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Brain responses to acute withdrawal in phenobarbital-dependent rats

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Abstract

Heat shock proteins (HSP) such as HO-1 and HSP27 have been implicated as functioning in a protective manner against oxidative and physical stress. The objective of the current study was to determine the role of HSPs in drug-withdrawal stress induced in phenobarbital-dependent rats. Increased expression of HO-1 and HSP27 was observed in the hippocampus and the cerebral cortex of phenobarbital-withdrawn rats. Gene expression was measured by Northern and Western blot analyses and in situ hybridization. The induction of HO-1 mRNA was suppressed by the administration of the NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo (*a,d*) cyclohepten-5,10-imine (MK801). Despite significant upregulation of glutamatergic transmission, neuronal cell degeneration was not apparent. These findings suggest that the induction of HO-1 and HSP27 during withdrawal from phenobarbital dependence may play a role in protection against glutamate toxicity.

Keywords: Phenobarbital; Dependence; Withdrawal; Stress; Heme oxygenase-1; Heat shock protein 27

1. Introduction

The induction of heat shock proteins (HSP) has been shown to occur in the brain upon exposure to oxidative stress such as ischemia (Nimura et al., 1996), hypoxia (Lee et al., 1997), thrombotic injury (Plumier et al., 1997), Parkinson's disease (Castellani et al., 1996) and Alzheimer's disease (Markesbery, 1997; Premkumar et al., 1995). Heme oxygenase-1 (HO-1), also known as HSP32, oxidatively cleaves heme at the α-meso carbon bridge, releasing carbon monoxide (CO), iron and biliverdin. Bilirubin, a reduced form of biliverdin, and CO act as scavengers of reactive oxygen species (Llesuy and Tomaro, 1994) and as activators of guanyl cyclase in the manner of a nitric oxide (NO)-like retrograde messenger (Verma et al., 1993).

Recently, an anti-inflammatory function attributable to CO via a pathway involving a mitogen-activated protein kinase has been described (Otterbein et al., 2000). Heat shock, which is sufficient to induce heat shock proteins including HO-1, protects cultured neurons from glutamate toxicity (Rordorf et al., 1991). Accumulated HO-1 protein has been shown to be associated with protection against oxygen toxicity (Dennery et al., 1997; Vile et al., 1994). These findings suggest that HO-1 may play a key role, at least in vitro, in protecting against oxidative stress.

Recent in vivo experiments have shown that HO-1 induction is protective against cell injury. Poss and Tonegawa have reported that mice lacking HO-1 are vulnerable to mortality and hepatic necrosis when challenged with endotoxin (Poss and Tonegawa, 1997b). Growth retardation, anemia, iron deposition and vulnerability are characteristics of human (Yachie et al., 1999) and murine (Poss and Tonegawa, 1997a,b) HO-1 deficiency. These studies suggest that HO-1 is an important enzyme in the protection of cells from oxidative stress in vivo as well. However, a functional role of HO-1 induction in the central nervous system remains to be determined.

We have focused on the possible involvement of glutamatergic neurotransmission in the development of phenobarbital dependence and its withdrawal syndrome (Tanaka

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et al., 1996, 1997). When dependent rats were deprived of phenobarbital, the blood concentration of phenobarbital decreased rapidly and the animals exhibited severe withdrawal symptoms, such as convulsions and weight loss (Aoki and Kuroiwa, 1982). In the brains of dependent rats, physiological homeostasis could be maintained by the elevated phenobarbital levels. Sudden drug withdrawal and a concomitant decrease in the phenobarbital concentration in the rat brain cause an increase in the extracellular levels of excitatory amino acids, such as glutamate and aspartate, in the hippocampus and the cerebral cortex (Tanaka et al., 1996). Phenobarbital withdrawal may induce a disturbance of homeostasis, leading to the appearance of withdrawal symptoms. It is interesting to know whether such withdrawal stress induces stress proteins. The purpose of the present study was to demonstrate the induction of HO-1 and other stress proteins in the brains of phenobarbital-withdrawn rats.

The results obtained in the present study show that heat shock proteins, such as HO-1 and HSP27, but not HSP70, are induced in the rat brain, where glutamatergic neurotransmission is strongly enhanced during withdrawal from phenobarbital. Additionally, these neuronal cells are likely to be viable despite excessive extracellular concentrations of excitatory amino acids in the brains of withdrawn rats. Moreover, we demonstrated that a NMDA receptor antagonist attenuates HO-1 induction as a result of phenobarbital withdrawal. These findings suggest that the induction of heat shock proteins is involved in the protection of neuronal cells against glutamate toxicity during withdrawal from phenobarbital dependence.

2. Materials and methods

2.1. Materials

[^{35}S]dCTP (specific activity 1000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Nonradioactive (+)-5-methyl-10,11-dihydro-5*H*-dibenzo (*a*,*d*) cyclohepten-5,10-imine (MK801) was a gift from the Merck Sharp and Dohme Research Laboratory (Rahway, NJ). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from the Institute of Isotopes of the Hungarian Academy of Sciences (Budapest, Hungary). pRHO-1 and HHCMH62 cDNA were obtained from Riken gene bank (Tsukuba, Japan) and ATCC (Manassaa, VA), respectively. GD5 (for glyceraldehyde 3-phosphate dehydrogenase: GAPDH) (Maehara et al., 1985) and pRC-H27 cDNA (Arata et al., 1995) were donated by Dr. K. Nose (School of Pharmaceutical Sciences, Showa University, Tokyo, Japan).

2.2. Preparation of phenobarbital-dependent rats

Male Wistar rats (5 weeks old, weighing about 100 g) were housed individually and given a powdered diet (F-2; Sankyo Labo-service, Tokyo, Japan) consisting of different concentrations of phenobarbital through two feeders according to the regimen described by Tagashira et al. (1978). The phenobarbital concentrations in the diet of the two feeders were as follows: 0.5 and 1 mg/g for the first week; 1 and 2 mg/g for the second week; 2 and 4 mg/g for the third and fourth weeks; and 4 mg/g for the fifth week. After 5 weeks of phenobarbital feeding, rats were withdrawn from phenobarbital by replacing the diet with a normal powdered diet.

2.3. Northern blot analysis

Total RNA was isolated by acid–guanidinium–thiocyanate–phenol–chloroform extraction, as described by Chomczynski and Sacchi (1987), from the cerebral cortex and the hippocampus. The cerebral cortex and hippocampus were dissected according to the method of Glowinski and Iversen (1966). Total RNA (20 μg) from each sample was size-fractionated by denaturing agarose gel electrophoresis prior to transfer onto Nytran membranes (Schleicher and Schuell, Dassell, Germany) as described previously (Tanaka et al., 1997). The cDNA probes were labeled with [^{32}P]dCTP by the random priming method (Megaprime DNA labeling system, Amersham Pharmacia Biotech, Buckinghamshire, UK). The cDNA probes used for HO-1, HSP27, HSP70 and GAPDH were the 0.9 kb *Hin*dIII/*Eco*RI fragment of pRHO-1 cDNA, the 0.4 kb *Eco*RI/*Fok*I fragment of pRC-H27 cDNA, the 1.7 kb *Eco*RI insert of HHCMH62 and the 0.5 kb *Pst*I fragment of GD5 plasmid, respectively. Quantitative analysis was performed with an image analyzer (Fujix BAS3000, Fuji Photo Film, Tokyo, Japan). The expression of HO-1 and HSP27 mRNAs was normalized by comparison with the expression of GAPDH mRNA.

2.4. In situ hybridization analysis

Rats were decapitated and the brains were immediately removed. The brains were embedded in a mixture of 20% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and Tissue Teck (2:1; Miles, Elkhart, IN), frozen on dry-ice, and kept at −70°C. Ten-micrometer sections were cut with a cryostat and mounted on 3% 3-aminopropyltriethoxysilane-coated slides. Hybridization histochemistry was performed as described previously (Tanaka et al., 1997). The HO-1 and HSP27 cDNA probes were labeled with [^{35}S]dCTP by the random priming method and purified by Sephadex G-50 column chromatography (Amersham Pharmacia Biotech). For autoradiography studies with LM1 emulsion (Amersham Pharmacia Biotech), exposure was carried out for 21 days at 4°C. The phenotypes of cells expressing HO-1 and HSP27 mRNAs were examined with hematoxylin–eosin-stained sections.

2.5. Western blot analysis

The cerebral cortex and the hippocampus were homogenized in five volumes of Tris buffer 50 mM Tris–HCl (pH 7.4), containing 50 mM KCl, 1 mM EDTA, 0.1 mM antipain, 0.25 mM *p*-aminophenyl methanesulfonyl fluoride, 0.1 mM leupeptin and 0.029 mM pepstatin. The protein concentration of the homogenates was adjusted to 10 mg/ml for cerebral cortex and 6.5 mg/ml for hippocampus, respectively. The homogenates were lysed using equal volume of 2 × sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% sodium dodecylsulfate, 10% 2-mercaptoethanol). Homogenates were then boiled for 5 min. Samples (15 μl/well) were electrophoresed and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% skimmed milk dissolved in PBS containing 0.05% Tween 20 for 3 h at room temperature. After being washed, the membrane was incubated with anti-rat HO-1 antibodies (1/500 dilution, StressGen, Victoria, BC, Canada) overnight at room temperature. Following incubation, the membrane was again washed and incubated with the second antibody coupled to alkaline phosphatase. Blots were developed using CDP-Star (New England Biolabs, Beverly, MA) as chemiluminescent susbstrate according to the supplier's instructions.

2.6. Hematoxylin–eosin-stain

Horizontal sections (10 μm) were cut with a cryostat, mounted on gelatin-coated slides and maintained at −70°C until use. Tissue sections on slides were thawed at room temperature and dried with cool air. Sections were fixed with 4% paraformaldehyde solution for 20 min. After being washed twice with PBS, the sections were stained with Mayer's hematoxylin solution for 3 min. Next, the sections were rinsed with tap water for 10 min, followed by distilled water. Sections were stained with 0.5% eosin for 30 s and were immediately dehydrated with 70%, 80%, 90%, 95% and 100% ethanol.

2.7. Statistical analysis

Statistical analysis was performed with a Mann–Whitney test.

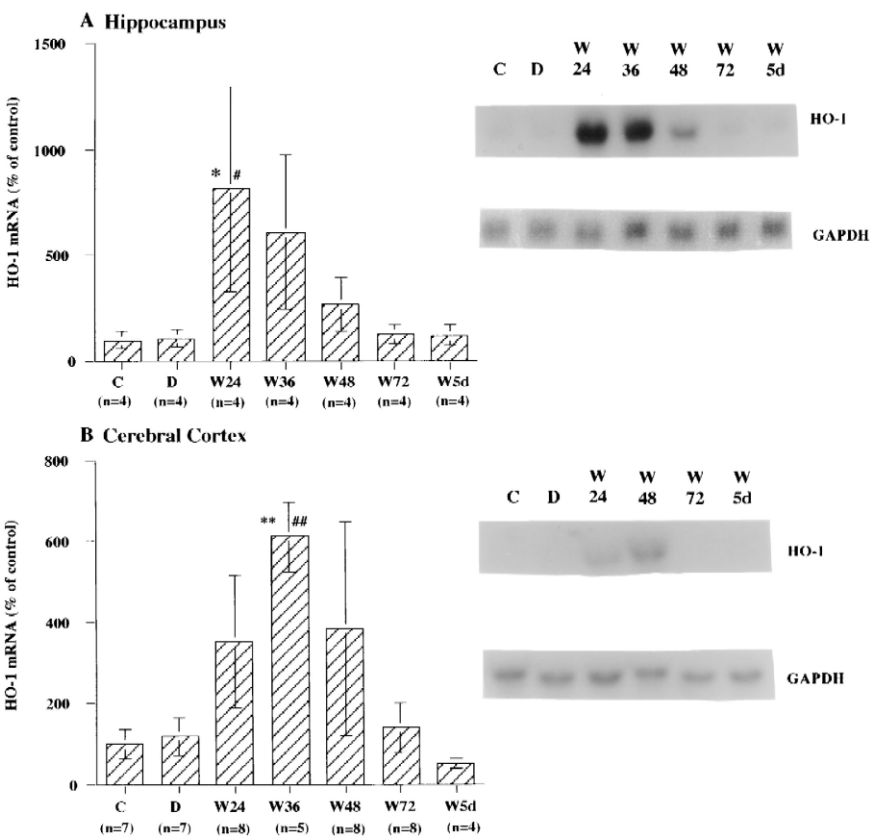

Fig. 1. HO-1 gene expression in the hippocampus and the cerebral cortex of control, phenobarbital–dependent and phenobarbital–withdrawn rats. Autoradiogram shows Northern blot analysis of HO-1 and GAPDH mRNA. Columns represent normalized HO-1 signals in the control (C), phenobarbital-dependent (D), and 12 h (W12), 36 h (W36), 48 h (W48), 72 h (W72), 5 days (W5d) phenobarbital-withdrawn rats. Values are expressed as mean percentages of the HO-1 mRNA in the control group and are presented as means ± S.E.M. Panels A and B show data for the hippocampus and cerebral cortex, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group, $\#P < 0.05$, $\#\#P < 0.01$ compared with the penobarbital-dependent group.

3. Results

3.1. Expression of HO-1 and HSP27 mRNA in the brain

We observed steady-state levels of HO-1 and HSP27 mRNAs in the hippocampus and the cerebral cortex. Detection of mRNA was by Northern blot analysis. Levels of HO-1 mRNA were low in the hippocampus and the cerebral cortex of control and phenobarbital-dependent rats (Fig. 1). HO-1 mRNA levels in the hippocampus at 24 h following phenobarbital withdrawal increased by 8.1 times over the control levels (Fig. 1a). HO-1 mRNA levels in the cerebral cortex, at 36 h following phenobarbital withdrawal, also increased by 6.1 times over the control levels (Fig. 1b). The enhanced HO-1 gene expression in these tissues returned to basal levels at 72 h following withdrawal.

Steady-state levels of HSP27 mRNA were also low in the hippocampus and the cerebral cortex of control and phenobarbital-dependent rats (Fig. 2). HSP27 mRNA levels in the hippocampus and the cerebral cortex increased between 24 and 72 h following phenobarbital withdrawal. The enhanced expression in the hippocampus and the cerebral cortex peaked at 13.8 and 14.6 times the control levels, respectively. Enhanced HSP27 gene expression was sustained for 72 h and returned to basal levels by 5 days following withdrawal. In contrast, steady-state levels of HSP70 mRNA did not change in the hippocampus or the cerebral cortex during withdrawal from phenobarbital (data not shown).

3.2. Expression of HO-1 protein

Fig. 3 shows the time-dependent changes in HO-1 protein levels in the hippocampus and cerebral cortex of phenobarbital-dependent rats following drug withdrawal. HO-1 protein was scarcely detectable in control and phenobarbital-dependent rats; however, HO-1 levels increased as a result of phenobarbital withdrawal and reached maximum levels 48 h later. Consequently, the induction of HO-1 protein followed an increase in HO-1 mRNA levels.

3.3. Relationship of the glutamate receptor to HO-1 mRNA expression

We have found that administration of the NMDA receptor antagonist MK801 attenuates c-*fos* induction in the brains of phenobarbital-withdrawn rats. Therefore, we ex-

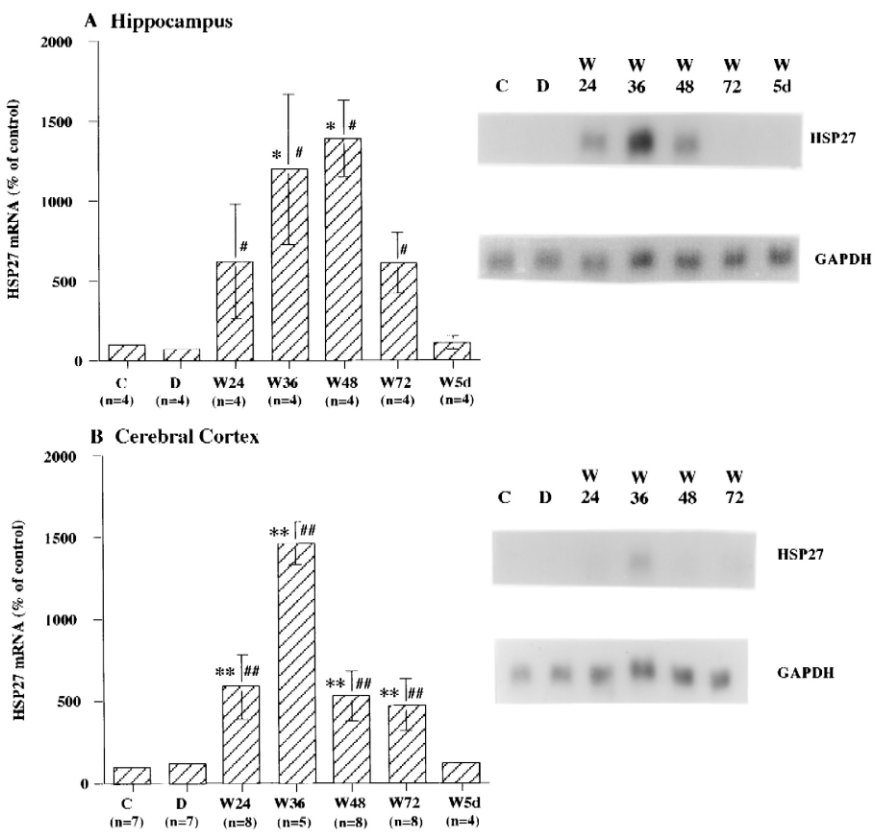

Fig. 2. HSP27 gene expression in the hippocampus and cerebral cortex of control, phenobarbital-dependent and phenobarbital-withdrawn rats. Northern blot analysis was carried out as described in the legend to Fig. 1. Values are expressed as mean percentages of the expression of HSP27 mRNA in the control group and are presented as means ± S.E.M. Panels A and B show data for the hippocampus and cerebral cortex, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group, $\#P < 0.05$ and $\#\#P < 0.01$ compared with the phenobarbital-dependent group.

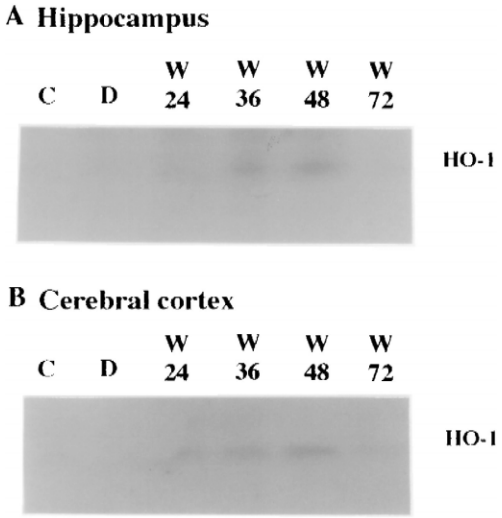

Fig. 3. HO-1 protein in the hippocampus and cerebral cortex of control, phenobarbital-dependent and phenobarbital-withdrawn rats. Protein samples were obtained from control (C), phenobarbital-dependent (D), and 12 h (W12), 36 h (W36), 48 h (W48), 72 h (W72) phenobarbital-withdrawn rats. Western blot analysis was carried out using anti-HO-1 antibody. Panels A and B show data for the hippocampus and cerebral cortex, respectively.

amined the role of NMDA receptors in HO-1 induction. As shown in Table 1, MK801 significantly suppressed HO-1 mRNA accumulation induced by phenobarbital withdrawal in the hippocampus and the cerebral cortex. MK801 produced a nearly complete inhibition of HO-1 induction 24 h after withdrawal. HSP27 mRNA was also decreased by MK801, but to a lesser extent. The decrease in HSP27 expression was not statistically significant because of the scatter in the data.

3.4. In situ hybridization histochemistry

The distribution of HO-1 transcripts was studied in the hippocampus of control rats (Fig. 4A) and in rats which had been withdrawn from phenobarbital for 36 h (Fig. 4B) by in situ hybridization. Low levels of HO-1 were detected in control rats. HO-1 levels increased in the hippocampus of rats 36 h following phenobarbital withdrawal (Fig. 4B). Extensive expression of HO-1 mRNA was found in all brain regions following withdrawal. In the hippocampus, strong HO-1 mRNA signals were detected in the pyramidal cells in the CA1 region (Fig. 4B). These signals were also found in glia-like cells but it was difficult to determine what kind of glial cells expressed these signals. The HSP27 signals were scarcely detectable in the brains of control rats (Fig. 5A). However, these signals appeared to increase 36 h after withdrawal. Intensive HSP27 mRNA expression was found in the pyramidal cells and glia-like cells of the CA1 region (Fig. 5B).

Table 1
Effect of MK801 on HO-1 and HSP27 mRNA expression
MK801 (5 mg/kg) was administered three times (12, 16 and 20 h after removal of phenobarbital from the feed). Total RNA was extracted from the hippocampus or cerebral cortex of 24 h phenobarbital-withdrawn rats. Gene expression is presented as a percentage of the saline group (means ± S.E.M.). Statistical analysis was performed with the Mann–Whitney test.

	HO-1 ($n = 5$)		HSP27 ($n = 3$)
	Hippocampus	Cerebral cortex	Hippocampus
Saline	100 ± 23.5%	100 ± 11.0%	100 ± 10.0%
MK801 treatment	30 ± 2.8%[a]	16 ± 3.3%[b]	51 ± 2.7%

[a] $P < 0.05$, compared with the saline group.
[b] $P < 0.01$, compared with the saline group.

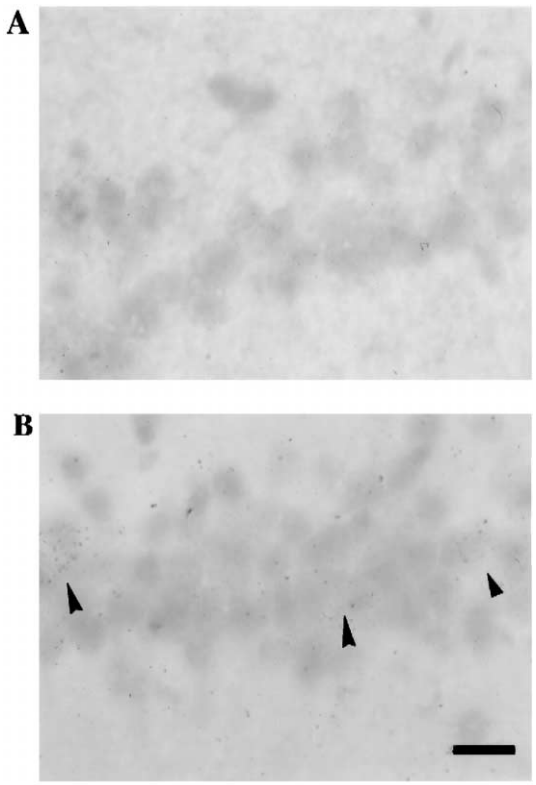

Fig. 4. Light-field photomicrographs of in situ hybridization histochemistry for HO-1 mRNA in the hippocampus. Panels A and B show photomicrographs of control and 36 h phenobarbital-withdrawn rat, respectively. In the 36 h phenobarbital-withdrawn rat, many grains of HO-1 mRNA are visible in the pyramidal cells of the CA1. Scale bars = 13 μm.

3.5. Cell survival

We have shown that concentrations of excitatory amino acids, such as glutamate and aspartate, increase significantly during phenobarbital withdrawal (Tanaka et al., 1996). The increased levels of excitatory amino acids have been shown to cause neuronal degeneration and cell death (Benveniste et al., 1984; Choi, 1988). As a result of these observations, we histologically analyzed brain preparations obtained from phenobarbital-withdrawn rats. Despite this

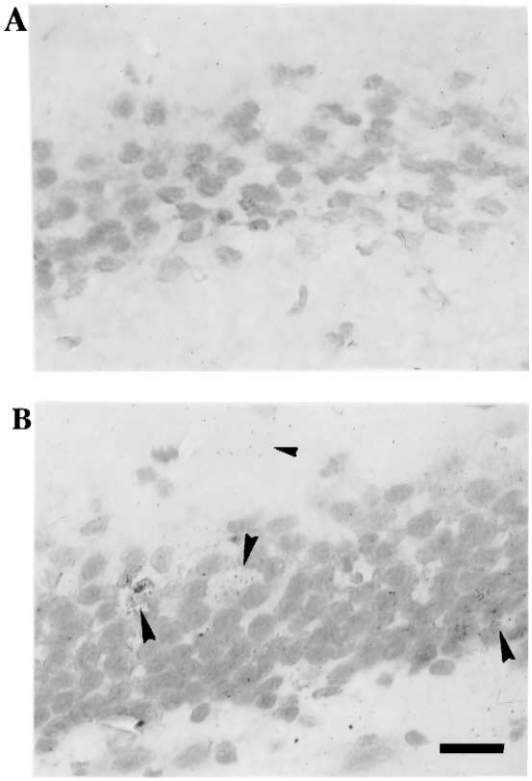

Fig. 5. Light-field photomicrographs of in situ hybridization histochemistry for HSP27 mRNA in the hippocampus. Panels A and B show photomicrographs of control and 36 h phenobarbital-withdrawn rat, respectively. In the 36 h phenobarbital-withdrawn rat, signals indicating HSP27 mRNA were predominantly found in the pyramidal cells of the CA1. Scale bars = 26 μm.

extremely high glutamate release (Tanaka et al., 1996), there was no evidence of degeneration in the hippocampal CA1 pyramidal neurons during the course of phenobarbital withdrawal (Fig. 6). We did not find neuronal cell death in the hippocampus, or in the neocortex (data not shown).

4. Discussion

Animals that were phenobarbital dependent were ataxic and laid on their abdomens or sides. Often, they demonstrated further severe hyperthymia-like behavior, e.g., running around and staggering into the side of the cage. Animals that were withdrawn from phenobarbital exhibited various abstinence signs, such as muscle fasciculation, writhing, marked irritability, aggressiveness, hyperkinesia, muscle rigidity, ataxia, tremors, and convulsions. There was a drastic loss of body weight between 48 and 72 h following phenobarbital withdrawal. Ninety percent of phenobarbital-withdrawn rats showed visible withdrawal convulsions within 48 h of withdrawal (Tanaka et al., 1997).

Using these phenobarbital-dependent animals, we demonstrated that heat shock proteins, such as HO-1 and HSP27, but not HSP70, were induced in the rat brain during withdrawal from phenobarbital. The increased expression of HO-1 and HSP27 was observed in the hippocampus and the cerebral cortex. In the present study, there was an individual variability in the HO-1 and HSP27 mRNA levels of rats withdrawn from phenobarbital. When we examined the changes in the extracellular levels of glutamate in the brain tissues of drug-withdrawn rats using in vivo microdialysis, there was an individual variability in the time at which glutamate levels began to increase and in convulsion development among phenobarbital-withdrawn rats (Tanaka et al., 1996). It seems that the time-dependent individual difference among rats in the response to pheno-

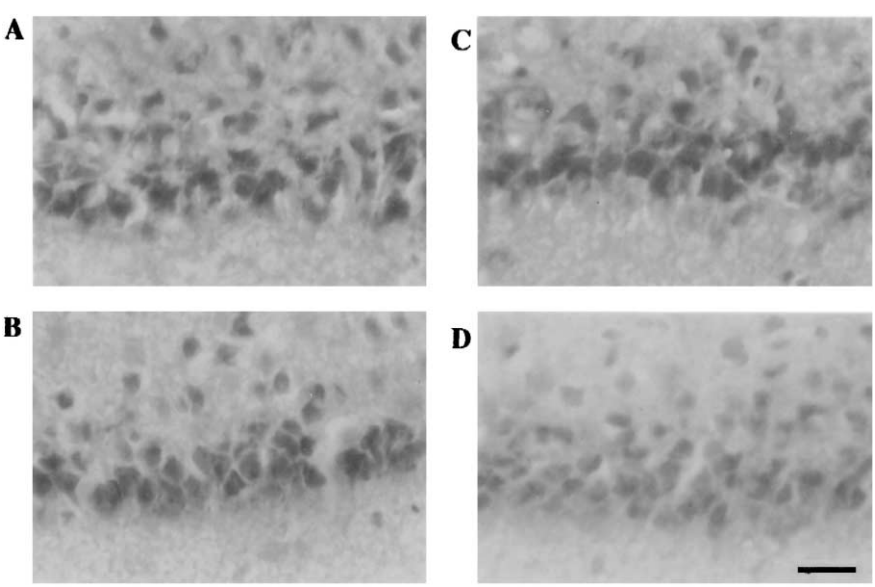

Fig. 6. Hematoxylin–eosin-stained sections of the hippocampus. Photomicrographs show hippocampal pyramidal neurons stained with hematoxylin–eosin during the course of withdrawal from phenobarbital. A, control; B, phenobarbital dependence; C, 24 h after withdrawal and 5 days after withdrawal. Scale bars = 13 μm.

barbital withdrawal could be attributable to the observed variability in the gene expression of HO-1 and HSP27 mRNA under the present experimental conditions. Furthermore, enhanced glutamatergic neurotransmission and upregulation of NMDA receptors results in the expression of immediate early genes, such as c-*fos* and c-*jun*, and in a concomitant increase in activator protein-1 (AP-1) DNA binding activity (Tanaka et al., 1997). The AP-1 binding site occurs in the enhancer/promoter region of the rat HO-1 gene (Muller et al., 1987). This fact, in addition to the current results and our previous results, suggests that increased AP-1 activity may be involved in the induction of HO-1 during withdrawal from phenobarbital.

The induction of HO-1 mRNA was suppressed by administration of the NMDA receptor antagonist, MK801. This finding supports the premise that the induction of HO-1 occurs in accordance with enhanced glutamatergic transmission, especially through NMDA receptors, during withdrawal from phenobarbital. Activation of the glutamate receptor leads to Ca^{2+} influx (Lerea and McNamara, 1993). An increased Ca^{2+} concentration in cells results in the activation of heat shock factor (Ding et al., 1996). The rat HO-1 and HSP27 genes possess the heat shock element within their promotor region (Oesterreich et al., 1996). Therefore, enhanced glutamatergic transmission following activation of heat shock factor may also be involved in the induction of HO-1 and HSP27. However, MK801 does not completely suppress withdrawal convulsions, as shown in our previous study (Tanaka et al., 1997). Because withdrawal convulsions are not always due to excessive stimulation of NMDA receptors alone, other types of glutamate receptors may also be involved in the withdrawal syndrome.

Lowenstein et al. demonstrated that glutamate failed to induce HSP72, which was inducible by heat shock, in cultured neurons (Lowenstein et al., 1991). This finding, supported by our present results in which HSP70 gene expression was scarcely inducible in the brain of phenobarbital-withdrawn rats, suggests that there is a distinct mechanism regulating the expression of HSP70 and that of HO-1 and HSP27.

It is well known that extreme Ca^{2+} influx leads to neuronal cell death. Brain ischemia, which results in an increase in the extracellular glutamate level up to 10 times greater than that found in controls, promotes neuronal cell death (Benveniste et al., 1984; Choi, 1988). We have demonstrated that phenobarbital withdrawal results in extracellular glutamate levels comparable to those found in ischemia (Tanaka et al., 1996). In addition to glutamate release, ischemia is associated with oxygen and glucose loss and energy depletion, which would contribute to cell death. Also, it has been reported that HO-1 induction occurs after brain ischemia. However, as shown in the present study, neuronal cell death was not observed in the rats subjected to phenobarbital withdrawal in spite of excessive glutamate release. These findings suggest that protective mechanisms, possibly due to the induction of heat shock protein, are activated during withdrawal.

The levels of heme and HO activity in the rat brain are reported to be 1.3 ± 0.1 nmol/mg protein and 280 pmol/mg protein/min, respectively. These values are comparable to those found in the liver (Ingi et al., 1996), suggesting that heme has a physiologic function in the central nervous system as well as in the liver. In the hippocampal cell line HT-22, glutamate-induced cell death is likely to be mediated by oxidative stress since antioxidants protect the cell from toxicity (Tan et al., 1998). Furthermore, it has been reported that CO, a product of HO oxidative cleavage, possesses anti-inflammatory activity (Otterbein et al., 2000). We have no direct evidence that heat shock proteins, the levels of which are increased by phenobarbital withdrawal, act as protective factors against glutamate toxicity. However, the recent data noted above suggest that heat shock proteins play a role in the protection and survival of neuronal cells (Rordorf et al., 1991; Sato et al., 1996). It has been reported that HSP27 functions as a molecular chaperone (Jakob et al., 1993). HSP27 has also been shown to function as a factor promoting actin polymerization and stress fiber formation (Lavoie et al., 1993). Recently, HSP27 induction has been shown in the rat brain in response to ischemia (Currie et al., 2000), hyperthermia (Bechtold and Brown, 2000) and seizure induced by kainic acid (Plumier et al., 1996). These findings, supported by our current results, suggest that HSP27 induction, as well as HO-1 induction, may be a stress response involving neuronal plasticity. Furthermore, signals for HO-1 and HSP27 were detected in glia-like cells in addition to CA1 neurons. However, it is very difficult to carry out in situ hybridization with immunohistochemistry in order to determine the cell type in which HSP was induced. Further studies are necessary to clarify the significance of HO-1 and HSP27 induction in the central nervous system.

In conclusion, our present results and previous data (Tanaka et al., 1996, 1997) suggest that the enhanced glutamatergic neurotransmission-mediated increases in the expression of c-*fos* and c-*jun*, following upregulation of AP-1 activity, are involved in the induction of HO-1 and HSP27 during the development of the phenobarbital-withdrawal syndrome. Irrespective of the enhanced glutamatergic transmission, neuronal cell degeneration was not observed. This finding suggests that the induction of heat shock proteins plays a protective role against glutamate toxicity during withdrawal from phenobarbital.

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