EXERCISE PRECONDITIONING REDUCES NEURONAL APOPTOSIS IN STROKE BY UP-REGULATING HEAT SHOCK PROTEIN-70 (HEAT SHOCK PROTEIN-72) AND EXTRACELLULAR-SIGNAL-REGULATED-KINASE 1/2

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Abstract—Exercise preconditioning induces neuroprotection after stroke. We investigated the beneficial role of heat shock protein-70 (HSP-70) and phosphorylated extracellular-signal-regulated-kinase 1/2 (pERK 1/2), as they pertain to reducing apoptosis and their influence on Bcl-xL, Bax, and apoptosis-inducing factor (AIF) in rats subjected to ischemia and reperfusion. Adult male Sprague–Dawley rats were subjected to 30 min of exercise on a treadmill for 1, 2, or 3 weeks. Stroke was induced by a 2-h middle cerebral artery (MCA) occlusion using an intraluminal filament. Protein levels of HSP-70, pERK 1/2, Bcl-xL, Bax, and AIF were analyzed using Western blot. Neuroprotection was based on levels of apoptosis (TUNEL) and infarct volume (Nissl staining). Immunocytochemistry was used for cellular expression of HSP-70 and pERK 1/2, after 3 weeks of exercise coincided with significant (P < 0.05) reduction in neuronal apoptosis and brain infarct volume. Inhibition of either one of these two factors showed a significant (P < 0.05) reversal in the neuroprotection. Bax and AIF were down-regulated, while levels of Bcl-xL were up-regulated in response to stroke exercise. Inhibiting HSP-70 or pERK 1/2 reversed this resultant increase or decrease. Our results indicate that exercise diminishes neuronal injury in stroke by up-regulating HSP-70 and ERK 1/2.

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Key words: ischemia/reperfusion injury, MCA occlusion, neuroprotection, pro-apoptotic protein, anti-apoptotic protein.

Neurological recovery after stroke depends strongly on the survival of brain cells. Expanding literature substantiates the beneficial effects of exercise preconditioning on stroke-induced brain injury in animal stroke models (Ding et al., 2005). However, the protective mechanisms in stroke, particularly pertaining to neuronal death induced by physical exercise, remain unclear.

It is well accepted that heat shock protein-70 (HSP-70) induced after physiological or pathological stress acts against subsequent damage by increasing the tolerance of affected cells (Giffard and Yenari, 2004; Matsumori et al., 2005). However, whether exercise preconditioning increases HSP-70 expression and whether the appearance of HSP-70 prior to ischemia/reperfusion (I/R) injury plays a role in the exercise-based neuroprotection remains unknown.

Mitogen-activated protein kinase (MAPKs) pathways play a pivotal role in signal transduction through both kinases and phosphatases. The extracellular-signal-regulated-kinase 1/2 (ERK1/2) pathway is the most extensively characterized MAPK pathway (Sharony et al., 2005). MAPK/ERK kinase 1/2 (MEK1/2) activates ERK1/2 by phosphorylation. While these kinases extensively promote cell death in both neurons and other cell types (Chu et al., 2004; Zhuang and Schnellmann, 2006), growing evidence implies ERK signaling has a beneficial and neuroprotective factor in many systems (Hetman and Gozdz, 2004). Activation of ERK1/2 is an important defense mechanism against transient hypoxia/ischemia, by counteracting cell death and enabling damage repair. Induction of ischemic tolerance following an episode of mild ischemia (preconditioning) is modeled by activation of ERK1/2 in cultured rat cortical neurons (Gonzalez-Zulueta et al., 2000), as well as in the ischemic heart (Tong et al., 2000) and ischemic brain (Shamloo and Wieloch, 1999; Jones and Bergeron, 2004). Investigating the effect of HSP-70 up-regulation and signal transduction on downstream apoptotic pathways was the focus of the present study.

Apoptotic neuronal death occurs in I/R injury. I/R injury exacerbates apoptotic cell death due to an imbalance between Bcl-2 family members that suppress (such as Bcl-2 and Bcl-xL) and those that promote (such as Bax and Bad) apoptosis in the brain (Wu et al., 2003). A caspase-independent apoptotic pathway, involving activation of apoptosiss-inducing factor (AIF), has also been identified (Joza et al., 2001; Cao et al., 2003). Recent studies have shown that MEK/ERK-mediated signals play a major role in ischemia-induced apoptosis through regulation of Bax/Bcl-2/Bcl-xL expression (Li et al., 2006; Mori et al., 2003; Szatwczky et al., 2006). In addition, previous studies have demonstrated a dual role of ERK in the regulation of survival and death via AIF (Ostrakhovitch and Cherian, 2005). The previous studies suggest a cascade of ERK1/2 activation and downstream anti- or pro-apoptotic pathways.
In order to better understand exercise-based neuroprotection, the present study first elucidates the beneficial effect of exercise preconditioning in reducing apoptotic cell death. Secondly, it describes the contributions of HSP-70 and ERK1/2 to neuronal survival by their regulation of apoptotic proteins in I/R injury.

EXPERIMENTAL PROCEDURES

Subjects
Sprague-Dawley rats (260–300 g, 3 month old, Charles River) were housed in the same animal care facility during a 12-h light/dark cycle throughout the study. Animal care and surgical procedures were carried out in accordance with guidelines approved by the NIH Guide for the Care and Use of Laboratory Animals revised in 1996 and the University Animal Investigation Committee. Efforts were made to minimize the number of rats used, as well as the suffering they experienced.

Motor exercise
Rats were randomly assigned either to treadmill exercise or non-exercise control groups. All rats (in the treadmill exercise group) ran on a four-lane treadmill (AccuPacer, AccuScan Instruments, Inc., Columbus, OH, USA) at a speed of 30 m/min for 30 min each day, 5 days/week. Rats that were not willing to run were excluded from further study (n=4).

Inhibition of HSP-70 and ERK1/2 activity
The neuroprotective role of HSP-70 in the rat brain was previously studied using anti-HSP-70 antibody or anti-HSP-70 oligonucleotide (Chang et al., 2004). A polycystic rabbit anti-mouse HSP-70 neutralizing antibody (SPA-812E, Assay Designs) dissolved in non-pyrogenic sterile saline was injected i.v. in a group of exercised rats at 48 and 24 h prior to ischemia. A rabbit anti-IgG antibody (20304E, Imgenex) was injected in another group of non-pyrogenic sterile saline was injected i.v. in a group of exercised rats, and non-exercised rats (n=5×4). The infarct region was defined as the area with reduced staining or containing dark pyknotic necrotic cell bodies. In order to minimize the error introduced by edema, an indirect method for calculating infarct volume was used (Ding et al., 2002a; Swanson et al., 1990; Ding et al., 2002b).

TUNEL staining for apoptosis
The distribution of neurons that underwent apoptotic transformation was assessed in the cortex and striatum 48 h after reperfusion in each group using TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining. TUNEL staining was performed on 10 μm cryostat sections using FD NeuroApop™ Kit, according to the manufacturer’s protocol. The sections were counterstained with Methyl Green. TUNEL-positive cells were morphologically recognizable as apoptotic neurons. The apoptotic neurons were counted within randomly selected areas using stereological procedures with a computer-assisted microscope and an image analysis system (AxioVision 4.5TM, Zeiss, Germany) (Swain et al., 2003). Briefly, an unbiased counting frame (0.18×0.1 mm²) digitally superimposed 20 non-overlapping areas (at 400×) from three different levels along the frontoparietal cortex and dorsolateral striatum. The numbers of labeled cells crossing points on the grid, as well as the total number of points (up to 96) falling within a sample, were counted. The number of TUNEL stained neurons per unit area was estimated by the ratio of the number of points falling upon vessels to the total number of points in the sample area.

Western blot for determination of protein expression
Western blot analysis was used to quantitatively detect protein expression of HSP-70, total ERK, and pERK1/2 in control rats for 1, 2, and 3 weeks of exercise (n=5×4) in MCA supplied regions, cortex, and striatum. Intravenous administration of neutralizing antibodies across the BBB was determined by Western blot in brain tissue. After 24-h reperfusion, protein expression of HSP-70, total ERK1/2, and pERK1/2, as well as Bax, AIF, and Bcl-xL, in ischemic territory was examined. The ischemic regions included the cortex and striatum, which were examined among ischemic exercised rats with or without treatment of HSP-70 antibody or ERK inhibitor, as well as in non-exercised ischemic rats (n=5×5). Equal amounts of protein (30 μg/well) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% skim milk in TBST and then incubated with the primary antibody (rabbit polyclonal anti-HSP-70 antibody, 1:40,000, SPA-812E, Assay Designs). The antibody is specific for the inducible HSP-70, namely HSP-72. Membranes were also incubated with other primary antibodies (rabbit polyclonal anti-ERK1/2 antibody, #9102, 1:1500; Cell Signaling; rabbit polyclonal anti-phosphorylated ERK1/2 antibody, 9101s, 1:2000, Cell Signaling; rabbit polyclonal anti-Bax, Sc-6236, 1:500; Santa Cruz; rabbit polyclonal anti-AIF, A-7549, 1:1000; Sigma; as well as a mouse monoclonal anti-Bcl-xL, SC-8392, 1:1000, Santa Cruz) overnight at 4 °C. After incubation with the secondary antibodies for 1 h at room temperature, detection of immunoreactive bands was performed with the ECL-system (GE healthcare, UK). Supernatants were used as whole-tissue lysates and protein concentration was determined using the Bradford assay (Bio-Rad). Protein equal loading was confirmed by intracellular protein β-actin (goat polyclonal anti-β-actin antibody, Sc-1616, dilution 1:1000, Santa Cruz). The intensity of protein expression was quantified using the ChemiGeniusQ Imaging Analysis System.
Immunocytochemical labeling for cellular expression of target proteins

Cryostat coronal brain sections at a thickness of 10 μm were processed for double immunocytochemistry to detect co-localization of HSP-70 and pERK1/2 in neurons in the frontoparietal cortex and dorsolateral striatum of rats exercised for 3 weeks. The cryosections were incubated with a polyclonal rabbit anti-HSP-70 antibody (1:250 SPA-812E, Assay Designs) at room temperature overnight. The first primary antibody was visualized by using Alexa Flour 555 conjugated anti-rabbit antibody (1:200 Invitrogen, Eugene, OR, USA). Sections were then incubated with a monoclonal anti-pERK1/2 (9101s, 1:2000, New England Biolabs, Beverly, MA, USA) at room temperature overnight, followed by FITC conjugated anti-rabbit antibody (1:200, Sigma-Aldrich, St. Louis, MO, USA) to visualize pERK1 labeling. Cellular correlation of the target proteins was observed and photographed using a confocal/multiphoton imaging system at 600x (Zeiss 510 NLO microscope equipped with three visible light lasers: 457, 488, 514, 543 and 633 nm and a Coherent Titanium-Sapphire femtosecond-pulsed laser). Immunopositive neurons were confirmed by their morphology, as well as double-staining by anti-NeuN, Neuronal Nuclei (mouse monoclonal antibody; CHEMICON International, Inc., Temecula, CA, USA). In exercised animals with (n=3) or without (n=3) administration of the rabbit anti-mouse HSP-70 neutralizing antibody (SPA-812E, Assay Designs), brain sections were introduced with a secondary antibody, the Alexa Flour 555 conjugated anti-rabbit antibody (1:200 Invitrogen, Eugene, OR, USA), to visualize anti-HSP-70 neutralizing antibody and determine whether the antibody penetrates the CNS.

Statistical analysis

All the data were described as mean± SE. Statistical analysis was performed with SPSS for Windows, version 15.0 (SPSS, Inc.). The differences among multiple groups were assessed using one-way analysis of variance (ANOVA) with a significance level at P<0.05. Post-hoc comparison between groups was further detected using the least significant difference (LSD) method.

RESULTS

Arterial blood pressure, blood pH, hematocrit, and blood gases of the animals remained comparable amongst the groups (data not shown). Approximately 5% of the animals died during this experiment, and consequently were not used in the results.

Protein expressions of HSP-70 and phosphorylated ERK1/2 after exercise

Western blotting analysis showed a significant (F_{3,12}=10.4, P<0.01) increase in protein expression of HSP-70 by 70% after 3 weeks of exercise, as compared to animals in the control group (Fig. 1A). There were no changes after 1 or 2 weeks of exercise. Data in exercised animals showed a significant (F_{3,12}=69.6, P<0.01) increase in phosphorylated ERK1/2 levels by 81.5% (Fig. 1B), but not total ERK1/2 levels (Fig. 1C). Furthermore, up-regulation of HSP-70 by pre-ischemic exercise was not altered by stroke or U0126 after stroke (Fig. 2A). Inhibition of ERK did not affect the alteration of HSP-70. This suggested that ERK1/2 activation was downstream to HSP-70 expression during I/R. Western blot of β-actin showed equal loading of protein in each lane of the gel.

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prior to I/R injury completely reversed ERK1/2 activation in exercised ischemic rats, in association with reduced infarct volume by exercise (see below).

Infarct volume

Brain infarction was defined as the area with reduced Nissl staining or containing dark, pyknotic-necrotic cell bodies. Infarct volume was determined by subtracting the non-infarcted region in the ipsilateral hemisphere from that in the contralateral hemisphere. Thus, it was presented as a percentage of the volume of the contralateral hemisphere. Thus, it was presented as a percentage of the volume of the contralateral hemisphere (Fig. 3). After 1 or 2 weeks of exercise, infarct volume resulting from ischemia/reperfusion injury was comparable to that in non-exercised ischemic animals (49.8 ± 1.9%) after 3 weeks of exercise (Fig. 3A). Post-hoc comparison between groups indicated that when either the HSP-70 or ERK1/2 activity was blocked, reduction in infarct volume by exercise was completely reversed (Fig. 3B). Since exercise partially reduced ERK1/2 expression in response to I/R (Fig. 2B) and complete inhibition of ERK1/2 with U0126 blocked exercise-induced neuroprotection, certain levels of pERK1/2 might play a pivotal role in exercise-associated neuroprotection. Furthermore, in comparison to HSP-70 antibody, rabbit anti-IgG antibody served as the isotype control and did not reverse exercise-reduced infarct volume (Fig. 3C).

Western blots demonstrated that exercise-increased HSP-70 in brain tissue was significantly (P<0.01) reduced to control levels by intravenous administration of neutralizing antibodies (Fig. 3D). Representative photomicrographs of immuno-labeling also indicated the anti-HSP-70 antibodies binding to the neurons in exercised brain tissue (Fig. 3E), as compared to the image from a negative control (the rats that did not receive administration of the antibody) (Fig. 3F), in which there are no immune reactions to the secondary antibody. This suggested a penetration of this agent across the BBB. This also suggested that the reduced HSP-70 by neutralizing antibodies could be the case to block neuroprotection in the present study, although blocking could occur without reducing levels of the target protein.

Cerebral apoptosis

Quantitative analysis demonstrated that the number of TUNEL positive cells undergoing apoptosis in rat cortex (Fig. 4A) (F(3,16) = 4.3, P<0.05) and striatum (Fig. 4B) (F(3,16) = 4.3, P<0.05) was significantly reduced by pre-ischemic exercise. Post-hoc analysis further indicated that inhibition of either HSP-70 or pERK 1/2 resulted in a reversal of exercise-induced neuroprotection, evidenced by loss of the previously observed reduction in apoptotic cells from ischemic rats without exercise in the cortex and striatum.

Cellular labeling for HSP-70 and pERK1/2

By using immunocytochemistry, the vast majority of cells were labeled for HSP-70, with a few neurons being double-labeled with pERK in rat cortex and striatum after exercise for 3 weeks (Fig. 5). Under a confocal microscope, HSP-70 was observed on the cell surface and cytoplasm, while pERK1/2 was mainly expressed intracellularly. All pERK1/2 positive cells also expressed HSP-70. However, many cells labeled HSP-70 were not pERK positive. These findings suggest that HSP-70 and pERK1/2 could exist in the same cell, and that early exercise-induced ERK1/2 phosphorylation determined by Western blot does not necessarily stimulate HSP-70 expression.

Exercise-altered apoptotic proteins and its regulation

In addition to TUNEL staining, we also measured expression of several apoptotic proteins, such as Bax and AIF in ischemic rats with or without physical exercise. At 24 h after reperfusion, Bax (F(3,16) = 15.1) and AIF (F(3,16) = 14.6) were significantly (P<0.01) increased in response to I/R compared to the non-infarcted region in the ipsilateral hemisphere from that in the contralateral hemisphere. Thus, it was presented as a percentage of the volume of the contralateral hemisphere.
Levels of brain infarct volume were significantly reduced (*\(P<0.01\)) by 3 wk of exercise as compared to stroke with no exercise, as well as 1 and 2 wk of exercise (A). When either ERK 1/2 or HSP-70 was inhibited, these benefits were completely reversed (*\(P<0.01\)), and there was no statistical difference between exercised and non-exercised rats (B). In comparison to HSP-70 antibody, rabbit anti-Ig G antibody served as the isotype control and did not reverse exercise-reduced infarct volume (C). Western blots demonstrated that exercise-increased HSP-70 (*\(P<0.01\)) in brain tissue was significantly (\#\(P<0.01\)) reduced to the control level by i.v. administration of neutralizing antibodies (D). Representative photomicrographs of immuno-labeling further indicate the anti-HSP-70 antibodies binding to the neurons (mainly on cell surface) in exercised brain tissue (E), as compared to the image from a negative control (the rats that did not receive administration of the antibody), in which there are no immune reactions to the secondary antibody (F). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
significantly \( (P < 0.01) \) increased in ischemic rats without exercise (Fig. 6A, B). ANOVA and post-hoc analysis indicated that this increase was significantly \( (P < 0.01) \) reduced by pre-ischemic exercise. In order to elucidate the effects of the HSP-ERK cascade in regulating Bax and AIF related apoptotic pathways, we blocked HSP-70 or pERK1/2, independently, in exercised rats prior to I/R or during ischemia and measured the expression of pro-apoptotic proteins. Post-hoc analysis showed that inhibition of HSP-70 reverses the exercise-reduced induction of Bax (Fig. 6A), and that inhibition of ERK1/2 or HSP-70 reverses the exercise-induced induction in AIF (Fig. 6B). These results suggest a beneficial effect of HSP-ERK signals in exercise-reduced pro-apoptotic protein after stroke. In rats subjected to a stroke, the Bcl-xL expression was significantly \( (F_{(3,16)} = 35.5, P < 0.01) \) enhanced by pre-ischemic exercise as compared to that in non-exercise ischemic rats (Fig. 6C). Post-hoc analysis indicated that this increase in anti-apoptotic protein expression was significantly \( (P < 0.01) \) reversed by inhibiting either HSP-70 or ERK1/2 activity. This result suggests that the exercise preconditioned brain resists I/R insults by up-regulating Bcl-xL.

**DISCUSSION**

HSP-70 and ERK 1/2 levels are increased with exercise. A functional association between these molecules is evidenced when one of the two is blocked and neuroprotection is lost (as seen by increasing levels of infarct volume and apoptosis). In addition to a reduction in infarct volume and apoptotic cell death, we showed, for the first time to our knowledge, that exercise substantially reduces pro-apoptotic proteins, such as Bax and AIF, as well as promotes anti-apoptotic proteins, such as Bcl-xL. The data suggest that exercise preconditioning simultaneously interrupts cell death signals and promotes survival signals in I/R injury. The apoptosis related events, which are altered by exercise, were regulated by the HSP-ERK cascade.

**Neuroprotection by heat shock protein in cerebral ischemia**

Heat shock protein of 70 kDA (HSP-70) is characterized by its highly inducible expression in response to various types of stress. Post-hoc analysis showed that inhibition of HSP-70 reverses the exercise-reduced induction of Bax (Fig. 6A), and that inhibition of ERK1/2 or HSP-70 reverses the exercise-induced induction in AIF (Fig. 6B). These results suggest a beneficial effect of HSP-ERK signals in exercise-reduced pro-apoptotic protein after stroke. In rats subjected to a stroke, the Bcl-xL expression was significantly \( (F_{(3,16)} = 35.5, P < 0.01) \) enhanced by pre-ischemic exercise as compared to that in non-exercise ischemic rats (Fig. 6C). Post-hoc analysis indicated that this increase in anti-apoptotic protein expression was significantly \( (P < 0.01) \) reversed by inhibiting either HSP-70 or ERK1/2 activity. This result suggests that the exercise preconditioned brain resists I/R insults by up-regulating Bcl-xL.
of stress, including heat shock, ischemia, oxidative stress, glucose deprivation, and exposure to toxins and heavy metals (Kiang and Tsokos, 1998). As a molecular chaperone expressed constitutively, induced HSP-70 facilitates optimal folding of nascent and denatured proteins during normal as well as stressful circumstances (Schlesinger, 1990). Over-expression of inducible HSP-70 has been shown to provide protection from cerebral ischemia both in animal stroke models and in cell culture hypoxia models (Giffard and Yenari, 2004). HSP-70 regulates apoptotic cell death by interfering with AIF, as well as increasing levels of anti-apoptotic proteins, such as the Bcl-2 family. HSP-70 induction has been identified in cells that survive cerebral ischemia, as well as in cells that survive after preconditioning with heat (Ohtsuka and Suzuki, 2000). A number of studies have shown that ischemic preconditioning induces HSP-70 expression in neurons (Chen et al., 1996; Chen and Simon, 1997; Masada et al., 2001; Lee et al., 2001) and in vasculature (Masada et al., 2001), leading to neuroprotection (Masada et al., 2001; Kirino et al., 1991; Chen and Simon, 1997). Furthermore, physical exercise was reported to induce HSP-70 expression (Hamilton et al., 2003; Lennon et al., 2004; Starnes et al., 2005), in association with cardioprotection against I/R injury (Hamilton et al., 2003; Lennon et al., 2004). The present study demonstrated that cerebral HSP-70 was induced by physical exercise. This protein over-expressed in neurons pertains to reducing neuronal apoptosis by up-regulating the levels of several anti-apoptotic proteins and down-regulating pro-apoptotic proteins. Although this study did not intend to investigate expression of HSP-70 in astrocytes or microglia, we cannot rule out the role of glial cell activation in HSP-induced neuronal survival. In fact, we previously reported an increased astrocyte proliferation after exercise (Li et al., 2005). The neuroprotective effect of inducible HSP-70 expressed in astrocytes has been reported from ischemic injury in vivo and in vitro (Sun et al., 2006). In our future study, we will particularly investigate whether and how glial cells mediate HSP-70 expression and induce neuroprotection.

Previous studies demonstrated varying degrees of protection by HSP-70, which reflect the limitation of HSP-70 against ischemic insult. Protection may not be observed if HSP-70 expression is at reduced levels or if the insult is severe (Giffard and Yenari, 2004). A recent study has further demonstrated less protection in wild type mice due to an insufficient quantity of HSP-70 induction (Matsumori et al., 2005). A 100% higher level of HSP-70 in wild type mice was not neuroprotective, as compared to 10-fold higher constitutive HSP-70 expression from transgenic mice, which induces neuroprotection. An earlier study also suggests that high levels of HSP-70 are necessary to protect the brain from denaturing stresses (Lee et al., 2001; Tsuchiya et al., 2003), and that HSP-70 is probably less effective in protecting against the most severe insults (Lee et al., 2001). In addition, the finding that HSP-70 transgenic mice show better neuroprotection than those in the wild type response to I/R injury may suggest that pre-ischemic induction of the protein is another key factor (Matsumori et al., 2005). In the present study, exercise induced a 70% higher expression of HSP-70. Inhibition of this rather mild HSP-70 expression with antibody, which may affect the intracellular pool of HSP-70 protein, completely blocked exercise-induced neuroprotection. This result strongly suggests that the increased HSP-70 levels

![Fig. 6.](image-url) Levels of various pro-apoptotic proteins detected using Western Blot analysis. Bax (A) and AIF (B) were significantly down-regulated with 3 wk of exercise. These levels were significantly reversed with inhibition of HSP-70 or ERK1/2. (C) In rats subjected to a stroke, the Bcl-xL expression was significantly enhanced by pre-ischemic exercise as compared to expression in non-exercise ischemic rats. This increase was completely reversed (*) with inhibition of ERK 1/2 or HSP-70. Representative immunoblots are presented. Western blot of β-actin showed equal loading of protein in each lane of the gel.
prior to I/R injury may be one of the key issues. Although the mechanisms underlying HSP-70 anti-apoptotic function in neuroprotection is unclear, our study demonstrated prior to the onset of I/R injury, intravenously administered HSP-70 neutralizing antibodies bind to HSP-70 antigen in the neurons and reduce the expression of antigen in brain tissue. The data suggest a penetration of the agent across the BBB. Areas of BBB disruption are the least likely to be rescued by treatment. Thus, the distribution via damaged BBB could be an important route for antibody entry. It was suggested that HSP-70 antibody had an effect by neutralizing HSP-70 produced in the brain, leading to an interference of anti-apoptosis.

The minimal effectiveness of HSP-70 in protecting against the most severe insults suggests that for optimal protection, other HSP-70 interacting proteins are required (Lee et al., 2001). Our previous studies in rats demonstrate that tumor necrosis factor, TNF-\(\alpha\), was up-regulated during 2 to 3 weeks of physical exercise, in association with reduced brain infarction and inflammatory injury in ischemic rats during reperfusion (Ding et al., 2005). We have further demonstrated that both anti-TNF antibody and inhibition of ERK1/2 reduce the exercise-induced neuroprotection (Guo et al., 2008). These results, together with the HSP-70 data in this study, suggest that increased HSP-70 expression and the co-appearance of TNF-\(\alpha\) and HSP-70 prior to I/R injury play a pivotal role in the exercise-based neuroprotection.

**Beneficial role of extracellular signal-regulated kinases (ERK)1/2 in ischemic injury**

Previous studies have shown that MEK/ERK-mediated signals play a major role in regulation of activity of Bax/Bcl-2-Bcl-x\(_L\) expression in ischemia-induced apoptosis (Ostrakhovitch and Cherian, 2005; Li et al., 2006; Mori et al., 2003; Sawatzky et al., 2006). Furthermore, these studies suggest that the dual role of ERK in the regulation of cell survival and death can be dependent on regulation of the Bax/Bcl-2-Bcl-x\(_L\) ratio (Zhuang and Schnellmann, 2006). In addition, previous studies have demonstrated a dual role of ERK in the regulation of survival and death via AIF. Activation of ERK1/2 causes release of multiple apoptotic proteins, including AIF in neurons (Stoica et al., 2005), while inhibition of ERK1/2 leads to AIF-mediated apoptosis in cancer cells (Ostrakhovitch and Cherian, 2005). The present study demonstrated ERK1/2 activation and downstream anti- or pro-apoptotic effects. ERK1/2 was activated during pre-ischemic exercise, and also was substantially activated in response to I/R insult. This I/R-induced activation was partially inhibited by pre-ischemic exercise. This limited ERK1/2 activation, in turn, plays a role in downstream anti- or pro-apoptotic pathways by regulating expression of Bcl-x\(_L\), Bax, and AIF in response to I/R insults, leading to a reduction in neuronal apoptosis and infarct volume. A block of ERK1/2 activation would completely overturn exercise-induced changes in anti- or pro-apoptotic proteins.

**HSP-ERK cascade in exercise-induced neuroprotection**

Only a few studies have examined the interaction between phosphorERK1/2 and HSP-70 (Gortz et al., 2005; Lee et al., 2005). HSP-70 exhibits regulatory functions to ERK1/2 phosphorylation in hyperosmolality-induced apoptosis. As mentioned above, both acute pathological (ischemia) and chronic physical (exercise) conditions can induce over-expression of HSP-70. The fairly high level of HSP-70 prior to I/R injury was essential in reducing brain infarction and apoptosis in exercised rats after stroke. This indicates that effects of HSP-70 on neuroprotection occur at pre-ischemic episodes. Although exercise and stroke both induced HSP-70 expression, only pre-ischemic expression seemed to confer neuroprotection. We also found a fairly high but significantly reduced expression of phosphorERK1/2 in exercise-induced neuroprotection. In addition, inhibition of ERK1/2 completely diminished exercise-induced neuroprotection, suggesting that certain levels of phosphorERK1/2 are essential in exercise-associated neuroprotection. Taken together, our studies suggested an HSP-ERK cascade in exercise-induced neuroprotection by demonstrating a complete reversal of neuroprotective effects after inhibition of any of the three molecules. Our studies also suggested that ERK1/2 activation was not the upstream mediator for HSP-70 in exercised rats in response to I/R injury, by demonstrating that blocking phosphorERK1/2 reverses the reduction in brain damage but does not change HSP-70 levels.

Neurologic recovery after stroke depends strongly on the survival of brain cells. Many neuroprotective agents to target cell death pathways have failed, whereas alternative strategies that target neuronal survival pathways have been favorably considered (Chan, 2004). Exercise-induced neuroprotection could provide a remedy in which up-regulated molecules simultaneously interrupt cell death and promote survival signals. Our research on elucidating the mechanistic issue underlying exercise-induced neuroprotection would lead to the development of new strategies to target cell death pathways as well as neuronal survival pathways.

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