Journal of Biochemistry and Molecular Biology, Vol. 38, No. 1, January 2005, pp. 111-114

JOURNAL OF Biochemistry and Molecular Biology

Short communication

Expression of Cu/Zn SOD Protein Is Suppressed in *hsp 70.1* Knockout Mice

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Received 6 May 2004, Accepted 19 July 2004

Heat shock proteins (HSPs) are known to protect cells from oxidative stress and other types of injuries. We previously reported the neuroprotective effect of HSP70 following cerebral ischemia and reperfusion using *hsp 70.1* knockout (KO) mice. However, the precise role of HSP70 in neuroprotection has not been established yet. The purpose of this study was to investigate the relationship between HSP70 and antioxidant enzymes using *hsp 70.1* KO mice. The activities of both SOD-1 and SOD-2 were significantly decreased in *hsp 70.1* KO mice than in the wild type (WT) littermates. SOD-1 protein level in the *hsp 70.1* KO mice was lower than that of WT. We speculate that HSP70 might be involved in regulation of expression of SOD-1 at the level of transcription or by post-transcriptional modification.

Keywords: Heat shock protein, *hsp 70.1*, Knock out, SOD-1, SOD-2

Introduction

Heat shock proteins (HSPs) are the major stress proteins induced in response to a variety of stresses including an oxidative stress (Yenari *et al.*, 1999). Recently, using gene transfer techniques and transgenic animal strains, 70-kDa HSP has been shown to play a role in neuroprotection during ischemic injury *in vitro* (Amin *et al*., 1996; Papadopoulos *et al*., 1996; Sato *et al*., 1996; Yenari *et al*., 1999) as well as *in vivo* studies (Rajdev *et al*., 2000; Lee *et al*., 2001). However, the precise role of HSP70 has not been understood. During cerebral ischemia and reperfusion, abundant oxygen radicals, especially superoxide anions are generated, and are thus responsible for the cellular damage (Chan, 1996). Recent studies have also provided evidence that oxygen free radicals can cause cellular damage and apoptotic cell death in the mitochondrial pathway following cerebral ischemia and reperfusion (Fujimura *et al*., 2000; Sugawara *et al*., 2002).

These reactive oxygen radicals are scavenged by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). There are two distinct types of SODs: a cytosolic, dimeric Cu/ZnSOD (SOD-1), and a mitochondrial, tetrameric MnSOD (SOD-2) (Halliwell and Gutteridge, 1998; Yun and Lee, 2003). Despite their different molecular weights and amino acid sequences, they catalyze the conversion of superoxide anions to less harmful products, H_2O_2. Therefore, the regulation and induction of the SOD protein would be of great interest during oxidative stress.

We recently reported that a reduced HSP70 protein level increases cellular damage during acute focal cerebral ischemia in *hsp 70.1* knockout (KO) mice (Lee *et al*., 2001). Using transgenic mice overexpressing SOD-1, previous studies also reported that SOD-1 overexpression can influence the induction of HSP70 mRNA, and is protective following cerebral ischemia (Kamii *et al*., 1994; Kondo *et al*., 1996).

However, the direct relationship between the levels of antioxidant enzymes and the HSP 70 has not been examined. In this study, we attempted to measure the activities of antioxidant enzymes, and also to compare the levels of SOD-1 and SOD-2 in both *hsp 70.1* KO and wild type (WT) littermates.

Materials and Methods

Production of hsp 70.1 KO mice We produced mice bearing a null-allele of *hsp 70.1* using the gene-targeting technique, which is

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described elsewhere in detail (Lee *et al*., 2001). The absence of *hsp 70.1* mRNA expression has also been confirmed previously (Lee *et al*., 2001). Male mice weighing 25-30 g were used throughout our study. The protocol for the care and use of animals throughout this procedure was approved by the animal care committee of Seoul National University.

Sample preparation Three KO and four WT mice were used to determine the activities of SOD, CAT, and GPx. The mice were sacrificed, and the cerebrum was obtained. Brain tissues were weighed and homogenized in ice-cold 50 mM Tris buffer (pH 7.4) containing 0.1 mM EDTA. Fractionations of brain homogenates were performed by differential centrifugation as described previously (Roy *et al*, 1984). After centrifuging the homogenates at 1,000 × *g* for 10 min at 4°C, the supernatant (Sup A) was used for CAT activity. Sup A was further centrifuged at 12,500 × *g* for 30 min at 4°C. The resulting supernatant (Sup B) containing microsome and cytosol was used for assays of SOD-1 and GPx activities and the pellet containing mitochondria was resuspended in the homogenizing buffer for SOD-2 activity assay.

CAT activity assay CAT activity in Sup A was determined by the decrease of the absorbance at 240 nm at 20°C (Abei, 1984; Lee *et al*., 2003). The enzyme activity was expressed as unit/mg protein (1 unit = 1 μmol of H_2O_2 degraded for a minute) using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

SOD-1 and SOD-2 activities assay SOD-1 and SOD-2 activities were assayed by the method of Misura & Fridovich (1972). The increase of the absorbance was monitored at 480 nm at 30°C. One unit of enzyme activity was defined as the amount of enzyme required to inhibit the rate of epinephrine oxidation by 50%.

GPx activity assay GPx activity in Sup B was assayed by the method of Tapple (1978) using cumene hydroperoxide as substrate. The oxidation of NADPH was monitored at 340 nm at 37°C and the calculations were made using a molar extinction coefficient of 6.22 M^{-1} cm^{-1}.

Protein measurement Protein concentration was determined by the method of Lowry *et al*. (1951), using bovine serum albumin as a standard.

Immunoblot analysis Three KO and four WT mice were used for Western blot analysis. The mice were sacrificed by decapitation, and the brains were quickly removed and dissected. These were then placed on ice in 10 volumes of cold homogenization buffer (50 mM Tris, 120 mM NaCl, pH 7.4) to which protease inhibitors (Complete Mini, GIBCO, Grand Island, USA) had been freshly added. The tissue was then homogenized and stored at −70°C. Protein concentrations were determined using the Bradford method (Bio-Rad, Richmond, USA). Protein extracts from brain tissue (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970; Ozdener *et al.*, 2002). The proteins were then transferred to nitrocellulose membrane (Bio-Rad, Richmond, USA), and the blots were probed with either anti-SOD1 antibody (sheep polyclonal IgG, Calbiochem, Darmstadt, Germany) or anti-SOD2 antibody (sheep polyclonal IgG, Calbiochem, Darmstadt, Germany). Signals were detected by enhanced chemiluminescence kit (Supersignal, Pierce, Rockford, USA). The results were scanned with a GS-700 imaging densitometer (Bio-Rad, Richmond, USA), and were quantified using the Multi-analyst software program (Bio-Rad, Richmond, USA). For an internal control to ensure equivalent protein loading and protein integrity, each blot was probed for alpha-tubulin.

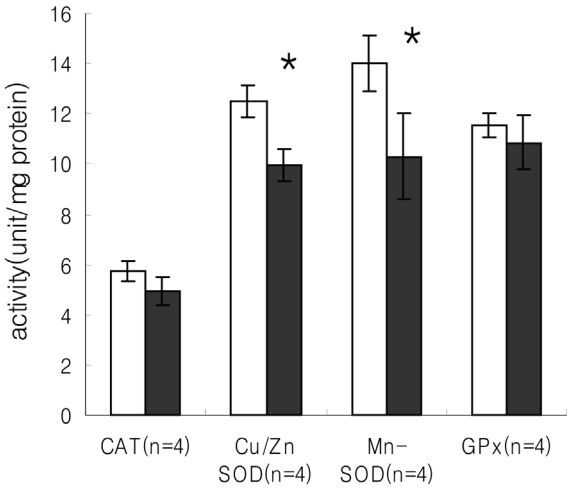

Fig. 1. Cu/ZnSOD(SOD-1), MnSOD(SOD-2), Catalase (CAT) and Glutathion peroxidase (GPx) activities in wild type (WT, open columns) and *hsp 70.1* knockout (KO) mice (closed columns). SOD-1 and SOD-2 activities in WT were significantly higher than those of KO (n=4, $p<0.05$, respectively), whereas no differences were found in CAT and GPx activities between 2 groups. Standard Deviations are presented as vertical bars (*: $p<0.05$, Wilcoxon rank sum test).

Statistical analysis All statistical analyses were carried out by Wilcoxon rank sum test, using the SAS program. A P value of < 0.05 was selected as a limit of statistical significance.

Results

Targeted deletion of the *hsp 70.1* gene did not affect the natural development of mice, nor did it produce any differences in phenotype between the *hsp 70.1* KO and WT mice. Particularly, the color, size, and gross morphology of the brains from the *hsp 70.1* KO mice were not different from those of their WT littermates, although the brains were more brittle in the *hsp 70.1* KO mice.

SOD-1and SOD-2 activities in *hsp 70.1* KO mice were significantly lower than those of WT mice ($p < 0.05$, respectively, Fig. 1). However, no significant differences were found in CAT and GPx activities between *hsp 70.1* KO and WT mice (Fig. 1). Western blot analysis showed a lower level of SOD-1 expression in *hsp 70.1* KO mice (Fig. 2A and 2B), whereas the level of SOD-2 expression was similar in both groups (Fig. 2A and 2B). There were no significant differences in α-tubulin immunoreactivity between two groups.

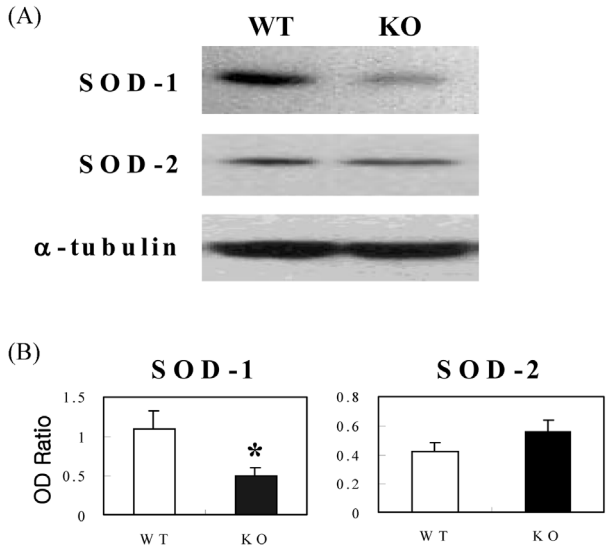

Fig. 2. (A) Western blot analysis of SOD-1 and SOD-2 in wild type(WT) and *hsp 70.1* knockout (KO) mice. An alpha-tubulin protein was used as an internal control and each band was standardized. (B) The optical density (OD) of SOD-1 and SOD-2 protein levels in WT (open columns) and KO (closed columns). The intensity of SOD-1 and SOD-2 protein levels was evaluated by measuring the relative OD. The relative OD of SOD-1 protein was significantly lower in the KO than in the WT group, whereas there were no differences in SOD-2 protein levels between 2 groups (n=3 mice, respectively; *, $p<0.05$, Wilcoxon rank sum test).

Discussion

To our knowledge, this study is the first that examined the direct relationship between the levels of antioxidant enzymes and the HSP70 in *hsp 70.1* KO mice. Our study showed that the expression of SOD-1 protein was low (Fig. 2A and 2B), and SOD-1 and SOD-2 activities were decreased in *hsp 70.1* KO mice (Fig. 1).

Previous studies suggested that regulation of HSP70 and SOD-1 was positively correlated. It has been reported that oxidative stress may induce expression of HSP70 gene (Yenari *et al*., 1999) as well as SOD-1 gene (Yoo *et al*., 1999). In SOD1-transgenic mice, there was prolonged expression of *hsp 70* mRNA after focal (Kamii *et al*., 1994) and global (Kondo *et al*., 1996) cerebral ischemia.

Induction of SOD-2 was reported to be related to HSP expression in rat cardiac myocytes (Liu *et al*., 1992; Das *et al*., 1995; Yamashita *et al*., 1997). Contrary to these results, our study showed that expression of SOD-2 protein was not affected although SOD-2 activity was decreased in *hsp 70.1* KO mice, suggesting that regulation of HSP70 and SOD-2 may not be positively correlated in our model. This was also in line with a previous study showing that transient hypoxic insults did not induce the synthesis of HSP72 in gerbil hippocampus, whereas it induced a progressive and sustained expression of SOD-2 (Garnier *et al*., 2001). The differing results could be due, in part, to multiple regulatory elements in expression and regulation of these enzymes. It is possible to hypothesize that the expression of SOD-2 is not regulated by HSP70 although *hsp 70.1* are partially involved in the activity of SOD-2 by assisting in the folding, transport, and assembly of these enzymes.

There has been controversy regarding the activity of CAT and heat stress. Some reported that endogenous CAT activity in the rat was increased after heat stress and could protect the isolated heart against ischemia-reperfusion injury (Currie *et al*., 1988; Karmazyn *et al*., 1990), while other studies found no correlation between the activity of CAT and the expression of HSP in myocardium (Auyeung *et al*., 1995), and in human umbilical vein endothelial cells (Gill *et al*., 1998). In the present study, the CAT or GPx activities in the brain in both groups were not different (Fig. 1). The reasons for these observations are not clear. It could be, however, that the regulation of CAT and GPx proteins in the brain are different from those of the heart, or the differences could arise simply from the varied experimental species.

One major question remaining to be addressed is whether induction of SOD-1 protein is regulated at the transcriptional level or at the post-transcriptional level. Unfortunately, this question can not be addressed at the present time. However, our results, as well as those of others, lead us to postulate that HSP70 might potentiate the protective effect of SOD-1/SOD-2 through its chaperone functions, and might also play a role in induction of SOD-1 gene.

In summary, our results demonstrate that activity and expression of SOD-1 are decreased, and activity of SOD-2 is decreased although expression of SOD-2 is not affected in *hsp 70.1* KO mice. Although our results are preliminary, we speculate that HSP70 contribute to regulating gene expression of SOD-1 at the level of transcription or by post-transcriptional modification. The interplay between HSP and antioxidant enzyme activities in acute focal cerebral ischemia using *hsp 70.1* KO mice, should be investigated in the future.

Acknowledgments This study was supported by BK21 project (0522-20010002) and Korea Science & Engineering Foundation (R04-2001-000-00197-0).

References

Aebi, H. (1984) Catalase in Vitro. *Methods Enzymol.* **105**, 121-126.

Amin, V., Cumming, D. V. and Latchman, D. S. (1996) Over-expression of heat shock protein 70 protects neuronal cells against both thermal and ischaemic stress but with different efficiencies. *Neurosci. Lett.* **206**, 45-48.

Auyeung, Y., Sievers, R. E., Weng, D., Barbosa, V. and Wolfe, C. L. (1995) Catalase inhibition with 3-amino-1,2,4-triazole does not abolish infarct size reduction in heat-shocked rats. *Circulation* **92**, 3318-3322.

Chan, P. H. (1996) Role of oxidants in ischemic brain damage.

Stroke **27**, 1124-1129.

Currie, R. W., Karmazyn, M., Kloc, M. and Mailer, K. (1988) Heat- shock response is associated with enhanced postischemic ventricular recovery. *Circ. Res.* **63**, 543-549.

Das, D. K., Maulik, N. and Moraru, I. I. (1995) Gene expression in acute myocardial stress. Induction by hypoxia, ischemia, reperfusion, hyperthermia and oxidative stress. *J. Mol. Cell. Cardiol.* **27**, 181-193.

Fujimura, M., Morita-Fujimura, Y., Noshita, N., Sugawara, T., Kawase, M. and Chan, P. H. (2000) The cytosolic antioxidant copper/zinc-superoxide dismutase prevents the early release of mitochondrial cytochrome c in ischemic brain after transient focal cerebral ischemia in mice. *J. Neurosci*. **20**, 2817-2824.

Garnier, P., Demougeot, C., Bertrand, N., Prigent-Tessier, A., Marie, C. and Beley, A. (2001) Stress response to hypoxia in gerbil brain: HO-1 and Mn SOD expression and glial activation. *Brain Res.* **893**, 301-309.

Gill, R. R., Gbur, C. J., Jr., Fisher, B. J., Hess, M. L., Fowler, A. A. 3rd, Kukreja, R. C. and Sholley, M. M. (1998) Heat shock provides delayed protection against oxidative injury in cultured human umbilical vein endothelial cells. *J. Mol. Cell. Cardiol.* **30**, 2739-2749.

Halliwell, B. and Gutteridge, J. M. C. (1999) Antioxidant defenses; in *Free Radicals in Biology and Medicine*, pp. 105-244, Oxford Univ. Press, Oxford, UK.

Kamii, H., Kinouchi, H., Sharp, F. R., Koistinaho, J., Epstein, C. J. and Chan, P. H. (1994) Prolonged expression of hsp70 mRNA following transient focal cerebral ischemia in transgenic mice overexpressing CuZn- superoxide dismutase. *J. Cereb. Blood Flow Metab.* **14**, 478-486.

Karmazyn, M., Mailer, K. and Currie, R. W. (1990) Acquisition and decay of heat-shock-enhanced postischemic ventricular recovery. *Am. J. Physiol.* **259**, 424-431.

Kondo, T., Murakami, K., Honkaniemi, J., Sharp, F. R., Epstein, C. J. and Chan, P. H. (1996) Expression of hsp70 mRNA is induced in the brain of transgenic mice overexpressing human CuZn-superoxide dismutase following transient global cerebral ischemia. *Brain Res.* **737**, 321-326.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, H. Y., Eum, W. S., Kim, D. W., Lee, B. R., Yoon, C. S., Jang, S. H., Choi, H. S., Choi, S. H., Baek, N. I., Kang, T. C., Won, M. H., Cho, S. W., Lee, K. S., Park, J. and Choi, S. Y. (2003) Isolation and identification of an antioxidant enzyme catalse stimulatory compound from Garnoderma Iucidum. *J. Biochem. Mol. Biol.* **36**, 450-455.

Lee, S. H., Kim, M., Yoon, B. W., Kim, Y. J., Ma, S. J., Roh, J. K., Lee, J. S. and Seo, J. S. (2001) Targeted hsp70.1 disruption increases infarction volume after focal cerebral ischemia in mice. *Stroke* **32**, 2905-2912.

Liu, X., Engelamn, R. M., Moraru, I. I., Rousou, J. A., Flack, J. E. 3rd., Daeton, D. W., Maulik, N. and Das, D. K. (1992) Heat shock: a new approach for myocardial preservation in cardiac surgery. *Circulation* **86**, 358-363.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. T. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Misura, H. P. and Fridovich, I. (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**, 3170-3175.

Ozdener, F., Kunapuli, S. P. and Daniel, J. L. (2002) Expression of enzymatically-active phospholipase Cã2 in *E. coli*. *J. Biol. Chem.* **35**, 508-512.

Papadopoulos, M. C., Sun, X. Y., Cao, J., Mivechi, N. F. and Giffard, R. G. (1996) Over-expression of HSP-70 protects astrocytes from combined oxygen-glucose deprivation. *Neuroreport* **131**, 429-432.

Rajdev, S., Hara, K., Kokubo, Y., Mestril, R., Dillmann, W., Weinstein, P. R. and Sharp, F. R. (2000) Mice overexpressing rat heat shock protein 70 are protected against cerebral infarction. *Ann. Neurol.* **47**, 782-791.

Roy, D., Pathak, D. N. and Singh, R. (1984) Effects of chlorpromazine on the activities of antioxidant enzymes and lipid peroxidation in the various regions of aging rat brain. *J. Neurochem.* **42**, 628-633.

Sato, K., Saito, H. and Matsuki N. (1996) HSP 70 is essential to the neuroprotective effect of heat- shock. *Brain Res.* **470**, 117-123.

Sugawara, T., Noshita, N., Lewen, A., Gasche, Y., Ferrand-Drake, M., Fujimura, M., Morita-Fujimura, Y. and Chan, P. H. (2002) Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. *J. Neurosci.* **22**, 209-217.

Tappel, A. L. (1978) Glutathione peroxidase and hydroperoxides. *Methods Enzymol.* **52**, 506-513.

Yamashita, N., Hoshida, S., Nishida, M., Igarashi, J., Taniguchi, N., Tada, M., Kuzuya, T. and Hori, M. (1997) Heat shock-induced manganese superoxide dismutase enhances the tolerance of cardiac myocytes to hypoxia-reoxygenation injury. *J. Mol. Cell Cardiol.* **29**, 1805-1813.

Yenari, M. A., Giffard, R. G., Sapolsky, R. M. and Steinberg, G. K. (1999) The neuroprotective potential of heat shock protein 70 (HSP70). *Mol. Med. Today.* **5**, 525-531.

Yoo, H. Y., Chang, M. S. and Rho, H. M. (1999) The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by paraquat and heat shock through the same heat shock element. *J. Biol. Chem.* **274**, 23887-23892.

Yun, Y. S. and Lee, Y. N. (2003) Production of superoxide dismutase by *Deinococcus radiophilus*. *J. Biochem. Mol. Biol.* **36**, 282-287.