Korean J. of Pharmacology
Vol. 32, No. 3, pp. 335~345, 1996

Oxidative Stress and HSP70 Expression Upon Cerebral Ischemia-Reperfusion in Mongolian Gerbil*

Young-Mee Park[1], Chul-Hoon Kim[3], Yun-Jeong Do[3]
Eun-Mi Choi[2] and Young-Soo Ahn[3]

[1]*Department of Biology and* [2]*Chemistry, Inchon University College of Natural Sciences, Korea, 402-749*
[3]*Department of Pharamacology, Yonsei University College of Medicine, Korea, 120-752*

ABSTRACT

A critical role of oxygen-derived free radicals has been implicated in ischemia/reperfusion (I/R)-induced brain damage. In this study, we have produced experimental I/R to the brains of Mongolian gerbil (*Meriones unguiculatus*) by a transient occlusion and release of the common carotid arteries. We have attempted to determine whether the oxidative stress is generated upon I/R and whether this oxidative stress is linked to the cell damage. Since hippocampus has been suggested as one of the most vulnerable regions of the brain to the oxidative stress, we analyzed samples from hippocampus in comparison with those from cortex. In addition, we have examined the expression of heat shock protein 70kD species (HSP70) in these regions in order to evaluate a possible role of this protein in I/R-induced brain damage. To determine whether the oxidative stress is produced upon I/R, we measured the glutathione oxidation, GSSG/ (GSH+ 2xGSSG), as an index of oxidative stress. We found an increase of the glutathione oxidation primarily in hippocampus upon I/R. To determine whether this oxidative stress is linked to the cell damage, we measured the degree of lipid peroxidation upon I/R. We found an increase of lipid peroxidation in both regions. However, the magnitude of increases was greater in hippocampus than in cortex. In addition, we found that changes in both the magnitude and the temporal patterns of glutathione oxidation closely correlated with those of lipid peroxidation. Our study provides biochemical evidences that the oxidative stress is generated upon I/R and this oxidative stress is linked to the oxidative cell damage. Our study also provides evidences that the degree of oxidative stress as well as oxidative cell damage is greater in hippocampus than in cortex. We could not find difference in the basal level of HSP70 expression between hippocampus and cortex, indicating that the intrinsic vulnerability of hippocampus cannot be explained by the lower level of HSP70 expression. We did find, however, that the induction of HSP70 expression upon I/R was impaired in the hippocampus. This impairment appeared to be at the transcriptional level. These results suggest that the measurement of HSP70 induction may be employed as a useful predictor of differential cellular susceptibilities to the I/R-induced brain damage.

Key Words: Oxidative stress, Ischemia/reperfusion, HSP70

*This study was supported in part by a Grant-in-Aid for Basic Medical Research from the Ministry of Education

INTRODUCTION

Ischemia, or the deprivation of blood flow, is a source of brain damage in a wide variety of pathological conditions including central nervous system trauma and stroke. A prudent therapy to reverse the progression towards ischemic brain damage is unquestionably reperfusion. Reperfusion following ischemia, however, has been suggested to accompany its own components of tissue damage (Cao *et al.*, 1988; Floyd 1990; Oliver *et al.*, 1990; Zweier *et al.*, 1988). One such component is reoxygenation, which sets the stage for oxygen-derived free radical production (Hearse *et al.*, 1973; Hearse & Humphery, 1975; Kirsch *et al.*, 1987). Using electron spin resonance trap technique, production of oxygen free radicals was directly demonstrated in rat brains upon I/R (Kirsch *et al.*, 1987; Cao *et al.*, 1988). Consequently, a critical role of oxygen free radicals has been proposed in ischemia/reperfusion (I/R)-induced brain damage (Chan 1992; Haba *et al.*, 1991; Hall 1989; Siejo *et al.*, 1989; Siejo & Katsura, 1992). Although the concept that oxygen radicals as important mediators of I/R- induced brain damage is generally accepted, experimental validation has been difficult and thus biochemical data to assess the radical mechanisms of brain damage has been desired.

For evaluating different animal models of I/R, it is clear that a number of factors are important in determining the extent of damage that occurs as a result of I/R. We chose to use Mongolian gerbil (*Meriones unguiculatus*) as an animal model to study the oxidative consequences upon brain I/R. Since this animal lacks a connection between the carotid and vertebro-basilar arterial circulation at age of 5 weeks or older (Donadio *et al.*, 1982; Matsuyama *et al.*, 1983), experimental brain I/R is easily produced by a transient occlusion and release of the common carotid arteries at the neck.

Under diverse oxidative stress conditions, the tripeptide glutathione is known to function as one of the major tissue radical scavengers and reductants (Schraufstatter *et al.*, 1985; Shan *et al.*, 1990). Determination of glutathione redox status, therefore, is important in assessing the degree of oxidative stress imposed in biological systems. Previously, glutathione was reported to be consumed and oxidized in rat brains upon I/R (Flamm *et al.*, 1978; Yoshida *et al.*, 1982). Using similar rat brain ischemia models, however, other investigators failed to observe the increase of the oxidized form of glutathione (Cooper *et al.*, 1980; Rehncrona *et al.*, 1980). Although the precise reason for this discrepancy is not clear, one of the possibility could be the difficulty to measure oxidized (GSSG) and reduced (GSH) forms of glutathione selectively. Accurate measurement of oxidized form of glutathione(GSSG) levels, in particular, has proved difficult, both because of the much lower amounts of this form normally present within cells and because of the absence of a convenient chemical feature such as that possessed by the reduced glutathione(Akerboom & Sies, 1981; Meister &Anderson, 1983). In this study, we could measure oxidized (GSSG) and reduced (GSH) forms of glutathione selectively by using sensitive HPLC method (Reed *et al.*, 1980).

Oxidative stress by oxygen free radicals would ultimately lead to the damage of important cellular components. In particular, accumulated information suggests a link between oxygen radical-mediated lipid peroxidation and the pathophysiology of brain upon I/R (Braughler & Hall, 1989; Hall 1989; Halliwell & Dizdaroglu, 1992). In this study, we compared the degree of glutathione oxidation and lipid peroxidation between hippocampus and cortex to investigate whether there is a difference in oxidative cell damage in these regions and to investigate a possible correlation between oxidative cell stress and oxidative cell damage.

Recently, the expression of heat shock protein of 70 kD species (HSP70) was shown to be induced in I/R affected rat or gerbil brains. Since one of the major functions of HSP70 is to protect cells from ill effect of environmental stress (Li *et al.*, 1992; Lindquist & Craig, 1988; Welch 1990), it was proposed that HSP70 plays some roles in protecting brain cells against oxidative stress induced by I/R (Aoki *et al.*, 1993; Nakata *et al.*, 1993). On the other hand, it was

also reported that the level of HSP70 expression differ in different cell types based on in-situ hybridization or immunohistochemistry study (Kato *et al.*, 1993; Liu *et al.*, 1993). These investigators found that HSP70 was preferentially localized within the cells that are susceptible to I/R insults. These observation led them to suggest that the induction of HSP70 is a marker of cells at greater risk. At present, it is not completely resolved whether the induction of HSP70 is physiologically relevant. If it is, it still remains to be solved whether HSP70 plays a protective role in I/R affected brain or it is rather a marker of cells at risk. In this study, we have attempted to investigate whether HSP70 is associated with the regional differences in susceptibily to I/R-induced brain damage. To this end, we compared the expression of constitutive and inducible forms of HSP70 in hippocampus and cortex both before and after experimental I/R.

MATERIALS AND METHODS

Animals

Adult, male mongolian gerbils weighing 60~80 g were kept at constant temperature (about 25℃) under a 12-h light and 12-h dark cycle. Food and water were allowed *ad libitum*..

Induction of experimental brain ischemia / reperfusion to gerbil brains

Forebrain ischemia was produced by bilateral carotid artery occlusion essentially as described by Uyama *et al.* (Uyama *et al.*, 1990). Briefly, the animals were placed onto a heating pad to maintain body temperature at 37℃. The common carotid arteries were exposed and aneurysm clips were placed on both arteries for 10 min. The animals were then reperfused for the indicated time intervals. Control animals were sham-operated but otherwise identically treated. The animals were then decapitated, the brains were removed, and the hippocampus and cortex were dissected. The samples were kept frozen (−80℃) until analyzed.

Measurement of oxidized and reduced forms of glutathione

Glutathione was analyzed by a minor modification of the HPLC method by Reed *et al.* (Reed *et al.*, 1980). Brain tissues was homogenized directly in 5% perchloric acid to precipitate proteins. The samples were derivatized with 1-fluoro-2,4-dinitrobenzene (FDNB) by adding an equal volume of 3% FDNB in absolute ethanol and were separated on 3-aminopropyl bonded reverse-phase ion exchange column. Two solvents were used to generate gradients. Solvent A was 4:1 methanol: water (v:v) and solvent B was prepared as follows: 272 g sodium acetate trihydrate, 122 ml water, and 378 ml glacial acetic acid were mixed and 200 ml of the resulting solution was added to 800 ml solvent A. Typically, an isocratic period of 10 min was followed by a gradient started at 5 to 25% and programmed linearly to 95% solvent B over a 20 to 40 min period. Duplicate aliquots of each sample were reacted with 20 mM N-ethylmaleimide and analyzed identically to correct for the oxidation of glutathione (GSH) to glutathione disulfide (GSSG) which might occur during sample preparation (Collison *et al.*, 1986).

Measurement of lipid peroxidation

The thiobarbituric acid method of Ohkawa *et al.* (1979) was used to measure the amount of lipid peroxides contained in samples. Briefly, each 100 ml of brain extracts was reacted with an equal volume of 8% sodium dodecyl sulfate. Subsequently, the reaction mixture consisting of two volume each of 0.8% 2-thiobarbituric acid and 20% acetic acid was added. This solution was placed in a boiling water bath and kept at 95℃ for 60 min. After stopping the reaction by cooling with tap water, the mixture was centrifuged at 15,000 g for 5 min to precipitate any interfering particulate materials. The amount of lipid peroxides was measured by spectrofluorometry (SPF-500 C; SLM instruments, Inc., Urbana, IL, USA) at emission wavelength of 553 nm with excitation at 515 nm. Protein contents were measured by the method of Lowry *et al.* (Lowry *et al.*, 1951).

Heat shock protein 70 induction in gerbil brains

The induction of heat shock proteins was achieved following 2 min of a transient ischemia. When the animals regained consciousness and righting reflex, they were returned to and maintained in their cages for 0, 3, 8, 24, 48 hrs. The animals were decapitated at the end of the indicated time periods, the brains were removed, and the hippocampus and cortex were dissected. Control animals were sham-operated. The samples were kept frozen at −80℃ until analyzed.

Western blot analysis:

Tissue extracts were solubilized in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with 10% acrylamide for separating gels as described by Laemmli (Laemmli 1970). Protein concentration was measured by the method of Lowry *et al.* (Lowry *et al.*, 1951) and equal amounts of proteins were subjected to SDS-PAGE. Immediately after the electrophoresis, the proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Bio-Rad) by electroblotting (E.C. Apparatus, St. Petersburg, Fla.). Proteins transferred to the membrane were probed with a mouse monoclonal antibody (mAb), N6 F3-5, which recognizes both the constitutive and inducible forms of HSP70 (gift from Dr. William Welch's lab. at UCSF, CA). Binding of the mAb to HSP70 was detected by incubation of the blot with an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Bio-Rad). 3-Hydroxy-2-naphtholic acid 2,4-dimethyanizide (Sigma)/ Fast Red TK dye (Bio-Rad) were used as substrates for the AP reaction.

Northern blot analysis:

Total RNA was isolated from dissected tissues by guanidium isothiocyanate lysis and CsCl ultracentrifugation(Chirgwin *et al.*, 1979). Aliquots of total RNA (10 μg) were fractionated on 0.8% agarose-formaldehyde denaturing gels and transferred onto nitrocellulose. The membrane was then baked in a vacuum oven for 3 h at 80℃ and hybridized with the 1.2 kb *BamHI-ClaI* fragment of the plasmid pH2.3 (kindly provided by Dr. R. I. Morimoto) labeled with (α-^{32}P)-dCTP. Hybridization was carried out at 42℃ for 24 h in 50% formamide / 5x Denhardt's solution / 1 M NaCl / 50 mM Tris-Cl, pH 7.4 / 0.1% SDS containing 100 mg/ml sonicated salmon sperm DNA. The blots were then washed four times in 2x SSC, 0.1 % SDS at 42℃ and twice in 0.1 x SSC, 0.1% SDS at 55℃ for 5 min per wash. The integrity of the RNA samples was confirmed by the presence of distinct ribosomal RNA bands under UV light in ethidium bromide-stained gels.

Statistics

Statistical analysis was performed using ANOVA (analysis of variance) and Scheffe test. Data were expressed as means ± standard error of the means. P values of <0.05 were considered as significant and indicated with asterisk symbols in the figures.

RESULTS

Measurement of oxidized (GSSG) and reduced (GSH) forms of glutathione from gerbil brains

Under diverse oxidative stress conditions, the tripeptide glutathione (GSH) is known to function as one of the major tissue radical scavengers and reductants. Since oxidative stress has been implicated in ischemia/reperfusion (I/R)-induced brain damage, it was reasonable to assume that the portion of GSH would be oxidized to GSSG upon I/R. We therefore have attempted to monitor the oxidative stress generated upon I/R by measuring the degree of the glutathione oxidation. One of the prerequisite to accomplish this goal was the assay system to quantitate the oxidized (GSSG) and reduced (GSH) forms of glutathione selectively. By taking advantage of HPLC assay system developed by Reed *et al.* (Reed *et al.*, 1980), we were able to quantitate oxidized (GSSG) and reduced (GSH) forms of glutathione contained in the brain tissue extracts. An HPLC chromatogram of 1-fluoro-2,4-dinitrobenzene (FDNB)-derivatized sample of brain tissue extract is shown in Figure 1A. Typical retention time for GSH and

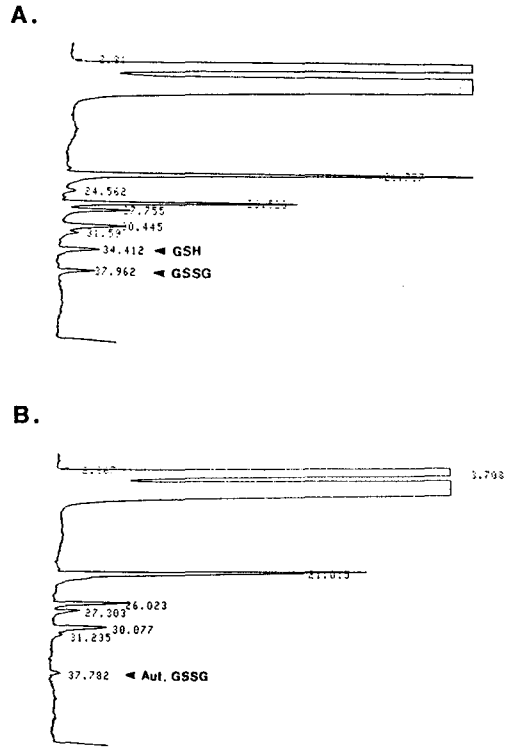

Fig. 1. An HPLC chromatogram of derivatized brain tissue extracts.
Brain tissues were extracted with 5 % perchloric acid and the supernatants were derivatized with 1-fluoro-2,4-dinitrobenzene (FDNB). The derivatized samples were separated by HPLC as described in Materials and Methods. A typical HPLC chromatogram is shown in panel A. The retention time of GSH and GSSG was 34 and 37 min, respectively (panel A). Typical HPLC chromatogram of the N-ethylmaleimide treated samples prior to the derivatization is shown for comparison (panel B). Note the reduction of GSSG peak as well as the disappearance of GSH peak in the N-ethylmaleimide treated sample.

GSSG was found to be 34 and 37 min respectively. We considered the possibility that a part of GSH might undergo oxidation during sample preparation procedure for HPLC analysis (Tietze, 1969). Thus, we pretreated a second aliqout of tissue extracts with N-ethylmaleimide (NEM) prior to the sample preparation procedure to block free -SH group of GSH. A decrease of GSSG peak in HPLC chromatogram of such NEM-reacted samples was observed, and the peak represented authentic GSSG in the sample (Fig. 1B). GSH was quantitated by substracting GSSG obtained as above from total glutathione. This result demonstrated that the portion of the GSSG peak was in fact the oxidized product of GSH than the authentic GSSG initially contained in the tissue. In addition, we found that the elution time of NEM reacted GSH was changed, resulting in the disappearance of GSH peak from its normal retention time. By converting the artificial GSSG to GSH equivalents, we were able to estimate the authentic GSH and GSSG initially contained in the tissue.

Effect of ischemia/reperfusion on glutathione oxidation in hippocampus and cortex

Since hippocampus has been suggested as one of the most vulnerable regions of the brain, we compared the degree of glutathione oxidation between hippocampus and cortex upon I/R. The amount of oxidized glutathione (GSSG)/ total glutathione equivalent (GSH+ 2 x GSSG) was taken as the glutathione oxidation index. We subjected the gerbil to a 10 min of ischemia by occluding the common carotid arteries of the neck and reperfused for 15, 30, 60, and 90 min subsequent to ischemia. Control animals received identical surgical treatment except for the occlusion of the common carotid arteries. We found that the glutathione oxidation index increased significantly in hippocampus at early phase of reperfusion, peaking at 30 min (Fig. 2).

Effect of ischemia/reperfusion on lipid peroxidation in hippocampus and cortex

Having found that the glutathione oxidation was greater in hippocampus, we next asked whether there is a difference in the oxidative cell damage between hippocampus and cortex. Since the important contribution of membrane lipid peroxidation on the pathogenesis of I/R-induced brain damage has been reported, we measured the lipid peroxidation as an index of oxidative cell damage. Lipid peroxidation was

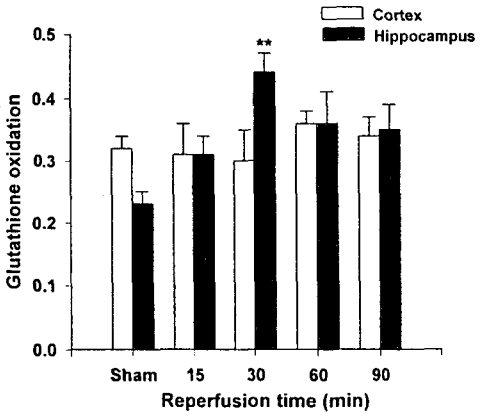

Fig. 2. Changes of glutathione oxidation status in hippocampus and cortex upon ischemia/reperfusion (I/R).
Gerbil brains were subjected to a 10 min ischemia by the common carotid occlusion and were allowed to reperfuse for the designated time periods. The brains were removed at the end of the reperfusion time and the hippocampi and cortices were dissected and subjected to the HPLC analysis of glutathione. Glutathione oxidation index was determined by the amount of oxidized glutathione (GSSG) over total glutathione equivalents (GSH + 2 x GSSG). Data obtained from hippocampus (dark bars) and cortex (light bars) are shown. Values are mean±S.E.M for 5 animals. Experiments were repeated at least three times. $^{**}P < 0.01$ vs. sham-operated controls.

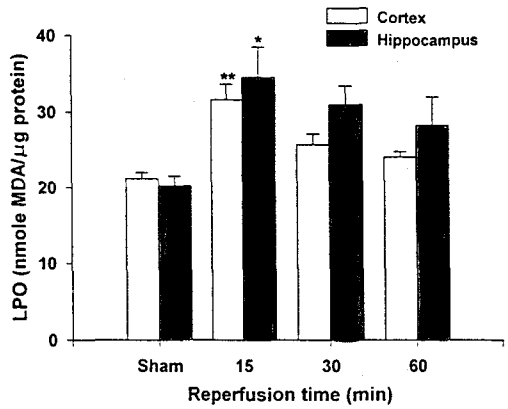

Fig. 3. Changes of lipid peroxidation in hippocampus and cortex upon ischemia/reperfusion (I/R).
Gerbil brains were subjected to a 10 min ischemia by the common carotid occlusion and were allowed to reperfuse for the designated time periods. The brains were removed at the end of the reperfusion time and the hippocampi and cortices were dissected and subjected to the lipid peroxidation analysis. The values of lipid peroxidation were expressed as nmole MDA per μg of tissue protein. Data obtained from hippocampus (dark bars) and cortex (light bars) are shown. Values are mean±S.E.M for 5 animals. Experiments were repeated at least three times. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham-operated controls. MDA, malondialdehyde.

estimated by thiobarbituric acid (TBA) reactivity assay (Ohkawa *et al.*, 1979) using spectrofluorometer as described in Materials and Methods. Our results show that the degree of lipid peroxidation increased in both hippocampus and cortex during an early phase of reperfusion, approximately peaking at 15 min (Fig. 3). However, the level of lipid peroxidation was greater in hippocampus than in cortex at all reperfusion period tested. In fact, both the magnitude and temporal patterns of the lipid peroxidation changes of hippocampus were similar to those seen in glutathione oxidation changes.

Comparison of HSP70 expression between hippocampus and cortex

Because HSP70 has been proposed to play some roles in protecting brain cells upon I/R-induced brain damage, we asked whether the preferential susceptibility of hippocampal cells is due, in part, to the lower level of constitutive HSP70 expression. The Coomassie blue-stained gel of the separated tissue proteins showed almost no differences in the level of constitutive HSP70 expression between hippocampus and cortex (data not shown), indicating the possibility unlikely.

We then examined the induction of HSP70 in these regions upon I/R by Western blot analy-

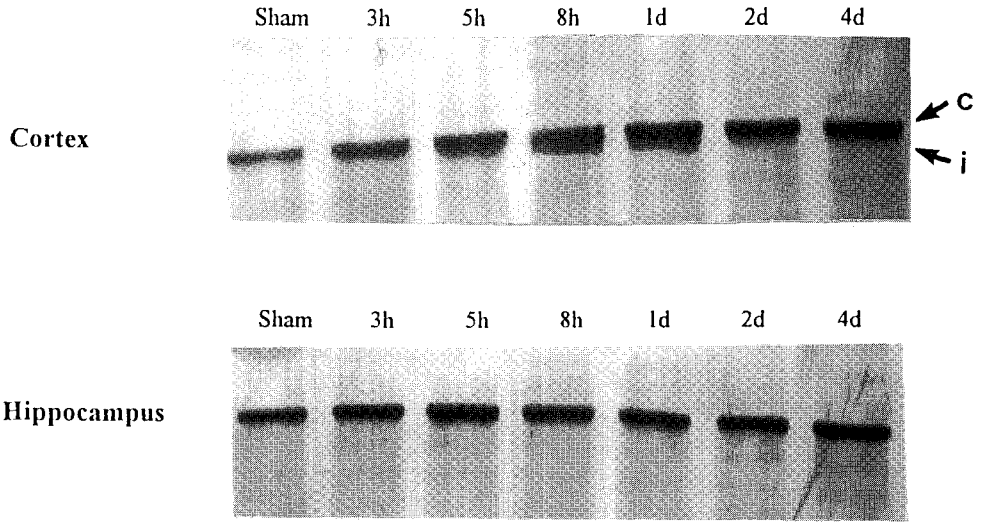

Fig. 4. Western blot analysis of hippocampus and cortex upon ischemia/reperfusion
Gerbil brains were subjected to a 2 min ischemia by the common carotid occlusion and were allowed to reperfuse for the designated time periods. Tissue extracts from hippocampus and cortex were separated by SDS-PAGE on 10% separating gel. The gel was transferred to a nitrocellulose membrane in a Trans-Blot apparatus (BioRad, Richmond, CA). The membrane was probed with a mouse monoclonal antibody (mAb), N6 F3-5, which recognizes both constitutive and inducible forms of HSP70 (c, constitutive; i, inducible)

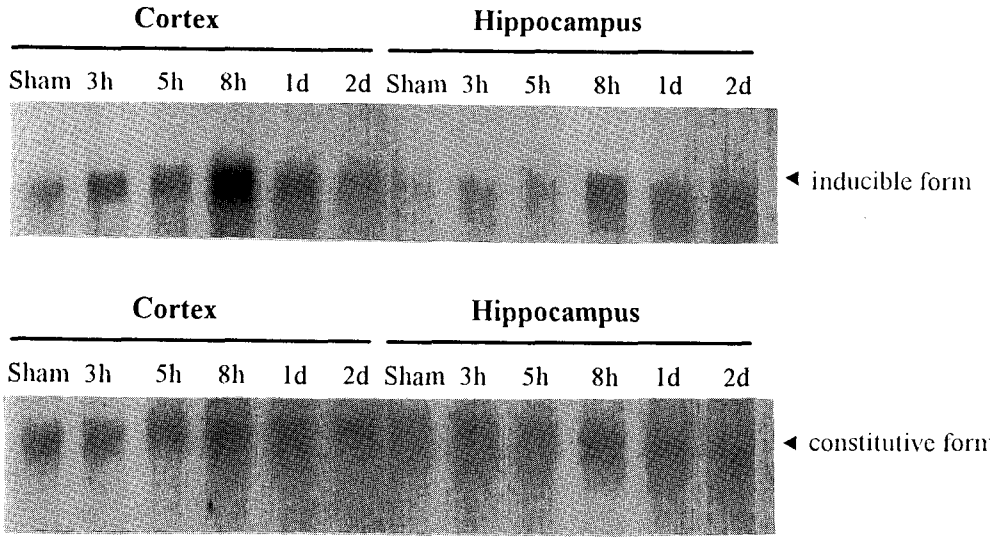

Fig. 5. Northern blot analysis
Gerbil brains were subjected to a 2 min ischemia by the common carotid occlusion and were allowed to reperfuse for the designated time intervals. Aliquots of total RNA (10 μg) from hippocampus and cortex were fractionated in 0.8% agarose-formaldehyde denaturing gels and transferred onto nitrocellulose. The blots were hybridized with a [α-^{32}P]-dCTP labeled, 1.2 kb *BamHI-ClaI* fragment of the human *hsp*70A (kindly provided by Dr. Morimoto) by random hexanucleotide priming. This probe hybridized with both constitutive and inducible forms of HSP70 mRNA.

sis. We tested 2, 5 and 10 min of ischemic period and observed essentially the same pattern of HSP70 expression as shown in Figure 4. The antibody we used to detect HSP70 was N6 F3-5, which recognizes both constitutive (c) and inducible (i) HSP70. In cortex, we found a strong HSP70 induction at about 5hr-1d reperfusion period that followed a transient ischemia (upper panel). However, the induction of HSP70 was not as obvious in hippocampus as in cortex, although there might be some induction in hippocampus at similar reperfusion period(lower panel). In order to detect the small amount of induced HSP70 protein in hippocampus, we had to load twice as much proteins (note the differences in sham-operated cortex and hippocampus). To examine if the impaired HSP70 induction shown in hippocampus merely represents an insufficient translational recovery, we carried out Northern blot analysis. Our results showed that the induction of HSP70 mRNA transcription was in fact poor in hippocampus (Fig. 5, upper panel). The pattern of constitutive HSP70 mRNA transcription was similar between hippocampus and cortex (Fig. 5, lower panel).

DISCUSSION

Our study shows that oxidative stress is generated upon ischemia/reperfusion (I/R) in gerbil brains and this oxidative stress is closely related to oxidative cell damage. The generation of the oxidative stress was demonstrated by the increase of glutathione oxidation upon I/R(kirsch *et al.*, 1987; Cao *et al.*, 1988). The close relationship between the oxidative stress and the oxidative cell damage was demonstrated by the fact that the changes of the glutathione oxidation index were faithfully reflected to those of the lipid peroxidation; changes in both the magnitude and temporal patterns of glutathione oxidation index and lipid peroxidation were similar. Our study also provides evidences that the degree of oxidative stress as well as oxidative cell damage is greater in hippocampus than in cortex. Greater increase of the glutathione oxidation index as well as lipid peroxidation was demonstrated in hippocampus. In addition, the results from our study suggest that this regional difference might be predicted by the differences in the capability to induce HSP70 expression upon I/R, rather than by the differences in the preexisting constitutive level of HSP70 expression.

It is well known that the exposure of biological membranes to the oxidative stress results in progressive degeneration of membrane structure and loss of activity(Esterbauer *et al.*, 1991; Vladimirov *et al.*, 1980). Since one of the most sensitive targets of oxidative attack in biological membrane is lipid and the generation of oxygen radicals has been demonstrated upon I/R, several investigators have attempted to link oxygen radical induced lipid peroxidation to the pathophysiology of I/R-induced brain damage. The logical hypothesis derived from these studies was that tissue antioxidants were being consumed to quench pathological free radical processes(Clemens & Panetta, 1994; Nagao *et al.*, 1995; Yamamoto *et al.*, 1983)

Although glutathione is one of the major tissue antioxidants and thus it is generally accepted that glutathione functions as a buffer against oxidative cell damage(Scharaufstatter *et al.*, 1985; Shanet *et al.*, 1990), there are not much experimental data available on the glutathione oxidation upon I/R induced brain damage. In this study, we have attempted to determine the changes of the glutathione oxidation in hippocampus and cortex upon I/R. By taking advantage of the sensitive HPLC technique (Reed *et al.*, 1980), we were able to measure oxidized and reduced forms of glutathione selectively. Our results show that the degree of gluathione oxidation increased upon I/R, providing biochemical evidence that oxidative stress is generated upon I/R. Our finding that the changes of the glutathione oxidation index were faithfully translated to those of lipid peroxidation provides experimental evidences that oxidative stress and cell damage generated upon I/R are closely correlated.

A protective role of HSP70 in diverse stress conditions including oxidative stress has been suggested(Li *et al.*, 1992; Lindquist & Craig, 1988; Welch, 1990). After we found a greater increase of the glutathione oxidation as well as

lipid peroxidation in hippocampus, we examined the possibility that the intrinsic vulnerability of hippocampal cells may result from the lower level of basal HSP70 expression. We could not find difference in the level of HSP70 expression between sham-operated hippocampus and cortex, indicating that the possibility is unlikely. We next examined whether there is a difference in the inducibility of HSP70 expression in these regions. We did find that the induction of HSP70 upon I/R was impaired in hippocampus. The impairment appeared to be at the transcriptional level.

Our results are consistent with the recent finding of Marcuccilli *et al.* (Marcuccilli *et al.*, 1996), who showed that rat hipppocampal neurons do not express heat shock factor 1 (HSF1) that is known to be a principal mediator of stress-induced heat shock gene expression. Since we did find some induction of HSP70 mRNA in hippocampus in our gerbil model, our result is somewhat different from that of these investigators. It is possible that the induction of HSP70 mRNA we observed was from cell types other than neuron since we did not distinguish cell types within the hippocampus. It is also possible that there is a species difference and that HSF1 may be present in the gerbil, but the signal transduction pathways activating the heat shock repsonse may be less active or impaired in hippocampal cells. Follow-up studies probing the differences in gene expression pattern would allow us to dissect molecular signals that I/R transmits the cell for protection against this form of stress. Although we cannot completely exclude the possibility that this regional difference is due to the insufficient translational recovery of hippocampal cells, this possibility is less likely because the expression of constitutive form of HSP70 as well as other cellular proteins was comparable between the two regions.

In summary, this study provides biochemical evidences that oxidative stress is generated upon I/R in gerbil brains and that this oxidative stress is closely related to oxidative cell damage. Our study also provides experimental evidences that hippocampus is more vulnerable to I/R-induced oxidative stress than cortex, verifying the view that hippocampus is one of the most vulnerable region of the brain to I/R-induced oxidative stress. In addition, our study implicates that the regional differences in cellular susceptibilities to oxidative stress may be predicted by the measurment of HSP70 inducibility rather than by the preexisting level of constitutive form of HSP70 protein.

REFERENCES

Aoki M, Abe K, Kawagoe J, Nakamura S and Kogure K: *The preconditioned hippocampus accelerates HSP70 heat shock gene expression following transient ischemia in the gerbil . Neuroscience Letters 155 :7-10, 1993*

Braughler JM and Hall ED: *Central nervous system trauma and stroke: I. biochemical considerations for oxygen radical formation and lipid peroxidation. Free Radical Biol Med 6: 289-301, 1993*

Cao W, Carney JM, Duchon A, Floyd RA and Chevion M: *Oxygen free radical involvement in ischemia and reperfusion injury in post-ischemic tissues. Neurosci Lett 88: 233-238, 1988*

Chan PH: *Antioxidant-dependent amelioration of brain injury: role of CuZn-superoxide dismutase. J Neurotrauma 9: S417-S423, 1992*

Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ: *Isolation of biologically active RNA from sources enriched in ribonuclesase. Biochemistry 18: 5294-5299, 1979*

Clemens JA and Panetta JA: *Neuroprotection by antioxidants in models of global and focal ischemia. Ann NY Acad Sci 738: 250-256, 1994*

Collison MW, Beidler D, Grimm LM and Thoma JA: *A comparison of protein S-thiolation(protein mixied-disulfide formation) in heart cells treated with t-butyl hroperoxide or diamide. Biochim Biophys Acta 855: 58-67, 1986*

Cooper AJ, Pulsinelli WA and Duffy TE: *Glutathione and ascorbate during ischemia and post-ischemic reperfusion in rat brain. J Neurochem 35: 1242-1245, 1980*

Donadio MF, Kozlowski PB, Kaplan H, Wisniewski HM and Majkowski J: *Brain vasculature and induced ishchemia in seizure-prone and non-seizure-prone gerbils. Brain Res 234: 263-274, 1982*

Easterbauer H, Zollner H and Schaur R: *Aldehydes formed in lipid peroxidation: mechanisms of formation, occurrence and determination. In Membrane Lipid Oxidation(ed. Vigo-Pelfry C) CRC Press, pp239-283, 1990*

Flamm ES, Demopoulos HB, Seligman ML, Poster RG and Ransohoff J: *Free radicals in cerebral ischemia. Stroke 9: 445-447, 1978*

Floyd RA: *Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J 4: 2587-2597, 1990*

Haba K, Ogawa N, Mizukawa K and Mori A: *Time course of changes in lipid peroxidation, pre- and postsynaptic cholinergic indices, NMDA receptor binding and neuronal death in the gerbil hippocampus following transient ischemia. Brain Research 540: 116-122, 1991*

Hall ED: *Free radicals and CNS injury. Crit Care Clin 5: 793-805, 1989*

Halliwell B and Dizdaroglu M: *The measurement of oxidative damage to DNA by HPLC and GC/MS techiques. Free Radical Research Communications 16: 75-87, 1992*

Kato H, Liu XH, Nakata N and Kogure K: *Immunohistochemical visualization of heat shock protein-70 in the gerbil hippocampus following repeated brief cerebral ischemia. Brain Research 615: 240-244, 1993*

Laemmli UK: *Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970*

Li GC, Li L, Liu RY, Rehman M and Lee MWF: *Heat shock protein hsp70 protects cells from thermal stress even after deletion of its ATP-binding domain. Proc Natl Acad Sci USA 89: 2036-2040, 1992*

Lindquist S and Craig EA: *The heat shock proteins. Annu Rev Genet 22: 631-677, 1988*

Liu Y, Kato H, Nakata N and Kogure K: *Correlation between induction of ischemic tolerance and expression of heat shock protein-70 in the rat hippocampus [Japanese]. No to Shinkei - Brain & Nerve 45: 157-162, 1993*

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: *Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275, 1951*

Marcuccilli CJ, Sameer KM, Morimoto RI and Miller RJ: *Regulatory differences in the stress response of hippocampal neurons and glial cells after heat shock. J Nuroscience 16: 478-485, 1996*

Matsuyama T, Matsumoto M, Fujisawa A, Handa N, Tanaka K, Yoneda S, Kimura K and Abe H: *Why are infant gerbils more resistant than adults to cerebral infarction after carotid ligation? J Cerebr Blood Flow Metab 3: 381-385, 1983*

Nagao T, Yamamoto YL, Inoue N, Ito Y: *Retrograde perfusion of the cerebral vein with antioxidant LY231617 reduces brain damage in the rat focal ischemia model. Neurol Med Chir 35: 861-868, 1995*

Nakata N, Kato H and Kogure K: *Inhibition of ischaemic tolerance in the gerbil hippocampus by quercetin and anti-heat shock protein-70 antibody. Neuroreport 4: 695-698, 1993*

Ohkawa H, Ohishi N and Yagi K: *Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-358, 1979*

Oliver CN, Strake-Reed PE, Stadman ER, Lin GJ, Camey JM and Floyd RA: *Oxidative damage to brain proteins, loss of glutamine synthase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc Natl Acad Sci USA 87: 5144-5145, 1990*

Reed DJ, Babson JR, Brodie PW, Ellis WW and Potter DW: *High-Performance Liquid Chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfide. Analytical Biochemistry 106: 55-62, 1980*

Rehncrona S, Folbergrova J, Smith DS and Siejo BK: *Influence of complete and pronounced incomplete ischemia and subsequent recirculation on cortical concentrations of oxidized and reduced glutathione in the rat. J Neurochem 34: 477-486, 1980*

Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RG and Cochrane CG: *Glutathione cycle activity and pyridine nucleotide levels in oxidant-induced injury of cells. J Clin Invest 76: 1131-1139, 1985*

Shan X, Yee, AWT and Jones DP: *Glutathione-dependent protection against oxidative injury. Pharmac Ther 47: 61-71, 1990*

Siejo BK, Agardh CD and Bengtsson F: *Free radicals and brain damage. Cerebrovasc Brain Metab Rev 1: 165-211, 1989*

Siejo BK and Katsura K: *Ischemic brain damage: focus on lipids and lipid mediators. Adv Exp Med Biol 318: 41-56, 1992*

Tietze F: *Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Application to mammalian blood and other tissues. Analytical Biochemistry 27: 502-522, 1969*

Uyama O, Shiratsuki N, Matsuyama T, Nakanishi T, Matsumoto Y, Yamada T, Narita M and M: *Sugita: Protective effects superoxide dismutase on acute reperfusion injury of gerbil brain. Free radical Biol Med 8: 265-268, 1990*

Vladimirov, Yu A, Olenev VI, Suslova TB & Cheremisina ZP: *Lipid peroxidation in mitochondrial membranes. Adv Lipid Res 17: 173-249, 1980*

Welch WJ: *The mammalian stress response: Cell physiology and biochemistry of stress proteins. Stress Proteins in Biology and Medicine, ed. Morimito R, Tissieres A and Georgopoulos C: Cold Spring Harbor, New York: Cold Spring Harbor Press, 1990.*

Yamamoto M, Shima T, Uozumi T, Sogabe T, Yamada K and Kawasaki T: *A possible role of lipid peroxidation in cellular damages caused by cerebral ischemia and the protective effect of alpha-tocopherol administration. Stroke 14: 977-982, 1983*

Yoshida S, Abe K, Busto R, Watson BD, Kogure K and Ginsberg MD: *Influence of transient ischemia on lipid-soluble antioxidants, free fatty acid and post ischemic reperfusion in rat brain. Brain Res 245: 307-316, 1982*

Zweier JL, Kuppusamy P and Lutty GA: *Measurement of endothelial cell free radical generation: Evidence for a central mechanism for free radical injury in post-ischemic tissues. Proc Natl Acad Sci USA 85: 4046-4050, 1988*

=국문초록=

모래쥐에서 뇌의 허혈/재관류에 의한 산화성 스트레스 형성과 HSP70의 발현

[1]인천대학교 자연과학대학 생물학과, [2]화학과, [3]연세대학교 의과대학 약리학교실

박영미[1] · 김철훈[3] · 도윤정[3] · 최은미[2] · 안영수[3]

허혈/재관류 뇌손상에서 활성산소류의 역할이 중요시되고 있다. 본 연구에서는 모래쥐의 총경동맥을 묶었다 풀어줌으로써 실험적 허혈/재관류 손상을 유도하고 산화성 스트레스 발생 유무와 이러한 산화성 스트레스가 세포손상으로 연결되는지를 알아보고자 하였다. 해마는 뇌조직 중에서도 특히 산화성 스트레스에 취약한 부분이므로 해마에서 얻은 조직을 대뇌피질에서 얻은 조직과 비교분석하였다. 또한, 이들 부위에서 heat shock protein 70(HSP70)의 발현이 허혈/재관류 손상에 미치는 영향도 검색하고자 하였다. 허혈/재관류에 의한 산화성 스트레스의 지표로써 글루타치온 산화정도, GSSG/(GSH+2xGSSG)를 측정하였을 때 주로 해마에서 산화지표가 상승됨을 관찰하였다. 한편 산화성 스트레스가 세포손상으로 연결되는지를 알아보고자 지질과산화물을 측정하였다. 두 부위 모두에서 지질과산화물 형성의 증가가 있었으며 대뇌피질에서보다 해마에서 더 증가됨을 알 수 있었다. 지질과산화물 형성의 정도나 시간적 변화양상이 글루타치온 산화의 그것들과 유사하였다. 이러한 결과들은 허혈/재관류에 의해 산화성 스트레스가 형성되며 동시에 이러한 산화성 스트레스가 세포 손상을 초래함을 보여준다. 또한 산화성 스트레스 및 산화성 세포손상 정도가 대뇌피질보다는 해마에서 더 큰 것을 알 수 있었다. 그러나, 피질과 해마에서 HSP70의 기초발현(basal level) 정도는 차이가 없었다. 이는 해마의 취약성이 HSP70 발현 결핍에 기인하지 않았음을 나타낸다. 반면 허혈/재관류에 의한 HSP70의 발현유도는 해마조직에서 제대로 이루어지지 않았고 northern blot결과 이는 전사단계에서의 부전에 의한 것으로 나타났다. 이러한 결과들로 볼 때 허혈/재관류에 의한 뇌손상에서 HSP70 유도정도를 측정하는 것이 세포의 취약성을 예측할 수 있는 지표로 유용하게 사용될 수 있을 것으로 사료된다.