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Effects of hypothermia on c-fos and zif/268 gene expression following rat forebrain ischemia

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Abstract

Purpose. To determine the effects of prophylactic mild hypothermia against transient forebrain ischemia, we examined the levels and localization of c-fos and zif/268 gene expression in the rat hippocampal formation at an early period during reperfusion.

Methods. For the histochemical evaluation, Wistar rats were divided into three groups: normothermia (37°C) during ischemia (n = 5), hypothermia (34°C) during ischemia (n = 5), and sham-operated controls (n = 5). The former two groups were subjected to 10-min forebrain ischemia using the bilateral carotid artery occlusion with arterial hypotension (35 mmHg) technique. After 60 min of reperfusion, the brains were removed and the in situ hybridization technique was used to detect c-fos and zif/268 mRNA expression. Additionally, to determine the histopathological changes of neuronal degeneration, animals were treated with hypothermia during ischemia (n = 4) and normothermia during ischemia (n = 4). They were allowed to survive for 7 days, and then the sections of hippocampal formation were examined by light microscopy.

Results. Transient ischemia increased c-fos and zif/268 mRNA signal densities from 1.8- to 6.1-fold compared with that in controls in the hippocampus. Mild hypothermia significantly inhibited the induction of c-fos and zif/268 ($P < 0.01$). In the CA1 subfield, hypothermia protected against delayed neuronal degeneration ($P < 0.05$), which was observed after normothermic ischemia.

Conclusion. The induction of transcription factor-related immediate early genes (IEGs) was a sensitive marker of the cellular response to ischemia. Mild hypothermia uniformly inhibited the induction of IEGs in the hippocampus, but the mechanisms underlying the protective effect of hypothermia against neuronal degeneration cannot be explained only by the IEGs.

Key words: Hippocampal formation, Hypothermia, c-fos, zif/268, Ischemia

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Introduction

Recent studies have shown that even small decreases in the brain or core temperature protect against the damage resulting from hypoxic-ischemic insult in both animals and humans [1–4]. The brain temperature for mild hypothermic resuscitation is maintained clinically at 33°–35°C. A previous study indicated that rat hippocampal CA1 neurons were consistently damaged when the brain temperature was maintained at 36°–37°C during ischemia but showed scarcely any damage when the brain temperature was maintained at 34°C [1]. Mild hypothermia at 34°–35°C during ischemic insults thus had a protective effect against the postischemic neuronal death of pyramidal cells of CA1 in the rat hippocampal formation. Several investigations have implied that a role of hypothermic protection against ischemic episodes is related to a suppression of major biochemical processes, such as a decrease in cerebral metabolism, a reduction of excitatory neurotransmitter release, and the attenuation of the intracellular accumulation of calcium ions [2,5].

The immediate early genes (IEGs) encode transcriptional regulatory factors that mediate long-term responses to transsynaptic signals. IEGs are rapidly and transiently expressed in response to a variety of extracellular stimuli, which include stimulation of the intact nervous system, brain injury, and ischemia. The induction of the expression of IEGs results in altered synthesis of neurotransmitters, receptors, and ion channel proteins and may control the physiological processes of signal transduction, cell growth, and differentiation. It has been well documented that brain ischemia induces the expression of several IEGs early during reperfusion in the rat hippocampal formation [6,7]. However, only a few investigations have dealt with changes in gene expression with brain resuscitation after ischemia [8,9]. The leucine zipper fos/jun IEGs are induced in the brain by a variety of physiological and pathological stimuli,

but IEGs related to transcription factors containing zinc finger motifs have not been studied extensively using pathological stimuli. In the present study, we therefore examined the effects of hypothermia on forebrain ischemia with regard to the expressions of c-fos and zif/268 mRNA in the rat hippocampus by in situ hybridization histochemistry, and examined the protective effect of mild hypothermia by histopathological outcome in the rat hippocampus.

Materials and methods

Fifteen male Wistar rats (280–320 g) were assigned at random to three groups of five: ischemia with normothermia (cranial temperature, 37.0°C), ischemia with hypothermia (cranial temperature, 34.0°C), and sham-operated controls. The former two groups were subjected to transient forebrain ischemia using the bilateral carotid artery occlusion combined arterial hypotension technique with modifications [10]. The animals were initially anesthetized with 4% isoflurane in oxygen, and tracheal intubation was performed. Subsequently, intramuscular pancuronium (0.5 mg) was given and the lungs were mechanically ventilated. The respiratory rate was adjusted to maintain normocapnia ($P\mathrm{aCO_2}$ 35–40 mmHg). Anesthesia was maintained with 1.5% isoflurane in 30% oxygen balanced in nitrogen. The animal's cranial temperature was measured with a 22-gauge needle thermistor (N452 precision thermistor probe; YSI, Yellow Springs, OH, USA) inserted into the parietal scalp, and was controlled with a homeothermic heating pad (LM-60; List Medical Electronic, Germany) at 37°C during the surgical procedure. A polyethylene catheter (PE-50; Portex, Kent, UK) was inserted into the tail artery to allow continuous monitoring of arterial pressure and the withdrawal of samples of arterial blood. A midline pretracheal skin incision was made, and a PE-50 catheter was placed in the superior vena cava via the right external jugular vein for drug administration. Both carotid arteries were exposed and separated carefully from the cervical sympathetic and vagus nerves. In the mild hypothermia treatment group, malleable ice packs were applied to the shoulders, neck, and skull, and the cranial temperature was controlled at 34°C. In all animals, after the target body temperature had been achieved, a 30-min equilibration period was allowed.

Transient ischemia was induced in rats by 10-min bilateral carotid artery clamping combined with a lowering of the mean arterial blood pressure to 35 mmHg by administration of trimethaphan (0.5–1.5 ml of a 10 mg·ml^{-1} solution). The mean arterial blood pressure was maintained by isoflurane (0.5–1.5%) and continuous administration of trimethaphan solution (0.2 ml·min^{-1}). The common carotid arteries were occluded with small aneurysmal clips, and the vessels were examined beyond the clip for the absence of flow. A pair of needle electrodes was inserted into the temporal muscle for electroencephalographic (EEG) recording, and brain ischemia was confirmed. After 10 min of ischemia, the administration of trimethaphan was stopped and the clips were removed. The animals were allowed to survive for 60 min under controlled ventilation with isoflurane anesthesia. The normothermic and hypothermic cranial temperatures were maintained during occlusion, and in the hypothermic group, the cranial temperature was returned to 37°C within 10 min after reperfusion with the use of a heating pad. After the reperfusion period, the animals were sacrificed under isoflurane anesthesia. In the sham-operated control animals, the common carotid arteries were exposed but not occluded. These animals were sacrificed 60 min after the equilibration period. The brains were removed and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), and coronal sections of the brains (30-μm thick) were cut with a cryostat and collected in 4 × SSC (600 mM NaCl and 60 mM sodium citrate) and processed for in situ hybridization as previously described [6].

For in situ hybridization, the sections were deproteinized with proteinase K and were acetylated. The antisense riboprobe for the rat c-fos mRNA was synthesized from c-fos cDNA (2.1 kbp), and antisense riboprobe for the rat zif/268 mRNA was synthesized from zif/268 cDNA (3.2 kbp). For semiquantitative data analysis, half of the sections were hybridized with the ^{35}S-CTP labeled c-fos or zif/268 cRNA probes. Each probe was dissolved in hybridization buffer [50% formamide, 10% (w/v) dextran-sulfate, 20 mM Tris-HCl (pH 7.8), 5 mM EDTA (pH 8.0), 0.3 M NaCl, 0.2% SDS, 500 μg/ml yeast tRNA, 1 × Denhardt's solution and 10 mM dithiothreitol], and the sections were incubated. After hybridization, the sections were washed in washing buffer (50% formamide, 2 × SSC) and were treated with RNase A solution. After being rinsed in 0.4 × SSC, the sections were dehydrated and exposed to Hyperfilm β-max. For the visualization of histochemical staining, the remaining half of the sections was processed for digoxigenin (DIG) probe hybridization. After hybridization and washing, the sections were transferred into buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl). They were then incubated in blocking reagent and with alkaline phosphatase-labeled anti-DIG antibody, and were reacted in a coloring solution containing 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in a buffer consisting of 100 mM Tris-HCl pH 9.5, 10 mM NaCl, and 50 mM $MgCl_2$. The sections were then examined by light microscopy.

For quantification of the levels of c-fos and zif/268 mRNA expression on Hyperfilm β-max, the densities of signals on areas corresponding to the dorsal hippocampal formation were analyzed by a computer-assisted digital image analysis system with MCID software (Imaging Research, Ontario, Canada). The light intensity was calibrated for each film using a filmstrip containing ^{14}C standards. The optical densities of signals in the CA1, CA3, and dentate gyrus of the dorsal hippocampal formation were determined in 10 sections chosen for each animal. The data are expressed as percentages relative to the control CA1 area. The experimental data were evaluated by one-way analysis of variance followed by post hoc testing by the Bonferroni method. A P value of 0.01 or less was considered significant.

To determine the protective effect of hypothermia, additional animals were investigated for normothermic ($n = 4$) and hypothermic ($n = 4$) ischemia by the same procedure as that described above. After a survival period of 7 days, the animals were deeply anesthetized with pentobarbital ($100\,mg \cdot kg^{-1}$) and killed. The brains were removed and fixed with 4% PFA in 0.1 M PB, and the 20-μm-thick sections of the dorsal hippocampus were stained with cresyl violet. The histopathological outcome following ischemia was examined by light microscopy, and the numbers of intact granule cells in the dentate gyrus and intact pyramidal cells in the CA1 and CA3 subfields at the dorsal hippocampal formation were counted with the use of a micrometer in each experimental condition. The experimental data (means ± SD) were analyzed using unpaired Student's t-tests. A P value of 0.05 or less was considered significant.

Results

Arterial blood gases and mean arterial blood pressure prior to and following ischemia were generally within the normal range and were similar among the three experimental groups (Table 1). In the hippocampal formation of the sham-operated control animals, a few c-fos mRNA-positive neurons with DIG-labeled cytoplasm were observed in the granule and pyramidal cell layers (Fig. 1C), and DIG-labeled zif/268 mRNA-positive neurons were observed in the pyramidal cells of the CA1 subfield and in the cells of the subiculum (Fig. 1F). Ischemia followed by 60 min of reperfusion under normothermic conditions induced massive c-fos gene expression in the dentate gyrus, with the strongest DIG staining found in the granule cell layer. Pyramidal cells in the CA1, CA3, and CA4 subfields also showed relatively high levels of c-fos gene expression. Some glia-like cells also expressed c-fos mRNA in the strata oriens, radiata, and lacunosa-moleculare (Fig. 1A). Strong zif/268 mRNA expression in the dentate gyrus and a moderate expression in pyramidal cells of the CA1 and CA3 subfields were detected (Fig. 1D).

Prophylactic hypothermia markedly inhibited the increase in c-fos-positive neurons throughout the hippocampal formation (Fig. 1B). In addition, hypothermia markedly inhibited the zif/268 mRNA-positive neurons in the dorsal hippocampal formation with a distribution pattern similar to that seen in the controls (Fig. 1E).

In sections hybridized with ^{35}S-labeled probes, autoradiography showed high c-fos mRNA signal density in the dentate gyrus and low levels of signals in the CA1 and CA3 subfields in the normothermic ischemia group, similar to the results using DIG-labeled probes. In regions of the central nervous system other than the dorsal hippocampal formation, the medial habenular nucleus in the epithalamus and the medial amygdaloid complex also showed strong signals. The parietal and piriform cortices showed weak signals (Fig. 2A). No signals were observed in the amygdaloid complex or cortex in the hypothermic or control groups (Fig. 2B,C).

Autoradiography with ^{35}S-labeled zif/268 mRNA probes showed the highest signal density in the dentate gyrus and relatively high signals in the CA1 and CA3 subfields in the normothermic ischemia group (Fig. 2D). The habenular nucleus in the epithalamus, the ventro-

Table 1. Physiological variables

Condition	pH		P_{CO_2} (mm Hg)		P_{O_2} (mm Hg)		MABP (mm Hg)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Normothermic ischemia ($n = 5$)	7.41 ± 0.02	7.35 ± 0.06	39.5 ± 3.7	38.5 ± 2.1	142.7 ± 29.7	139.5 ± 18.2	135.1 ± 6.3	139.5 ± 10.7
Hypothermic ischemia ($n = 5$)	7.41 ± 0.03	7.36 ± 0.05	39.0 ± 3.2	40.3 ± 1.1	136.5 ± 33.5	127.8 ± 15.7	129.6 ± 12.2	128.5 ± 8.0
Sham ($n = 5$)	7.42 ± 0.04	7.34 ± 0.08	38.3 ± 4.1	37.8 ± 5.2	133.4 ± 22.1	139.4 ± 12.8	133.9 ± 14.5	130.7 ± 10.1

Values are means ± SD. Preischemic values (Pre) were obtained immediately prior to the 10-min ischemic insult. Post-ischemic values (Post) were obtained after the 50-min postischemic reperfusion period. In the sham-operated group, blood gas analyses were performed at the same time intervals. There were no statistically significant differences between groups in preischemic data, and between groups in postischemic data by one-way analysis of variance. MABP, Mean arterial blood pressure.

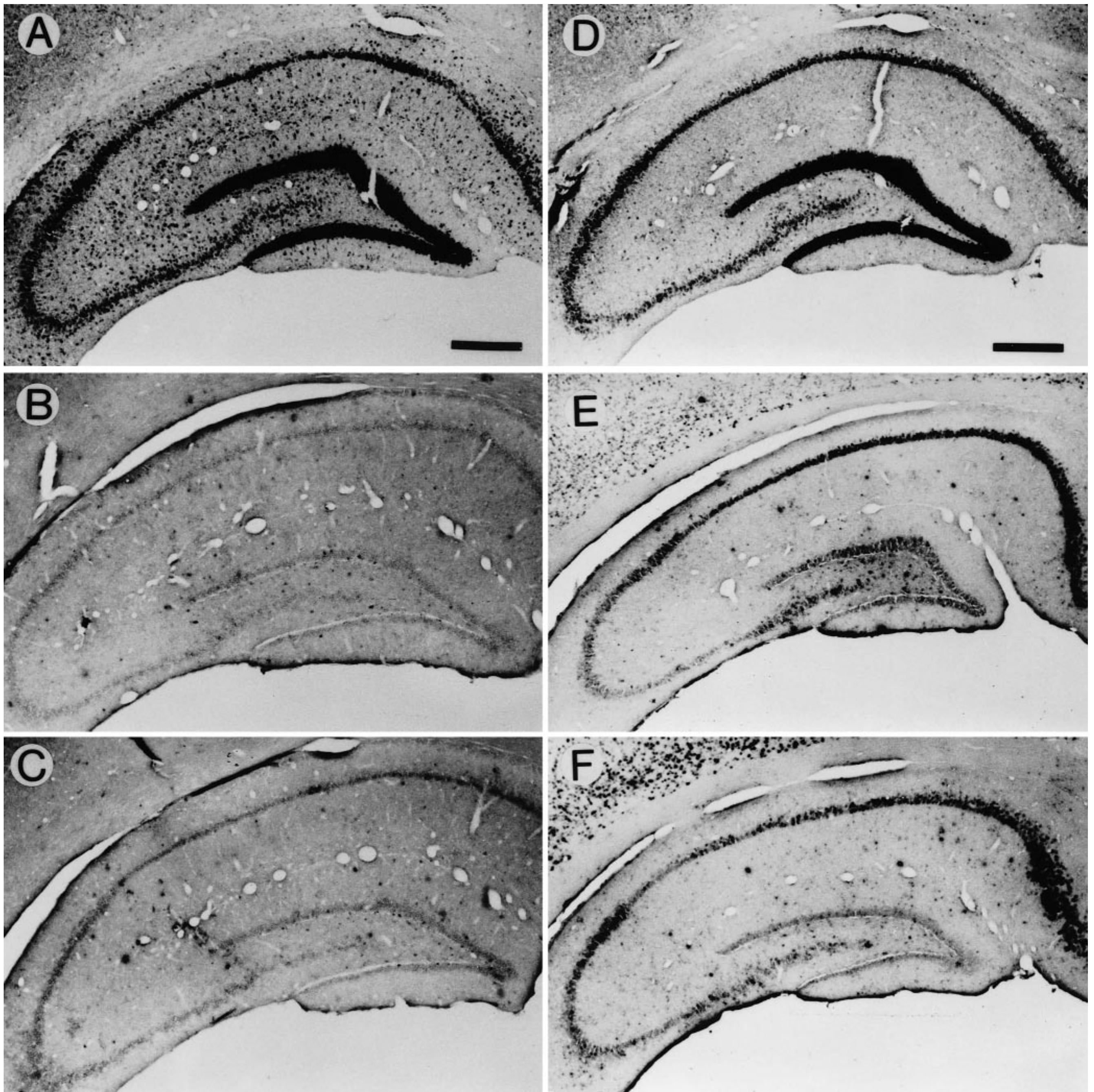

Fig. 1. Photomicrographs showing c-fos (**A**, **B**, **C**) and zif/268 (**D**, **E**, **F**) mRNA-expressing cells detected by in situ hybridization with DIG-UTP-labeled probes as described in the text. **A**, **D**: 60 min after the completion of normothermic ischemia. **B**, **E**: 60 min after the completion of hypothermic ischemia. **C**, **F**: Control animals. *Bars* = 1 mm

medial nucleus in the hypothalamus, and the amygdaloid complex also showed strong zif/268 mRNA signals in the normothermic ischemia group. However, no signals were observed in the dentate gyrus, hypothalamic region, or amygdaloid complex in the hypothermic ischemia and control groups (Fig. 2E,F). The entorhinal, parietal, and piriform cortices showed high signal densities in all three groups.

To determine the distribution of c-fos and zif/268 mRNA in the dorsal hippocampal formation more precisely, we performed a computer-assisted counting of signal densities. Significant increases in c-fos mRNA levels were detected in the normothermic ischemia group in all regions of the hippocampal formation examined. The density of the c-fos mRNA signal increased 6.1-fold in the dentate gyrus, 2.0-fold in the

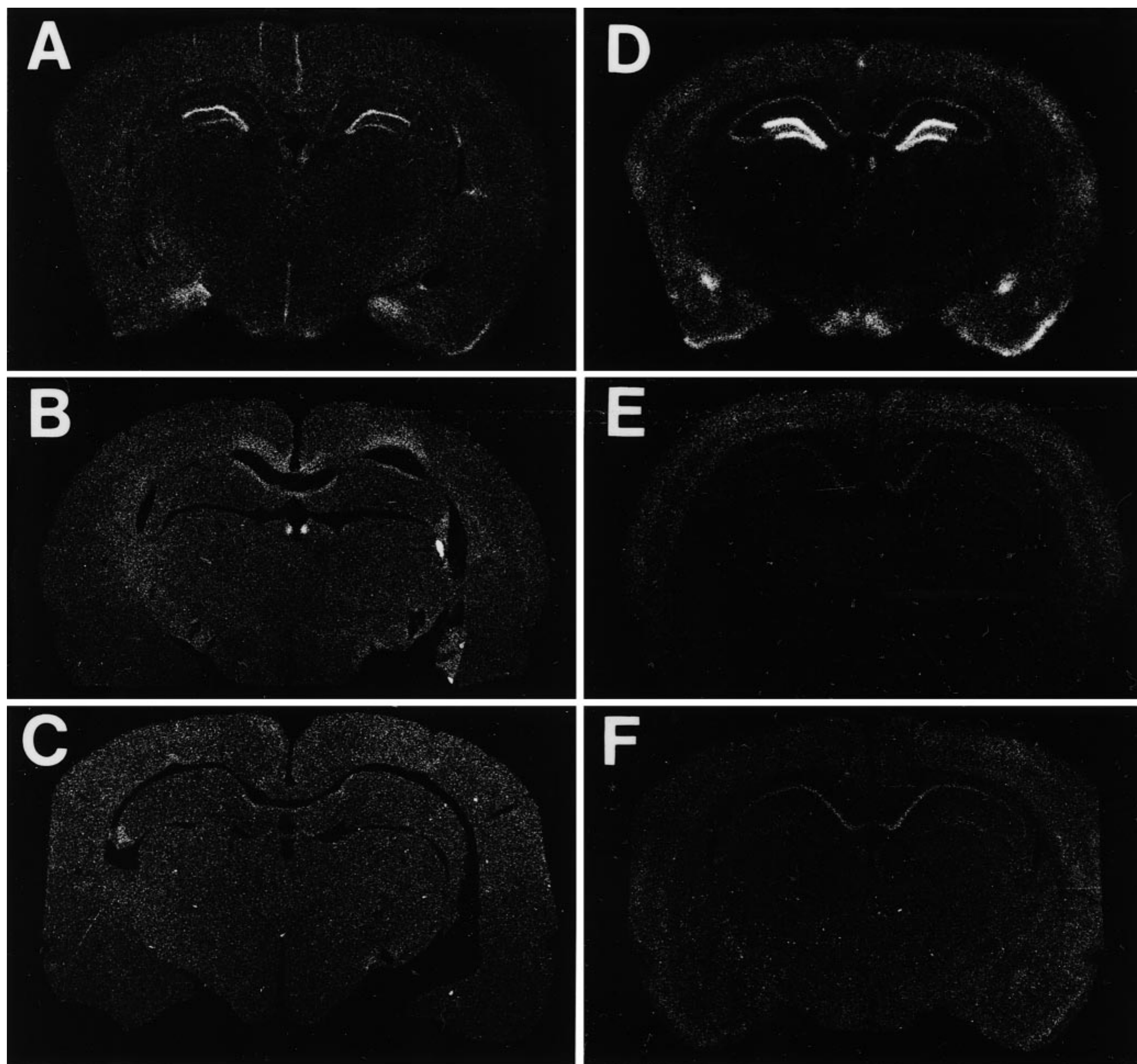

Fig. 2. Representative autoradiograms showing c-fos (**A**, **B**, **C**) and zif/268 (**D**, **E**, **F**) mRNA expression in the rat hippocampal formation. Sections were processed for in situ hybridization with ^{35}S-labeled riboprobes and exposed to film so the autoradiographic grains appear light against a dark background. **A**, **D**: 60 min after the completion of normothermic ischemia. **B**, **E**: 60 min after the completion of hypothermic ischemia. **C**, **F**: Control animals. Note the strong induction of c-fos and zif/268 mRNA in the dentate gyrus of the hippocampal formation following normothermic ischemia, and the lack of c-fos and zif/268 mRNA induction following hypothermic ischemia

CA1 subfield, and 1.8-fold in the CA3 subfield, as compared with that in the CA1 subfield of the control animals. However, there were no significant differences in signal density between the hypothermic ischemia and the control groups (Fig. 3A). Concerning zif/268 mRNA expression, the signal density revealed increases of 5.8-fold in the dentate gyrus, 2.2-fold in the CA1 subfield, and 2.6-fold in the CA3 subfield in the normothermic ischemia group as compared with that in the CA1 subfield of the controls. In hypothermic conditions, the ischemia-induced gene expression was significantly inhibited in all of the regions of the hippocampal formation examined. There were no significant differences in signal density between the hypothermic and control groups, which is similar to the results regarding c-fos mRNA expression (Fig. 3B).

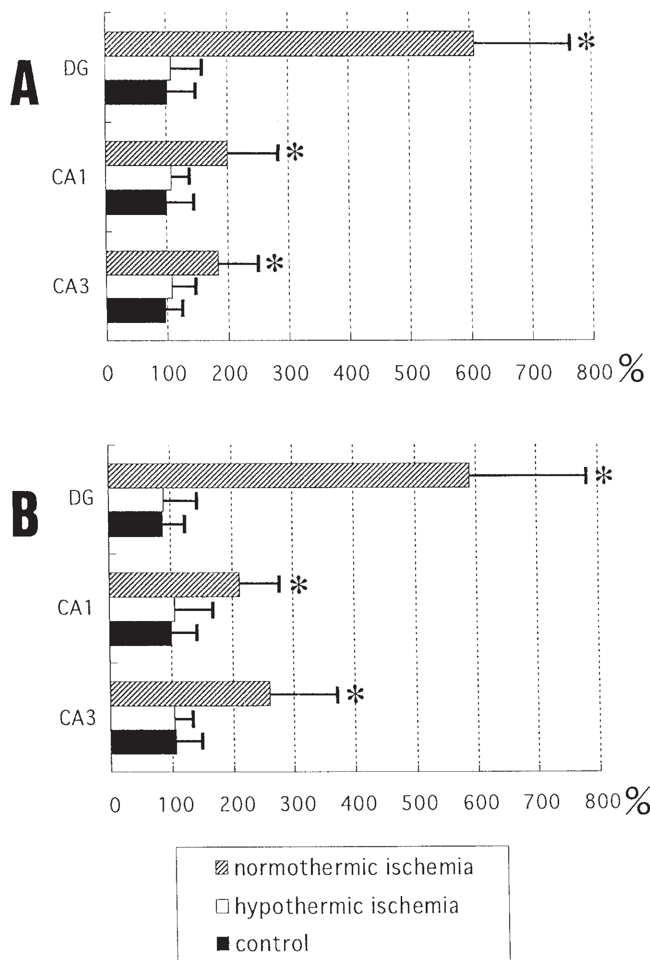

Fig. 3. Results of the quantitative analysis of c-fos (**A**) and zif/268 (**B**) mRNA expression after transient forebrain ischemia. The level of each mRNA was measured as signal density on a macroradiogram, and the results are expressed as percentages of the values obtained in the CA1 of control animals. *Asterisks* indicate significant differences in normothermic ischemia compared with hypothermic ischemia and sham-operated control ($P < 0.01$). Values are means ± SEM of five animals per group. *DG*, Dentate gyrus

Table 2. Numbers of intact neurons counted per 0.5-mm linear length in the corresponding region of the dorsal hippocampal formation under a light microscope using a micrometer

Condition	Hypothermic ischemia (n = 4)	Normothermic ischemia (n = 4)
DG	387.5 ± 55.2	412.4 ± 62.8
CA1	192.5 ± 48.2	17.8 ± 4.5*
CA3	136.5 ± 42.8	127.5 ± 39.7

Values are means ± SD. * $P < 0.05$ versus hypothermic ischemia DG, Dentate gyrus.

The histopathological changes 7 days after ischemic insult were consistent with those described in previous reports. The 10 min of forebrain ischemia resulted in the delayed neuronal degeneration of the hippocampal CA1 pyramidal cells (Fig. 4C,D; Table 2), whereas the dentate gyrus and CA3 cells looked intact. Some CA1 pyramidal cells were darkly stained, whereas others were swollen and stained only slightly (Fig. 4D). This damage was prevented when the rats were hypothermic during the ischemic event (Fig. 4A,B; Table 2).

Discussion

It is well known that transient forebrain ischemia causes delayed neuronal death in the hippocampal formation and caudate putamen. Pre-ischemic brain hypothermia exerts a protective effect against transient brain ischemia, and in the present study we examined the changes in the early onset of transient IEG induction after ischemia.

In the present study, the rats treated with prophylactic hypothermia showed no delayed neuronal death, whereas the rats with normothermic ischemia displayed marked cell death in the CA1 subfield 7 days after the ischemic episode. The mechanisms of the protective effects of hypothermia, however, have not been fully determined. The neuronal death after ischemia seems to be at least partially apoptotic, as judged by the fragmentation of genomic DNA into internucleosomal pieces. This programmed cell death requires new protein synthesis, which induces a variety of pathogenic processes in ischemia, resulting in neuronal degeneration [11]. Hypothermia reduces the release of ischemia-induced excitatory amino acids [5] and modifies the levels of intracellular mediators [2]. It may also suppress the overshoot of signal transmission leading to cell death-related gene transcription and result in rescue from cell death. The present findings indicate that prophylactic hypothermia uniformly inhibited ischemia-induced c-fos mRNA expression throughout the hippocampal formation, to levels similar to those seen in the control animals. The zif/268 mRNA induction in the dentate gyrus and the CA1 and CA3 areas was also inhibited by hypothermia.

Most IEGs encode transcriptional factors that act on several steps of signal transduction. After synaptic transmission due to transmitter receptor coupling, the IEGs function in the steps of conversion of signals into intracellular components and arrange the cellular milieu for the phosphorylation of several kinds of proteins. Particularly, expression of c-fos induced by pathophysiological conditions has been used for metabolic mapping at the nuclear level and to explore the topographical distribution of nervous system activity [12,13]. The inhibition of ischemia-induced presynaptic activation by mild hypothermia may lead to the inhibition of transcription factor-related IEG expression. However, there are no studies showing that blocking prolonged

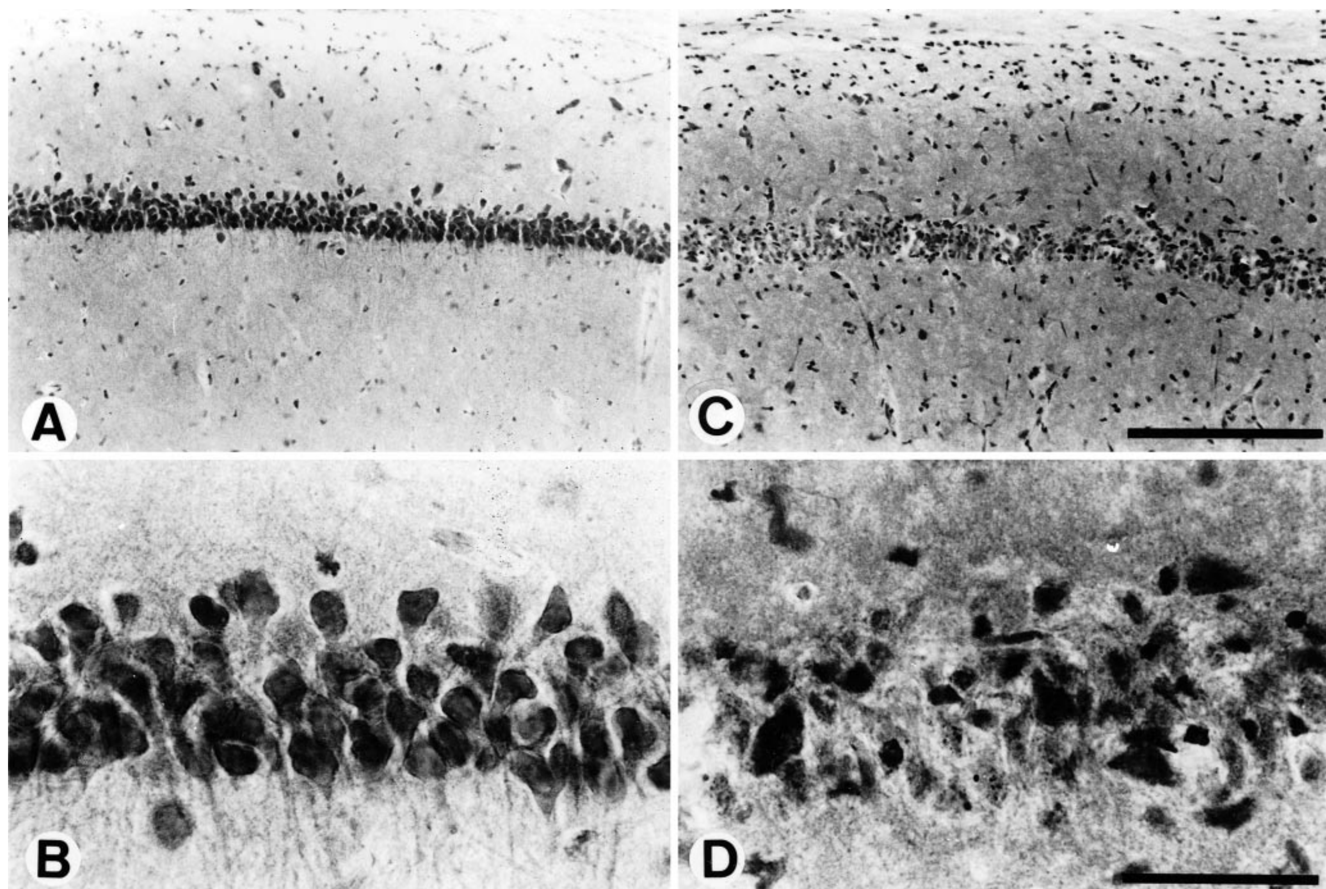

Fig. 4. Photomicrographs showing the hippocampal CA1 pyramidal cells 7 days after the transient ischemic insult. **A**, **B**: Hypothermic ischemia. **C**, **D**: Normothermic ischemia. After normothermic ischemia, some pyramidal cells of the CA1 subfield were darkly stained, whereas others were swollen and stained only slightly. Almost no uniform type of cell decomposition was detected in the CA1 subfield (**C**, **D**). This damage was almost prevented when the rats were hypothermic during the ischemic event (**A**, **B**). **A**, **C**: *Bars*, 500 μm. **B**, **D**: *Bars*, 100 μm

IEG expression attenuates cell death. The relationship between the suppression of the expression of IEGs and the neuronal protective effect was not clear, but our data indicate that in the early stage after ischemia, mild hypothermia exerted a blocking effect against neuronal responses such as IEG expression.

We observed strong c-fos and zif/268 mRNA expression in the granule cells of the dentate gyrus after ischemia followed by 60 min of reperfusion under normothermic conditions. The pyramidal neurons in the CA1 and CA3 subfields also showed relatively high levels of c-fos and zif/268 mRNA expression. Transient forebrain ischemia was recently reported to induce the early onset of transient IEG induction [6,7]. Our histopathological study revealed the consistent degeneration of pyramidal neurons throughout the CA1 region, but other regions were not degenerated. With respect to the vulnerability to ischemic insult, pyramidal cells of the CA1 are more vulnerable than granule cells of the dentate gyrus. Therefore, the induction of c-fos and zif/268 gene expression appears to be nonspecific. The mechanisms of hippocampal IEG induction are unknown. The acute induction could be caused by a transsynaptic activation of the hippocampus from the entorhinal cortex [14]. It is possible that ischemia-induced cortical spreading depression depolarizes entorhinal neurons that project to the hippocampus, and this activates hippocampal neurons and induces the IEGs via the *N*-methyl-D-asparate (NMDA) receptor [13]. The question whether an IEG expression is protective or associated with cell death within a particular neuron or region of the brain was not answered by our experiments. With regard to postsynaptic responses to ischemia, neurons in the CA1 and other hippocampal regions are thought to show different longer-term responses, such as nitric oxide synthase (NOS)-positive neuron activity. It is well known that stimulation of the perforant pathway of Schaeffer collaterals produces long-term potentiation on the pyramidal cells of the CA1 and granule cells of the dentate gyrus. Although nitric oxide (NO) is involved in the production of LTP in those two kinds of neurons [15], NOS may be formed

in the granule cells but not in the pyramidal cells of the CA1. In experiments of cortical cell culture, it has been speculated that neuronal NOS (nNOS) neurons themselves are resistant to a variety of toxic insults [16]. Although nNOS neurons are relatively spared from cell death in NMDA neurotoxicity, they mediate widespread neurotoxicity by producing NO [17]. Under hypothermic conditions, NO in the pyramidal cells of the CA1 may be decreased in correspondence to decreased NOS activity in the granule cells from which NO is produced and diffuses to adjacent neurons such as the pyramidal cells of CA1. However, further examination is necessary to elucidate the regional specific vulnerability to ischemia.

In conclusion, in the early stages after ischemic insult, IEG expression was sensitive to the intracellular response to ischemia. In the hippocampal formation, hypothermia blocked the activation of transcription factor-related IEGs. However, the mechanisms underlying the hypothermic protective effect against ischemia-induced neuronal degeneration is not explained only by IEG expression. Further investigations of the mechanisms acting against ischemia, such as the neuronal apoptotic process, are needed.

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