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The influence of delayed postischemic hyperthermia following transient focal ischemia: Alterations of gene expression

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Abstract

We have recently shown that moderate hyperthermia, even if delayed, markedly enlarges the volume of an acute ischemic infarct. In the current study, we used in situ hybridization autoradiography to assess the effects of delayed hyperthermia on the regional expression of messenger RNA (mRNA) for the immediate early genes c-fos and c-jun, the inducible heat-shock protein 70 (hsp70) and glial fibrillary acid protein (GFAP) following 1 h of transient middle cerebral artery occlusion (MCAo) produced in rats by the insertion of an intraluminal suture. Sham-occluded rats were also studied. One day after MCAo, rats were placed into a heating chamber, where cranial temperature was either maintained at 37–38°C (normothermic group) or was elevated to 40°C (hyperthermic group) for 3 h. At either 2 or 24 h thereafter, brains were studied by in situ hybridization. Low-level constitutive c-fos and c-jun expression in sham-occluded rats was unaffected by delayed temperature manipulation. Prior MCAo decreased c-fos and c-jun mRNA in the affected striatum and overlying cortex. In rats studied 2 h after delayed hyperthermia, however, c-fos mRNA was markedly *increased* in ipsilateral cingulate cortex. By contrast, the pattern of c-jun mRNA was similar in rats with prior MCAo irrespective of delayed normothermia or hyperthermia: increased expression involved ipsilateral cingulate and paramedian cortical areas. Bilateral increases in hsp70 expression were produced by hyperthermia alone, and hsp70 mRNA was densely increased throughout the ischemic cortex and striatum following MCAo, while delayed hyperthermia altered this pattern by extending the zone of increased hsp70 message to cingulate and paramedian cortical areas at 2 h. GFAP mRNA was decreased within the previously ischemic field but increased in surrounding regions. The induction of c-fos and hsp70 message in tissue regions abutting zones of enhanced injury in brains with delayed postischemic hyperthermia indicates that these zones have been additionally stressed: these gene responses may possibly contribute to the protection of these threatened regions.

Keywords: Gene expression; Messenger RNA; In situ hybridization; Autoradiography; Middle cerebral artery occlusion; Immediate early genes; Heat shock protein; Glial fibrillary acidic protein; Rat

1. Introduction

A persuasive body of evidence has accumulated within recent years, demonstrating that moderate hyperthermia is capable of markedly exacerbating the degree of neural injury resulting from acute brain ischemia or trauma; this evidence has been recently summarized [15]. The results of experimental studies have been reinforced by recent clinical reports showing that fever during the first week of hospitalization following acute stroke is an independent risk factor associated with worse prognosis [5], and that body temperature in acute stroke patients on admission to hospital is highly correlated with stroke severity, infarct size, and increased mortality [37].

In two recent studies from our laboratory, we have noted a dramatic accentuation of brain injury resulting from the superimposition of moderate hyperthermia *one day after* a focal or global ischemic insult [8,19]. In the focal-ischemia study, rats were subjected to 60 min of transient middle cerebral artery (MCA) occlusion followed 1 day later by a

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3-h period of hyperthermia. This delayed hyperthermia markedly increased infarct volume [19].

The present study extends this work by exploring the molecular mechanisms by which delayed hyperthermia might influence the outcome of prior transient focal ischemia. We again employed a model of 1-h MCA occlusion produced by the insertion of an intraluminal suture, followed 1 day later by a 3-h period of hyperthermia to 40°C. We used in situ hybridization autoradiography to assess the effects of these manipulations on the regional expression of message for the immediate early genes c-fos and c-jun, as well as heat-shock protein 70 (hsp70) and glial fibrillary acid protein (GFAP). This in situ hybridization study expands upon, and complements, a preliminary study of this problem (reported in abstract form) in which dot blots were used to analyze mRNA in four brain regions of interest [20].

2. Materials and methods

Male Sprague–Dawley rats weighing 280–320 g, obtained from Charles River Laboratories, Wilmington, MA, were used in this study. The animals were fasted overnight but allowed free access to water. Experimental protocols were approved by the University of Miami School of Medicine's Animal Research Committee. Anesthesia was induced with 3% halothane. Rats were intubated endotracheally and were ventilated mechanically with a mixture of 0.75% halothane, 70% nitrous oxide, and a balance of oxygen. Atropine sulfate (0.3 mg) was injected intraperitoneally. Polyethylene catheters were inserted into a femoral artery and vein. Arterial blood was sampled periodically for blood gases, which were regulated at normal levels by ventilatory adjustments. Arterial blood pressure was continually monitored. Cranial temperature was monitored by a pre-calibrated 33-gauge thermocouple (CN9000, Omega, Stamford, CT) inserted into the right temporalis muscle and was controlled at 37.5°C by a small high-intensity lamp above the head. Rectal temperature was separately monitored and controlled at 37.0–37.5°C by a heating lamp above the body.

2.1. Production of focal cerebral ischemia

A 60-min period of middle cerebral artery (MCA) occlusion was produced by the intraluminal suture technique as described by [44], in a fashion identical to that reported in our previous study [19]. The right common carotid artery was exposed. The external carotid artery and its branches, as well as the pterygopalatine artery, were occluded. Heparin, 0.1 ml (150 IU/ml) was administered intravenously. A 3–0 monofilament nylon suture, whose tip had been rounded by heating, was threaded retrogradely through the external carotid artery stump into the internal carotid artery and thence into the origin of the MCA, a distance of 18–19 mm, depending upon the body weight. Following 60 min of MCA occlusion, the intraluminal filament was withdrawn to permit reperfusion. Incisions were closed, and rats were allowed to awaken and then returned to their cages. Sham-operated animals underwent all surgical procedures except for occlusion of the MCA.

2.2. Delayed temperature modulation

This procedure was carried out 24 h after transient MCA occlusion. Prior to this point, rats were fasted for 12 h but allowed free access to water. They were then reanesthetized as described above, intubated and ventilated mechanically. Temperature probes were reintroduced into the temporalis muscle and rectum as described above. (Invasive brain temperature monitoring was avoided due to the possible risk of gene induction [17,32]). The head was then secured in a stereotaxic apparatus. Rats were then allowed to awaken and were placed into a heating chamber for temperature modulation. The cable of the cranial temperature probe permitted free movement (CMA/120 System, Carnegie Medicin AB, Stockholm, Sweden).

The heating chamber was similar to that used in our previous study [19]. It measured 70×45×50 cm (length× width×height) and was equipped with a heating lamp, humidifier and oxygen supplier. The ambient temperature controlled the heating lamp via a servo-regulated temperature controller (CN76000 Autotune, Omega, Stanford, CT). The chamber also contained a shaded area in which the animal could escape the heating lamp.

Rats with prior MCA occlusion (I), and sham-operated animals (S), were divided into subgroups according to the delayed temperature protocol. Each subgroup consisted of five rats. In the normothermic (N) subgroups, cranial temperature was maintained at 37–38°C for a 3-h period. In the hyperthermic (H) subgroups, cranial temperature was maintained at 40°C. The duration of heating was timed from the point that temperatures reached the target temperature. During this time, animals received humidified oxygen. Following the 3-h thermoregulation period, rats were returned to their home cages. At either 2 h or 24 h after normothermic or hyperthermic temperature modulation, rats were reanesthetized with halothane, and brains were removed and examined for gene expression by in situ hybridization.

2.3. In situ hybridization

Brains were removed quickly and frozen by immersion in dry ice-cooled 2-methyl butane and stored for sectioning at −80°C. After the brains were brought to −20°C, they

were cryosectioned onto Probe-On Plus slides (Fisher Scientific) under RNAse free conditions and stored dessicated at −80°C until use.

Fresh frozen sections were thawed to room temperature and fixed for 5 min in 4% formaldehyde in phosphate-buffered saline. Sections were acetylated for 10 min at room temperature in 0.25% acetic anhydride and 0.1 M triethanolamine HCl (pH 8), then dehydrated through a series of graded ethanols, delipidized in 100% chloroform for 5 min, rinsed in 100% ethanol, and allowed to air-dry.

Sections were hybridized to ^{35}S-labeled riboprobes generated by in vitro transcription of the anti-sense (for positive probe) and sense (for negative probe) strands of cDNA clones subcloned into transcription vectors using the Progmega Riboprobe System. The rat inducible hsp70 cDNA clone was supplied by F.R. Sharp [30]. A 248bp ApaI–XhoI fragment from the 3′ end of the gene was subcloned into pGEM7zf for use as a probe because this region shows the least homology to HSP72 and does not cross-hybridize at the stringency used in this study, as verified by Northern analysis (data not shown).

The mouse genomic clone, pc–fos mouse 3* [12], was obtained from ATCC. A 3.2 KB Bam H1–EcoR1 fragment was subcloned into pGEM7zf.

GFAP was detected using a human cDNA clone, HHCPF23 [2], obtained from ATCC. This clone is a 2.3 KB fragment in pBluescript SK-. Jun was probed for using the mouse cDNA clone in pGEM-2, also obtained from ATCC [38]. Jun, fos and GFAP antisense probes were sheared by limited alkaline hydrolysis to an average size of 200bp prior to use.

The denatured probe (2×10^7 dpm/ml) was added to a solution containing 100 μg/ml salmon testes DNA, 250 μg/ml each of yeast total RNA and tRNA, 50% formamide, 20 mM Tris HCl (7.4), 1 mM EDTA, 300 mM NaCl, 10% dextran sulfate, and 1×Denhardts. The hybridization solution was added to the sections, covered with coverslips, and hybridized under humid conditions at 55°C for 20 h. After removal of the coverslips, the sections were washed at room temperature in a series of decreasing amounts of SSC (standard saline citrate) with 1 mM DTT to a final wash of 0.1x SSC. The slides were treated with 20 μg/ml RNAse A for 30 min at 37°C. The final high stringency wash was carried out in 0.1x SSC, 1 mM DTT for 1 h at 65°C. Sections were dehydrated through a series of ethanols containing 300 mM ammonium acetate, ending with 100% ethanol.

Sections were exposed to Amersham Hyperfilm at 4°C for various amounts of time and were developed in Kodak D-19 for 4 min and fixed in Kodak rapid fixer. Following film exposure, slides were dipped in Kodak NTB2 emulsion diluted 1:1 with water and exposed for various times at 4°C. Following 2.5-min development in 15°C Dektol Developer (Kodak), slides were counterstained in 1% cresyl violet and coverslipped for microscopic dark field photography. Negative control (sense strand) probes showed no hybridization signals (data not shown).

3. Results

3.1. Physiological variables

Physiological values in the normothermic and hyperthermic groups are shown in Table 1. The preischemic and intraischemic values for arterial blood gases and pH, mean arterial pressure, and intraischemic plasma glucose were similar among the groups.

3.2. c-fos expression – sham-occluded animals

Low homogeneous levels of mRNA were evident bilaterally throughout the neocortex and all sectors of the hippocampus, including the dentate gyrus. Prominent c-fos expression was noted bilaterally in the primary olfactory cortex, together with lower levels of expression in striatum and thalamus bilaterally. This pattern was identical in sham-occluded animal groups with either delayed normothermia (SN) or delayed hyperthermia (SH) (Fig. 1) and was similar in brains examined at either 2 h or 24 h after these manipulations.

3.3. c-fos expression – animals with prior MCAo

In the majority of rats (three of five) with MCA occlusion followed by delayed normothermia and 2-h survival (IN-2), c-fos expression was markedly decreased throughout the ipsilateral striatum and the overlying lateral and dorsolateral regions of neocortex, but with patchy foci of increased expression within the middle layers of the lateral cortex ipsilateral to prior MCAo (Fig. 1). Constitutive levels of c-fos mRNA were present in the ipsilateral cingulate cortex and in the contralateral neocortex (Fig. 1). In one rat, however, decreased levels of c-fos mRNA were confined to the posterior dorsolateral striatum; and a fifth rat resembled its sham normothermic control (SN-2).

Ischemic rats with delayed hyperthermia studied at 2 h (IH-2) also showed markedly decreased c-fos mRNA in the striatum and the lateral and dorsolateral cortex ipsilateral to prior MCAo. (In one rat, decreased c-fos mRNA was confined to the ipsilateral striatum). However, the majority of IH-2 rats (four of five) differed importantly from their ischemic–normothermic group counterparts (IN-2) in having markedly *increased* c-fos mRNA in the ipsilateral cingulate cortex (Fig. 1). In two of these rats, c-fos mRNA was increased as well in portions of ipsilateral thalamus, and individual rats variously showed increased c-fos expression in ipsilateral hippocampus (but with decreased expression in the dentate gyrus), the inner layers of ipsilateral lateral and dorsolateral neocortex,

Table 1
Physiological variables

	MABP (mmHg)	PCO_2 (mmHq)	PO_2 (mmHg)	pH	Glucose (mg/dl)
Ischemia, normothermia, 2-h survival (n=5)					
Preischemia	113±10	35.0±3.4	147±27	7.46±0.03	
Ischemia	112±7	36.5±3.1	137±22	7.41±0.05	103.1±34.0
Ischemia, hyperthermia, 2-h survival (n=5)					
Preischemia	114±14	28.5±6.2	151±23	7.50±0.03	
Ischemia	114±10	36.5±2.0	141±14	7.46±0.02	94.6±13.4
Sham, normothermia, 2-h survival (n=5)					
30 min after operation	110±12	31.6±2.4	121±21	7.46±0.05	
60 min after operation	106±12	35.3±3.5	130±13	7.46±0.02	105.9±21.5
Sham, hyperthermia, 2-h survival (n=5)					
30 min after operation	110±9	33.9±4.3	132±20	7.48±0.03	
60 min after operation	108±10	33.7±1.9	127±9	7.46±0.02	126.4±28.9
Ischemia, normothermia, 24-h survival (n=5)					
Preischemia	113±13	35.0±3.1	125±20	7.47±0.02	
Ischemia	111±10	39.1±2.5	121±12	7.44±0.03	110.1±10.6
Ischemia, hyperthermia, 24-h survival (n=5)					
Preischemia	111±12	35.0±3.4	123±28	7.48±0.03	
Ischemia	111±12	37.6±6.1	130±17	7.44±0.04	132.1±13.9
Sham, normothermia, 24-h survival (n=5)					
30 min after operation	117±9	33.5±4.7	123±34	7.47±0.02	
60 min after operation	116±12	37.6±2.3	127±38	7.44±0.01	115.8±22.9
Sham, hyperthermia, 24-h survival (n=5)					
30 min after operation	109±10	33.9±1.8	122±6	7.47±0.04	
60 min after operation	103±8	37.1±2.1	107±29	7.43±0.03	95.1±11.2

Values are mean±S.D.
One-way ANOVA revealed no significant inter-group difference for any variable.

ipsilateral septal nuclei, and ipsilateral amygdaloid complex.

In ischemic normothermic rats with 24-h survival (IN-24), the majority (four of five rats) showed a pattern of c-fos expression largely similar to the predominant pattern of the IN-2 group: decreased c-fos expression affected the dorsolateral two-thirds of ipsilateral striatum and the overlying lateral and dorsolateral regions of neocortex, sparing cingulate cortex and hippocampus (Fig. 1). In one rat, however, decreased c-fos expression was confined to the striatum. In the majority of 24-h ischemic rats with delayed hyperthermia (IH-24), c-fos expression was similar to the predominant IN-24 pattern, with markedly decreased expression involving the entire striatum and overlying lateral and dorsolateral neocortical regions. In both the affected IN-24 and IH-24 rats, ipsilateral hemispheral swelling was evident. One IH-24 rat showed no abnormality of c-fos expression. All of the increased c-fos mRNA evident in the cingulate gyrus and other regions of the 2-h ischemic–hyperthermic group (IH-2) had subsided by 24 h.

3.4. c-jun expression – sham-occluded animals

Both SN and SH brains showed low-level mRNA within the dentate granule cells and sectors CA1 and CA3 of hippocampus bilaterally, against an otherwise nonspecific background (Fig. 2). This pattern was identical in SN and SH brains at both 2 h and 24 h following temperature manipulations.

3.5. c-jun expression – animals with prior MCAo

In rats with 1-h MCA occlusion followed by delayed temperature manipulation, the pattern of c-jun mRNA was essentially similar in brains with both delayed normothermia and hyperthermia, and at both the 2 h and 24 h study points (Fig. 2). In all cases, the pattern differed from the corresponding sham (SN and SH) groups in containing a zone of *increased* c-jun mRNA involving the cingulate and paramedian cortex ipsilateral to prior ischemia. In the 2-h subgroups, this finding was present in two of five brains with delayed normothermia (IN-2) and in four of five brains with delayed hyperthermia (IH-2); in each group, it was associated with decreased expression throughout the ipsilateral lateral and dorsolateral neocortex and striatum. The majority of both normothermic and hyperthermic rats studied at 24 h (i.e. IN-24 and IH-24 groups) also showed ipsilaterally decreased c-jun involving the ischemic cortex and striatum (Fig. 2).

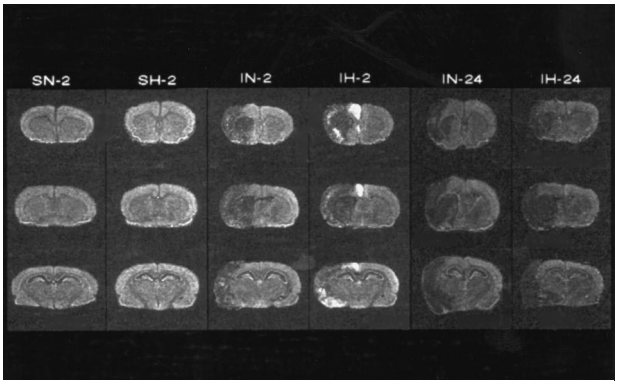

Fig. 1. In situ hybridization autoradiographs of c-fos mRNA at three coronal levels in representative brains of various experimental groups. The abbreviations SN and SH refer to rats with sham-occlusion of the MCA followed by delayed normothermia or hyperthermia, respectively. Groups IN and IH refer to MCA occluded rats with delayed normothermia or hyperthermia, respectively. The designations '-2' and '-24' refer to 2 h and 24 h survival durations following delayed temperature manipulation. Figure panels display in situ hybridization images of relative optical density in reverse-video at a uniform threshold and gain to permit valid inter-group comparisons. MCA-occluded rats show decreased c-fos mRNA in both ipsilateral striatum and lateral/dorsolateral neocortex (groups IN-2, IH-2, IN-24 and IH-24). However, MCA occlusion followed by delayed hyperthermia increases c-fos mRNA markedly in the ipsilateral cingulate cortex, inner layers of dorsolateral and lateral neocortex, and amygdaloid complex (group IH-2). This has subsided by 24 h (IH-24).

3.6. hsp70 expression-sham-occluded animals

In sham brains studied at either 2 h or 24 h after normothermic temperature manipulation (SN-2 and SN-24), hsp70 mRNA was observed at low levels throughout the dentate gyrus and all sectors of the hippocampus bilaterally, against an otherwise non-specific background (Fig. 3). By contrast, in brains of sham rats studied at either 2 h or 24 h after delayed hyperthermia (SH-2 and SH-24), moderately increased hsp70 expression was apparent bilaterally throughout all layers of neocortex, as well as the dentate gyrus and all sectors of hippocampus. Hippocampal expression greatly exceeded that of the SN brains (Fig. 3). The respective hsp70 patterns at 2 h versus 24 h were identical.

3.7. hsp70 expression-animals with prior MCAo

In ischemic rats with delayed normothermia and 2-h survival (IN-2), two differing patterns of hsp70 mRNA were observed (Fig. 3, columns IN-2(a) and IN- 2(b)). In three of five brains (pattern (a)), hsp70 mRNA was densely increased throughout the ipsilateral neocortex (lateral and dorsolateral zones) and the entire subjacent striatum, thus demarcating the entire zone of antecedent ischemia [19]. In the remaining two brains (pattern (b), Fig. 3), a more restricted zone of increased hsp70 expression was present, affecting only the lateral cortex and dorsolateral striatum ipsilateral to prior ischemia. In most (four of five) ischemic–normothermic brains with 24-h survival (IN-24), this increased hsp70 expression persisted at both cortical and striatal sites, sparing the cingulate cortex. The hippocampus was unaffected in four of five of the latter brains, but in the fifth there was a focal region of increased

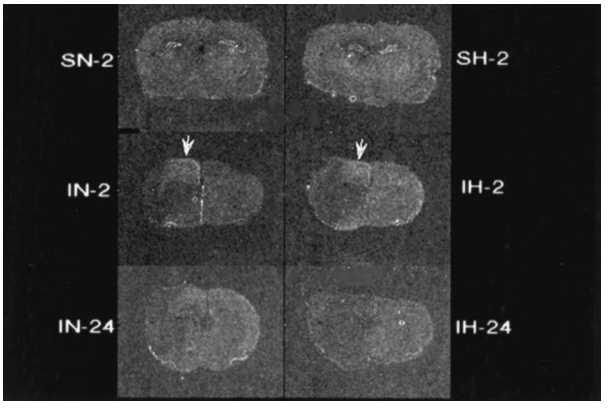

Fig. 2. In situ hybridization autoradiographs showing c-jun mRNA in representative sham-occluded and MCA-occluded rats with delayed normothermia or hypothermia. Group designations are as described in Fig. 1. Modestly increased c-jun expression is evident in the paramedian/cingulate cortex ipsilateral to prior MCA occlusion in rats with both delayed hyperthermia or normothermia (arrows). By contrast, c-jun mRNA is decreased in the dorsolateral and lateral neocortex and underlying striatum – the zones of prior ischemia.

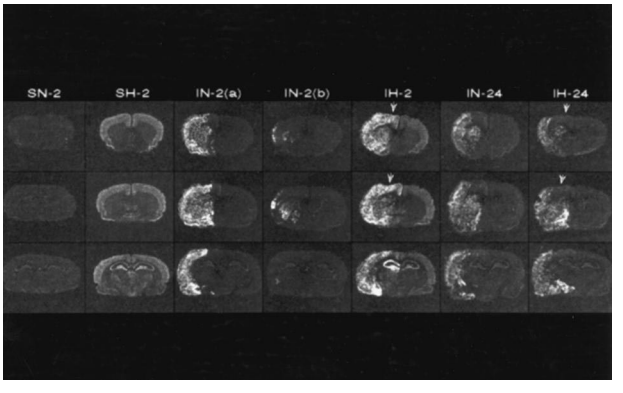

Fig. 3. In situ hybridization autoradiographs of hsp70 mRNA in sham-occluded rats and in rats with prior MCA occlusion receiving either delayed normothermia or hyperthermia, shown at three coronal levels. Abbreviations are as given in Fig. 1. In MCA-occluded rats with delayed normothermia studied 2 h later (group IN-2), two differing patterns of hsp70 mRNA are shown, designated as IN-2(a) and IN-2(b). In the absence of ischemia, hyperthermia increases hsp70 expression diffusely in neocortex and hippocampus (SH-2). Prior MCA occlusion, irrespective of delayed temperature manipulation, induces widespread ipsilateral hsp70 expression, more extensive than the histological infarct known to result from these insults. However, the increased hsp70 mRNA is more consistent in the IH than the IN group and extends in IH brains to involve paramedian/cingulate cortex (arrows) and ipsilateral hippocampus at 2 h following delayed hyperthermia (IH-2). By 24 h (IH-24), the increased cingulate and hippocampal expression noted at 2 h has subsided (IH-24, arrows).

hsp70 expression involving the lateral portion of the CA1 sector.

In brains with ischemia followed by delayed hyperthermia and 2-h survival (IH-2), the pattern of hsp70 expression differed from the corresponding normothermic group (IN-2) in showing more extensive zones of ipsilaterally increased expression throughout the neocortex, extending to involve the cingulate and paramedian cortex (in three of four brains). The ipsilateral striatum was involved as in IN-2 rats, and the hippocampus showed prominently increased hsp70 mRNA throughout areas CA1 and subiculum-zones not affected in the ischemic normothermic group.

hsp70 expression in the 24-h ischemic subgroups (IN-24 and IH-24) resembled one another in showing increased hsp70 mRNA throughout the ipsilateral cortex and stratum, as well as hippocampus (Fig. 3). Importantly, however, the conspicuously increased hsp70 mRNA present in the cingulate and paramedian cortex of 2-h ischemic–hyperthermic rats was not evident in animals surviving 24 h (Fig. 3, 1H-24). In one IH-24 brain, increased hsp70 mRNA was confined to a portion of the lateral cortex ipsilateral to prior MCAo.

3.8. GFAP expression-sham-occluded animals

GFAP mRNA patterns in brains of sham rats were identical, irrespective of delayed normothermia (SN) or hyperthermia (SH), and independent of survival time (2 h versus 24 h). In all cases, GFAP expression was confined to the ependymal surfaces of the lateral ventricles and subpial sites, against an otherwise non-specific background (Fig. 4).

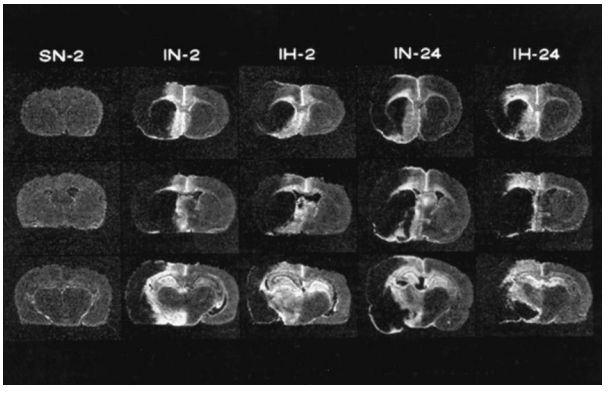

Fig. 4. In situ hybridization autoradiographs of GFAP mRNA at three coronal levels in sham-occluded and MCA-occluded rats with delayed normothermia or hyperthermia. Abbreviations are as described in Fig. 1. Sham-occluded rats show low-dose level GFAP expression in ependymal regions, irrespective of temperature manipulation. While prior MCA occlusion leads to increased GFAP expression in a variety of zones adjacent to the regions of frank ischemia, including contralateral sites, these regions do not differ substantially in rats with delayed normothermia versus hyperthermia. Regions of increased GFAP message include ipsilateral and contralateral cingulate cortices. Of note, GFAP expression is reduced within the zones of most severe prior ischemia.

3.9. GFAP expression-animals with prior MCAo

In almost all ischemic rats studied 2 h after delayed normothermia (IN-2), brains showed zones of prominently increased GFAP expression outlining the lateral margin of the thalamus (extending into hypothalamus) and the medial margin of the striatum and septal area ipsilateral to prior MCAo (Fig. 4). One-half of rats also showed increased GFAP mRNA in the paramedian and cingulate regions of ipsilateral neocortex, together with a smaller strip of the adjacent, contralateral cingulate cortex, and portions of the hippocampus bilaterally (ipsilateral $>>$ contralateral) (Fig. 4). By contrast, GFAP expression was *decreased* below control levels throughout the lateral and dorsolateral cortex and striatum ipsilateral to prior MCAo in three of the five IN-2 brains. In one brain, striatal GFAP expression was focally increased.

GFAP mRNA patterns in rats studied 2 h after delayed hyperthermia (IH-2) in general resembled those of the IN-2 group: all brains showed bands of increased expression involving the ipsilateral septum and medial margin of striatum and the lateral margin of thalamus and hypothalamus. Four of five IH-2 brains showed elevated GFAP mRNA involving paramedian cortex bilaterally and ipsilateral thalamus and hippocampus, and decreased GFAP message within the previously ischemic lateral and dorsolateral cortices and striatum itself (Fig. 4).

These patterns of increased and decreased GFAP expression persisted at 24 h after both delayed normothermia (IN-24) or hyperthermia (IH-24) (Fig. 4). In both groups, the majority of rats continued to show increased GFAP mRNA in the ipsilateral paramedian/cingulate cortex, at the medial margin of the ipsilateral striatum and septal area, and in the ipsilateral hippocampus. Foci of increased GFAP message involving ipsilateral thalamus were present in four of five IN-24 brains and in two of five IH-24 brains (Fig. 4). Decreased GFAP mRNA persisted within the dorsolateral and lateral cortical regions ipsilateral to prior MCAo.

4. Discussion

In our prior study [19], we characterized the histological changes resulting from a 60-min period of transient MCA occlusion compounded by delayed hyperthermia: In MCA-occluded rats without delayed hyperthermia, histological examination following 4-day survival showed well-demarcated zones of pancellular necrosis consistently affecting the caudoputamen, together with patchy infarction of the adjacent dorsolateral cortex and a zone of eosinophilic, shrunken neurons at the infarct margins [19]. By contrast, brains of MCA-occluded rats with delayed hyperthermia

(40°C) showed an extensive zone of neocortical and striatal infarction. The volumes of cortical infarction and total infarction were increased by six- and three-fold, respectively, by the addition of delayed 40°C hyperthermia to prior MCA occlusion.

Using this same model in the present study, we have now shown that a 3-h period of 40°C hyperthermia, when imposed 1 day following 60-min MCA occlusion, leads 2 h later to dramatic increases in c-fos expression, chiefly affecting cingulate cortex, deeper laminae of the ipsilateral dorsolateral/lateral cortex, and other regions (Fig. 1). Hyperthermia in the absence of prior ischemia (i.e. groups SH-2 (Fig. 1) and SH-24) however, fails to affect c-fos expression. These results confirm and extend our preliminary dot blot study [20], which showed significant upregulation of c-fos mRNA at 2 h after hyperthermic temperature modulation in the ipsilateral fronto–cingulate cortex. As the regions exhibiting increased c-fos expression in this study correspond to areas lying at the periphery of the prior ischemic field, these data suggest that *the interaction of delayed moderate hyperthermia with antecedent ischemia* imposes a stress on areas lying immediately peripheral to the zone of most severe prior ischemia.

The voluminous literature on altered gene expression in brain ischemia has recently been masterfully summarized [24] (see also Kinouchi et al., 1994 [21]). Immediate early genes (IEGs), which recognize specific DNA binding sites and regulate the expression of late-response genes, include the transcription factors belonging to the Fos and Jun families. Focal ischemia gives rise to IEG induction which is maximal at 0.5–1 h but which, depending upon the ischemic conditions, may persist for many hours [24]. In mild focal ischemia, IEG expression is limited to the ischemic region, but if more severe, may extend beyond it [3,4,32].

In permanent focal ischemia, other workers [21,42] have described the robust induction of c-fos mRNA throughout the ischemic cortex with short-duration (1 h) permanent MCA occlusion, but decreased c-fos within the ischemic core-cortex by 4 h, and persistence of increased c-fos message only in cingulate cortex and the peri-infarct surround by 24 h [21]. This widespread early c-fos induction has been hypothesized to be due to spreading depression (i.e. ischemic depolarizations) [42]-a possibility consistent with several reports showing that MK-801, a potent NMDA antagonist, blocks expression of fos and jun [14,40]. If this is so, the profound induction of c-fos in the cingulate cortex in the 2-h ischemic–hyperthermic group of the present study (IH2, Fig. 1) may indicate that delayed hyperthermia, when superimposed upon a zone of prior focal ischemia, leads to focal depolarizations. This hypothesis is readily amenable to experimental testing. It is also possible that the increased c-fos message itself contributes to the histological protection of these areas, as we know from our earlier studies that the cingulate gyrus is spared from histological injury in this model [9,19]. A recent study has reported that the prolongation and enhancement of c-fos expression by fasting was associated with a substantial degree of histological neuroprotection following focal vascular occlusions [29]. This supports the assertion that c-fos may be neuroprotective.

The *declines* in c-fos mRNA observed 1 day following MCA occlusion in the present study affected the cortical and subcortical regions (chiefly dorsolateral and lateral cortex and underlying striatum) known to lie within the moderate-to severely ischemic field in this focal–ischemia model [9,19]. However, in ischemic–normothermic rats (IN-24, Fig. 1) the extent of decreased c-fos expression exceeded the area expected to become infarcted following a 60-min period of MCA occlusion [9,19]. Thus, c-fos expression appears sensitive to degrees of ischemia less than those needed to produce frank infarction.

In the present study, modestly increased c-jun expression was noted at 1 day following 60-min MCAo in rats with either delayed normothermia or hyperthermia (Fig. 2, groups IN-2 and IH-2), but this had subsided 24 h thereafter (IN-24, IH-24). The ipsilateral paramedian/cingulate cortex was affected (Fig. 2). This finding suggests that prior ischemia alone is sufficient to stimulate expression of this immediate early gene.

As shown in prior studies, protein synthesis is inhibited within the core of a focal ischemic lesion, Thus, IEGs induced at the transcriptional level may not be translated after ischemia [24]. In the ischemic penumbra, however, in which CBF is somewhat higher and in which recurrent metabolic stress and ionic dyshomeostasis occur [6,7], this situation may differ. As noted above, NMDA antagonism blocks both ischemic depolarizations and IEG induction and protects tissue from ischemic injury [6,14,16,40]. However, the extent to which IEG expression contributes to neuronal death and/or survival is not understood [24].

The heat shock protein hsp70 was upregulated in the neocortex bilaterally by hyperthermia alone (Fig. 3, SH-2), and was strongly though variably induced throughout the ischemic hemisphere 1 day following MCA occlusion (Fig. 3, 1N-2(a), IN-2(b), IH-2). Rats with delayed hyperthermia, however, differed from their normothermic counterparts in showing consistent hsp70 expression, as well, involving the ipsilateral paramedian/cingulate cortex (IH-2); this had subsided 24 h later (Fig. 3, IH-24). The latter distribution reinforces the view that the ipsilateral cingulate gyrus is stressed when delayed hyperthermia is superimposed on brain somehow ‘sensitized’ by prior transient ischemia; it is possible that this hsp70 response may help to protect the cingulate gyrus from histological injury.

The heat shock gene hsp70 belongs to a large group of genes which may be induced by stressful events and may participate either in the injury process or in adaptive or protective tissue responses [31,34]. A fundamental characteristic of heat shock genes is the presence of heat shock elements in their promoter regions, to which various heat

shock factors may bind. Abundant evidence has established that hsp genes are induced in the brain by increasing body temperature [11,31]. Immunocytochemical studies have confirmed the induction of HSP70 not only in neurons but in glia. HSP protein is thought to be involved in the folding and stabilization of other proteins, and in the facilitation of their transport across cell membranes.

The large literature on hsp expression in cerebral ischemia has been recently reviewed [31,34,35,41]. In general, following short-duration permanent MCA occlusion, hsp70 is expressed more widely than the expected extent of infarction [42]. However, with permanent MCA occlusion of sufficient duration, hsp70 mRNA comes to be expressed chiefly within those neurons at the periphery of an ischemic focus and, to a small degree, within the core of the infarct itself. By contrast, HSP70 *protein* is expressed in endothelial cells within the infarct and in glial cells within and at the margins of the infarct [23]. On the basis of these studies, it was proposed that the ischemic penumbra could be delineated as that zone, lying peripheral to the infarct, in which hsp70 mRNA and HSP70 protein are expressed in neurons alone. In regions such as the caudoputamen, which are the most severely affected by early infarction with short-duration MCA occlusion, induction of hsp70 is suppressed [1,22]. These findings are reinforced by other studies which have carefully characterized the temporal sequence of HSP72 protein expression following graded periods of temporary MCA occlusion [27]. At 48 h following very brief MCA occlusion, HSP protein is expressed first in the caudoputamen, then progressively in lateral cortex; following 30-min MCAo, there is dense HSP protein expression throughout the lateral and dorsolateral cortex. However, with longer durations (60–120 min) of occlusion, HSP protein becomes progressively restricted to the dorsolateral-most portion of the previously ischemic cortical field [27].

The fact that MCA occlusion also leads to hsp70 mRNA in regions lying outside the primary ischemic field (e.g. hippocampus and thalamus) is consistent with either milder degrees of ischemia affecting those structures, or to the possible influence of diffusible substances within the CSF. Of interest in this regard, recent studies of hsp70 mRNA in transgenic mice with MCAo suggest that the reduction in oxidative stress in transgenic mice overexpressing CuZn-superoxide dismutase permits the more prolonged expression of hsp70 following MCA occlusion [18].

The 50-kDa glial fibrillary acidic protein (GFAP) constitutes the major intermediate filament of astrocytes [13,26]. Astrocytes are highly reactive in response to pathological insults [33], and increased GFAP is a hallmark of reactive astrocytosis [10]. While GFAP expression is well described in the setting of cerebral ischemia, frank necrosis appears not to be required for reactive astrocytosis [36,39].

In the present study, we noted the consistent upregulation of GFAP in widespread ipsilateral as well as contralateral zones adjacent to and remote from, but not within, the zone of ischemic injury. Previous studies have reported a virtually identical distribution of GFAP within non-damaged areas, including the contralateral hemisphere, following MCA occlusion [28,43]. Postulated mechanisms underlying GFAP induction in this setting include extracellular ionic and neurotransmitter changes, ischemic depolarization, and the release of cytokines [43]. Importantly, Kraig et al. [25] have demonstrated conclusively that GFAP increases in response to recurrent spreading depression. The GFAP mRNA findings of our study are consistent with neurotransmitter-, cytokine-, or ischemic depolarization-induced stimulation of tissue zones adjacent to the ischemic field or neurally connected zones. The latter may explain the prominent GFAP expression noted in the *contralateral* hippocampus in MCA-occluded rats with subsequent hyperthermic challenge (Fig. 4, groups IH-2 and IH-24).

In summary, we have shown that a period of moderate hyperthermia (40°C) occurring 1 day after a relatively brief (60-min) period of transient MCA occlusion gives rise, not only to an exacerbation of histological injury [19] but to complex alterations of local gene expression. The induction of the immediate early gene c-fos and the heat shock gene hsp70 in threatened tissue regions abutting zones of enhanced injury in ischemic brains with delayed hyperthermia indicates that these zones have been stressed by the superimposition of delayed hyperthermia upon a prior ischemic insult, and it is possible that these gene responses may contribute to the neuroprotection of threatened tissue regions. As a vast number of genes are induced by pathological states, however, the identification of those genes uniquely identified with hyperthermia-induced enhancement of neural injury must await further study.

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