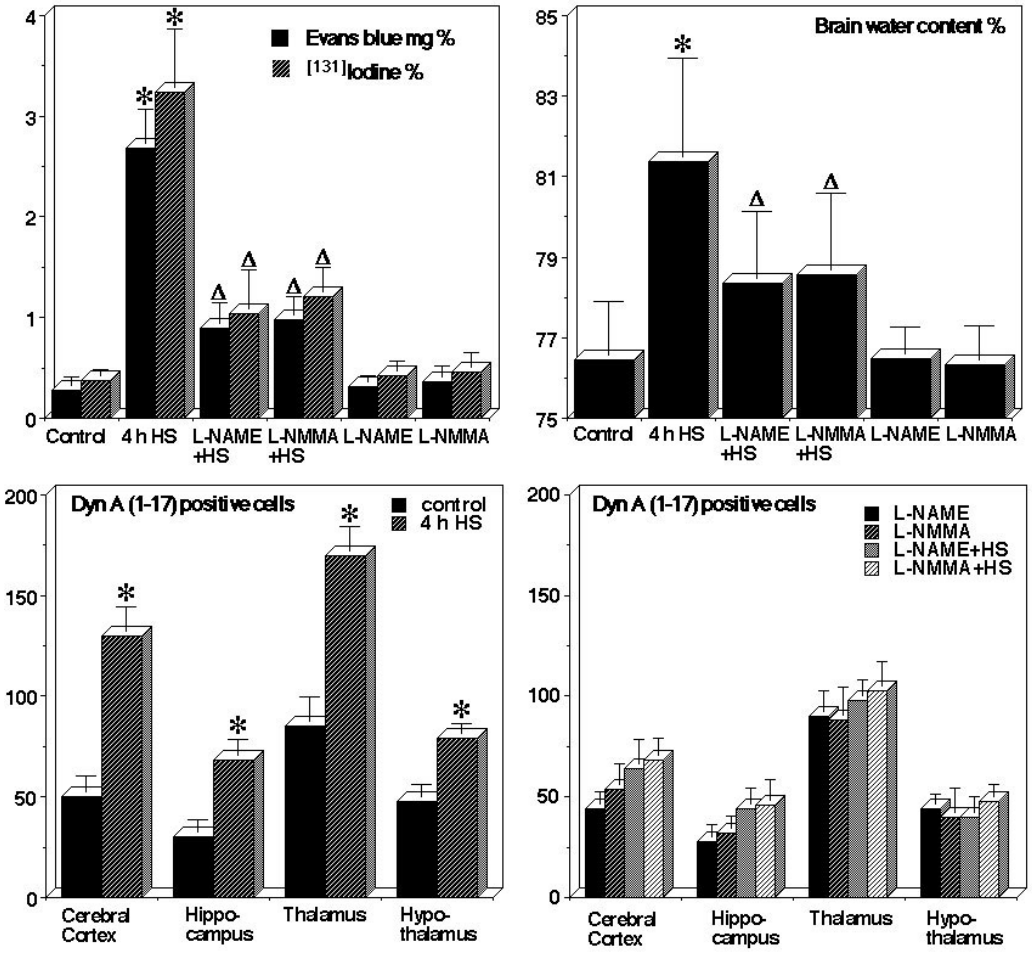

Fig. 2. Blood-brain barrier (BBB) permeability, brain edema and dynorphin immunoreactivity in untreated heat stressed rats and their modification with nitric oxide synthase (NOS) inhibitors. Untreated heat stressed rats exhibited profound increase in the permeability of the BBB (Evans blue and radioactive iodine) and brain water. This increase in the BBB permeability and brain edema formation is significantly reduced by two potent NOS inhibitors, L-NAME and L-NMMA (Upper panel). Semiquantitative data on dynorphin A immunoreactive cells in heat stress and their modification with L-NAME and L-NMMA are shown in the lower panel. Subjection of rats to heat stress (*HS*) significantly increased the number of dynorphin immunopositive cells in several brain regions. Pretreatment with L-NAME or L-NMMA markedly reduced this upregulation. * = $P < 0.01$, compared to control, △ = $P < 0.01$, compared to 4 h heat stress, ANOVA followed by Dunnet's test for multiple group comparison

dye in the control group. However, these drug treatments significantly attenuated the extravasation of Evans blue dye into the brain of rats subjected to 4 h heat stress (Fig. 1b). A marked reduction in the Evans blue staining in these drug-treated and stressed rats were also evident in the ventricular walls, dorsal surface of hippocampus as well as in the thalamus and hypothalamus. It appears that L-NMMA was more effective in attenuating Evans blue extravasation in the brain of heat stressed rats compared to the L-NAME.

Quantitative assessment of the tracers extravasation

Measurement of Evans blue staining in the brain showed a marked increase in the dye concentration in the brain compared to the control group (Fig. 2). A significant increase in the extravasation of radiotracer was also observed in the brain of heat stresses rats (Fig. 2). Pretreatment with L-NAME or L-NMMA in normal animals did not induce any marked changes in the extravasation of either Evans blue or [131]Iodine compared to the control group. However, when the drug treated rats were subjected to 4 h heat stress, the extravasation of these protein tracers were significantly reduced (Fig. 2).

Table 2. Dynorphin A (1–17) immunoreactivity in the rat brain following hyperthermia and its modification with nitric oxide synthase inhibitors in normal and heat-stressed rats

Rats were subjected to 4 h heat stress in a biological oxygen demand incubator (BOD) at 38°C (relative humidity 45–47%, wind velocity 20–25 cm/sec). L-NAME or L-NMMA were administered in separate group of rats (30 or 35 mg/kg, i.p.) daily for 7 days. On the 8th day, animals were subjected to heat stress. For details see text

Experiment type	Cerebral cortex					Hippocampus			Hypothalamus
	Posterior VI	Cingulate VI	Parietal VI	Occipital	Temporal	Dentate gyrus	CA1	CA3	Pre optic ant.
Control (5)	–	–/+	++	–	++	–	+	++	+/–?
L-NAME (4)	+	+	+	–/+?	+	–	–	++	++
L-NMMA (4)	+	+	++	–/+?	+	–	–	+/–	++
4 h heat stress (6)	+++	+++	+++	+++	+++	++	+++	+++	+/–?
L-NAME + HS (5)	++	++	++	–	–	–	+	+/–	++
L-NMMA + HS (5)	+	+	+/–	–	–	–	–	+/–	++

+, mild; ++, moderate; +++, intense; –, absent; +/–, variable; *?*, not clear; *VI*, layer VI in the cortex. Figures in parentheses indicate number of animals

Brain edema formation

Measurement of brain water content revealed profound increase in animals subjected to 4 h heat stress compared to the control group (Fig. 3). There was a mean increase of 4.5% brain water content in the heat stressed rats. This increase in brain water is comparable to more than 18% of volume swelling (Elliott and Jasper, 1949). Pretreatment with L-NAME or L-NMMA significantly attenuated the increase in brain water content following heat stress. Thus, the L-NAME or L-NMMA treated stressed rats exhibited only a 2% increase in the brain water content that corresponds to about 8% volume swelling compared to the control group. However, pretreatment with L-NAME or L-NMMA in normal rats did not influence the brain water content significantly (Fig. 2).

Dynorphin A (1–17) immunoreactivity

Normal animals

Dynorphin A immunoreactivity is observed in nerve cells and nerve fibres in several brain regions in the normal rat as described previously by other workers (see Fallon and Cioffi, 1984). In the cerebral cortex, the peptide immunostaining was mainly present in the cellular layer VI. There were some minor variations in the intensity of dynorphin A immunoreactivity in the cerebral cortex. The parietal cortex and occipital cortex were particularly rich in the dynorphin immunostaining compared to the cingulate and the temporal cortices. In the hippocampus, the peptide immunostaining was mainly seen in the dentate gyrus and in the CA 3 region. In the thalamus, very few dynorphin A immunoreactive nerve cells were present. However, some nerve fibres in the thalamus were Dynorphin A positive. The hypothalamus, exhibited some Dynorphin A immunostained nerve cells and nerve fibres mainly located into the pre-optic anterior hypothalamus (Table 2).

4 h heat stressed animals

Subjection of animals to 4 h heat stress resulted in a profound upregulation of dynorphin A (1–17) immunostaining in many regions of the cerebral cortex, hippocampus, thalamus and hypothalamus (Table 2). This upregulation of dynorphin immunoreactivity was seen in distorted neurons or in the regions

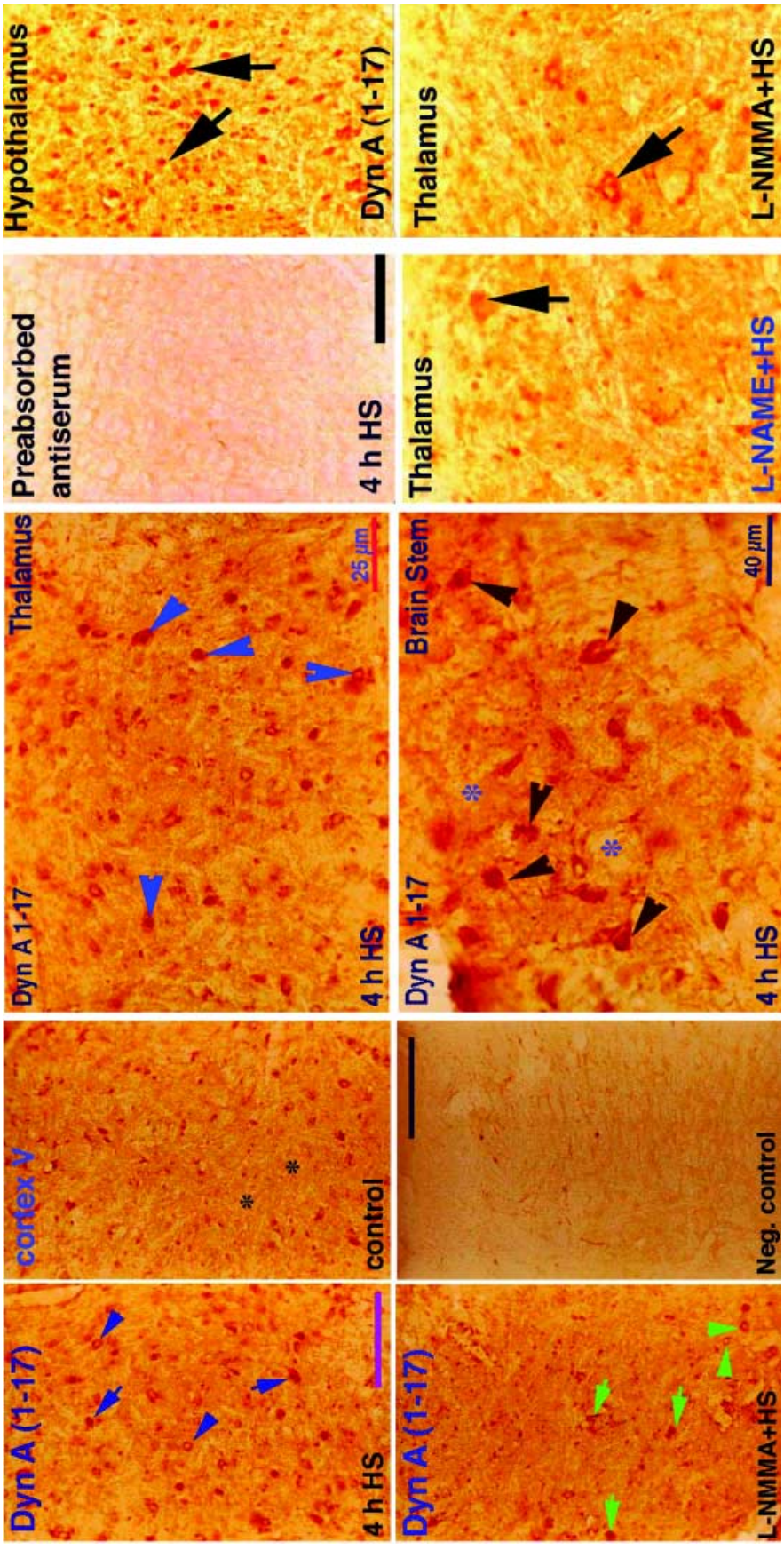

Fig. 3. Representative examples of dynorphin A (1–17) immunoreactivity in one control, one heat stressed rat and its modification with L-NAME or L-NMMA treatment. Dynorphin A (*Dyn*) immunoreactivity is significantly increased (arrows) in cortex, thalamus, hypothalamus and brain stem following heat stress compared to the control rats (cortex). Pretreatment with L-NAME or L-NMMA markedly attenuated the heat stress (HS) induced upregulation of dynorphin immunoreactvity as shown in the cortex (*L-NMMA*), and thalamus (*L-NAME* and *L-NMMA*). The dynorphin immunoreactivity could not be seen when the antiserum was either omitted (negative control) or the preabsorbed antiserum with synthetic Dyn A was applied (for details see text)

associated with sponginess and edema. Many dynorphin positive neurons can be seen in the heat stressed animals in the areas which normally do not exhibit the peptide immunoreaction in normal animals (Fig. 2). In the cortex, there is a significant upregulation in the dynorphin immunoreactivity in the layers III to V. This upregulation of dynorphin immunoreactivity was mainly pronounced in cingulate, occipital, parietal and temporal cortices. The pyriform cortex also exhibited an upregulation of dynorphin A immunoreactivity, not commonly seen in the control group. In thalamus, that normally exhibits only a few dynorphin positive fibres, upregulation of the peptide was seen in many nerve cells (Fig. 3). Similarly, in the brain stem, where dynorphin A immunoreactivity was not present in normal animals, exhibited profound upregulation of the peptide in many nerve cells (Fig. 2). In the hypothalamus, the dynorphin immunostaining was seen widespread in the heat stressed animals in the whole anterior and posterior regions compared to the control group which often exhibited few dynorphin stained nerve cells and fibers in the pre-optic anterior region (Fig. 2).

Effect of NOS inhibitors

In normal animals, pretreatment with either L-NAME or L-NMMA did not influence the dynorphin immunoreactivity (Table 1). However, this drug treatment significantly attenuated the dynorphin upregulation in the brain of rats subjected to 4 h heat stress (Fig. 2). Thus, in L-NAME or L-NMMA treated and heat stressed animals did not show much dynorphin A immunostaining in the cerebral cortex, hippocampus, thalamus and hypothalamus (Table 1). This effect was most pronounced in animals treated with L-NAME (Fig. 2).

Ultrastructural changes

Morphological examination of several brain regions exhibiting an upregulation of dynorphin A immunoreactivity in heat stress, revealed profound cell injury. Thus, marked edema, membrane disruption, axonal damage, myelin vesiculation and distortion of nerve and glial cells were evident in these regions of the brain in heat stressed rats compared to the control group. A representative example of ultrastructural damage in the layer VI of the parietal cerebral cortex in heat stress rat is shown in Fig. 4a. Membrane damage, edema and axonal injury are clearly seen in this heat stressed animal.

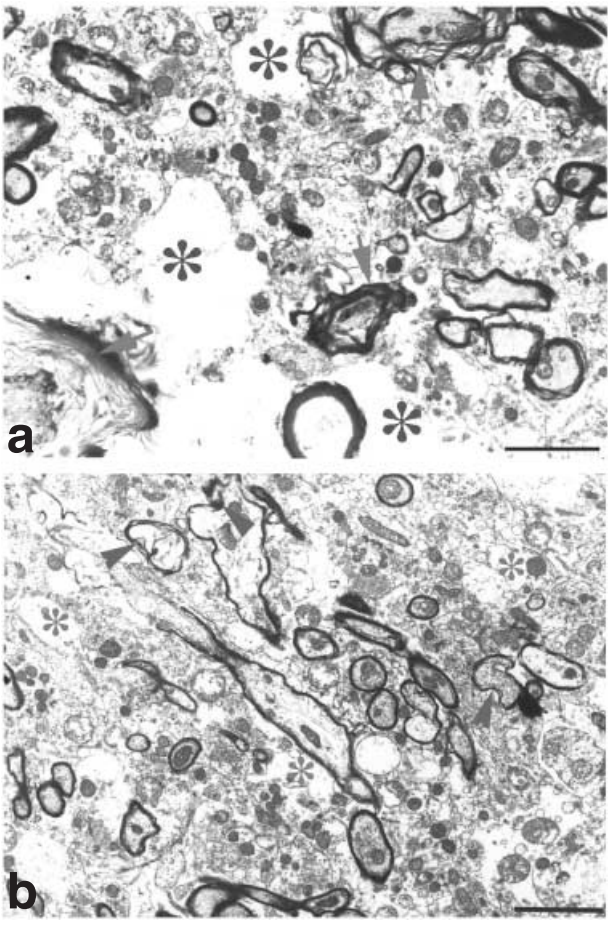

Fig. 4. Low power transmission electron micrograph from the parietal cerebral cortex (layer V) of one 4 h heat stressed rat (**a**) and its modification with L-NAME pretreatment (**b**). Marked cellular injury edema and membrane damage (*) can be seen in the untreated heat stressed rat (**a**), a feature mainly absent in the L-NMMA treated heat exposed animal (**b**) (bar = 800 nm)

Pretreatment with either L-NAME or L-NMMA markedly attenuated the ultrastructural changes in the several brain regions of the heat stressed rats. Thus, in these drugs treated rats, edema, membrane damage, neuronal, glial or axonal injury was less common compared to the untreated heat stressed animals. Ultrastructural changes in the layer VI of parietal cortex of one heat stressed rat pretreated with L-NAME are shown in Fig. 4b. Edema, membrane damage and axonal changes are much less evident in this drug-treated heat stressed animals compared to the untreated heat exposed rats (Fig. 4a). It appears that L-NAME pretreatment has far superior effects in attenuating the ultrastructural changes in the heat stressed rats

compared to the L-NMMA treated animals. This effect appears to be most pronounced in the cerebral cortex, hippocampus, and hypothalamus.

Discussion

The salient new findings of the present investigation clearly show that 4 h heat stress has the capacity to induce an upregulation of dynorphin A (1–17) immunoreactivity in several brain regions. In many brain regions, dynorphin A immunoreactivity is seen in the areas that normally do not exhibit the peptide activity. This indicates that dynorphin immunoreactivity can be induced by hyperthermia, not reported earlier. It is interesting to note that the induction of dynorphin immunoreactivity following heat stress is seen in the distorted nerve cells located in the edematous regions of the brain. These observations suggest that dynorphin can contribute to the cell injury in hyperthermia.

The pathophysiology of heat stress includes breakdown of the BBB leading to the extravasation of protein tracers in several brain regions (Sharma and Dey, 1987). Extravasation of proteins in the extracellular compartment of the brain will results in vasogenic edema formation and cell injury (Sharma and Cervós-Navarro, 1990a). The present results further show that the regions that exhibited profound BBB disruption, edema formation and cell injury also showed marked upregulation of dynorphin A immunoreactivity. This indicates that upregulation of dynorphin in heat stress is related to cell injury.

The detailed mechanisms by which dynorphin can induce cell injury are not clear. An increase in dynorphin immunoreactivity suggests that the peptide can influence a direct action on the nerve cells via specific opioid receptors, or through some non-opioid mechanisms (Faden, 1990, 1993). The present results do not suggest the involvement of any particular opioid receptor sub-types or non-opioid receptor mediated effects of dynorphin in causing cell injury in heat stress. It seems likely that both opioid and non-opioid mechanisms are responsible for dynorphin induced cell damage in heat stress.

In the brain and spinal cord, dynorphin interacts particularly with κ-opioid receptor (Mansour and Watson, 1993). The peptide also has the capacity to bind with μ and δ-receptors (James and Goldstein, 1984). Dynorphin in high concentrations activates κ-opioid receptors (Faden, 1993). Activation of k-opioid receptors results in adverse cell reaction (Faden, 1990). This is apparent from the fact that κ-opioid receptor antagonists, such as nor-BNI exerts its power neuroprotective effects in the spinal cord following injury (Faden, 1993). Dynorphin has the capacity to inhibit other transmitters release by depressing calcium conductance (Cherubinin and North, 1985). This effect is supposed to be mediated by κ-opioid receptors since activation of μ and δ-receptors increase potassium conductance (Miahara and North, 1986). However, very little is known about the possible second messengers by which dynorphin receptors may interact.

That dynorphin has the capacity to induce cell injury is further evident from the results obtained with the administration of synthetic peptide directly into the brain or spinal cord. Thus, the synthetic peptide dynorphin when applied topically or administered intrathecally induces severe vasoconstriction resulting in ischemia and cell damage (Hu et al., 1999; Tang et al., 2000; Hauser et al., 2001). This suggests that the peptide has the capacity to induce cell injury in the spinal cord. This assumption gets further support from the findings that intrathecal administration of dynorphin antiserum into the spinal cord following trauma results in an increased motor performance (Faden, 1990). Similarly, topical application of dynorphin A (1–17) antiserum either 2 min before (Winkler et al., 2002) or 2 min after injury (Sharma et al., 1995) results in marked neuroprotection. There are reasons to believe that the dynorphin antiserum has the capacity to bind the antigens in vivo to neutralise the effects of endogenous dynorphin in the spinal cord (Han and Xie, 1984). Thus, an increased dynorphin immunoreactivity in this study, that probably reflects an increased synthesis or release of this peptide, seems to be capable of inducing adverse cell reactions in the brain following heat stress.

The details mechanisms by which dynorphin can induce neurotoxicity are still speculative. There are evidences that dynorphin can stimulate glutamate release in the CNS by acting through N-methyl-D-aspartate (NMDA) receptors (Tang et al., 2000). This idea gets further support with the findings that dynorphin antiserum has the capacity to antagonise NMDA receptors in vivo (Hauser et al., 2001). The effects of dynorphin antiserum are far more potent in reducing allodynia induced pain than that of the classical NMDA receptor antagonist MK-801 (Nichols et al., 1997). This observation suggests that dynorphin

can induce neurotoxicity through a glutamate mediated effects in the CNS.

Activation of glutamate receptors is known to induce generation of free radicals and formation of nitric oxide (Azbill et al., 1997). It appears that dynorphin upregulation in hyperthermia can influence nitric oxide formation. This is evident with the fact that hyperthermia induces profound upregulation of NOS in several brain regions at the same time (Sharma et al., 1998b). There are now abundant experimental and clinical evidences which suggest that upregulation of nitric oxide is injurious to the CNS following different pathological states including hyperthermia (Sharma et al., 1996; Alm et al., 1998; Sharma et al., 1998a, 2000b; Steinbush et al., 2000). Previous studies from our laboratory show that pretreatment with an antioxidant compound H-290/51 significantly prevented NOS upregulation and cell injury (Alm et al., 1998, 2000). These observations clearly suggest that upregulation of nitric oxide in hyperthermia is associated with the cell injury.

Another most important finding of this investigation is that pretreatment with NOS inhibitors significantly attenuated dynorphin immunoreactivity in the brain. This is evident with the fact that pretreatment with two potent NOS inhibitors, L-NAME and L-NMMA significantly attenuated dynorphin immunoreactivity in the CNS. This observation suggests that blockade of NOS is somehow responsible for downregulation of the dynorphin in the brain following hyperthermia. The detail mechanism of how NOS inhibitors induced downregulation of the dynorphin A immunoreactivity in hyperthermia is not clear from this investigation.

Hyperthermia is associated with profound oxidative stress (Sharma and Westman, 1998, 2000). Thus, hyperthermia induced oxidative stress seems to play key roles in generation of free radicals and formation of nitric oxide. It appears that formation of nitric oxide is one of the important factors in dynorphin upregulation in hyperthermia. Alternatively, an upregulation of dynorphin may also contribute to hyperthermia induced nitric oxide formation. That nitric oxide plays an important role in dynorphin A upregulation is evident from the findings that pretreatment with NOS inhibitors markedly attenuated the dynorphin A immunoreactivity in the brain of heat stressed rats. This suggests that hyperthermia induced nitric oxide formation is one of the important stimulant for dynorphin A upregulation in the brain. A direct inhibitory effect of NOS inhibitors on dynorphin immunoreactivity is less likely. This is supported from the fact that pretreatment with NOS inhibitors in normal rats did not significantly attenuate the dynorphin A immunoreactivity.

Upregulation of NOS requires activation of intracellular Ca^{2+} (Hauser et al., 2001). An increase in intracellular Ca^{2+} is responsible for cell death (Balentine, 1988). There are reports that dynorphin induced motor neurons death in culture studies is accompanied by an increase in intracellular Ca^{2+} (Tang et al., 2000; Hauser et al., 2001). Thus, it seems likely that dynorphin may stimulate nitric oxide synthesis via accumulation of intracellular Ca^{2+}. It may be that dynorphin and/or nitric oxide in hyperthermia induces an intracellular accumulation of Ca^{2+} leading to cell death. Pretreatment with a potent calcium channel blocker nimodipine that induced neuroprotection in hyperthermia supports this hypothesis (Sharma and Cervós-Navarro, 1990b). Taken together, our results suggest that an interaction between dynorphin and nitric oxide seems to play important roles in the CNS dysfunction in hyperthermia.

The dynorphin also participates in inflammatory responses. Sydbom and Terenius (1986) reported an increased release of histamine from mast cells caused by dynorphin in a dose dependent manner. Chahl and Chahl (1986) found that dynorphin induce plasma extravasation. These observations are in good agreement with our study in hyperthermia. Thus an increased immunoreactivity of dynorphin in several brain regions following hyperthermia seems to be responsible for breakdown of the BBB permeability. Activation of histamine and other neurotransmitter such as serotonin, prostaglandin and nitric oxide by dynorphin will induce breakdown of the BBB permeability and brain edema leading to cell injury.

In conclusion, our results suggest that hyperthermia has the capacity to upregulate dynorphin immunoreactivity in the brain. Inhibition of NOS activity in heat stress attenuates the dynorphin immunoreaction and cell injury. This indicates that dynorphin induced neurotoxicity in hyperthermia is mediated via mechanism involving nitric oxide, not reported earlier.

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