Endurance exercise training inhibits activity of plasma GOT and liver caspase-3 of rats exposed to stress by induction of heat shock protein 70

Toshibo Mikami, Satoshi Sumida, Yoshitomo Ishibashi, and Shigeo Ohta. Endurance exercise training inhibits activity of plasma GOT and liver caspase-3 of rats exposed to stress by induction of heat shock protein 70 (HSP70), which protects cells against various stresses. In this study, we investigated whether endurance exercise training enhances liver level of HSP70 and, if so, whether HSP70 contributes to hepatic protection against stress in vivo. Mice of an exercise-training group performed 60 min of treadmill running 5 days/wk for 4 wk. The resting level of liver HSP70 was 4.5 times higher in the trained than in sedentary mice. After 4 wk of exercise training, both groups of mice were exposed to the following stresses: 1) heat stress, 2) cold stress, 3) oxidative stress, 4) ethanol stress, and 5) exercise stress by compelling the mice to run on a treadmill until exhausted. After exposure to the stresses, the liver was immediately isolated. Elevation of liver HSP70 in the trained mice was evident, whereas no elevation was found in the sedentary mice. On exposure to heat, diethyldithiocarbamate and ethanol, activities of glutamic oxalacetic transaminase in plasma, and liver caspase-3, a key enzyme of apoptotic processing, were elevated in the sedentary mice but not in the trained mice. These results suggest that exercise training enhanced the resting level of liver HSP70 and hepatic protection against various stresses, at least partly attributing to the suppression of caspase-3 activity by the increase in HSP70.

Skeletal muscle and heart are directly affected by exercise. During exercise, the liver may also be exposed to stressors such as elevations of body temperature, the formation of reactive oxygen species (ROS), the suppression of blood circulation, and a decrease in glycogen. These stressors are known to induce the expression of HSP70. Thus it is interesting to investigate how chronic exercise affects liver levels of HSP70. Although Salo et al. (43) and Kregel and Moseley (23) reported a transient increase in liver HSP70 after an acute bout of exercise, no data are available concerning the effect of chronic exercise training on liver HSP70 expression as reported in skeletal and myocardial muscle.

Stresses such as heat, cold, and oxidants are known to induce apoptosis. Apoptosis is a process that mediates the elimination of damaged or unwanted cells during development and tissue homeostasis in eukaryotic organisms. The molecular machinery that drives the apoptotic programs consists of activating a family of cysteine proteases, caspases, which cleave specific intracellular substrates to produce the characteristic features of this form of cell death (50). Several recent studies have shown that HSP70 suppresses caspase-3 activity after apoptosis induction by a wide variety of stimuli (3, 25, 42) and that the survival-promoting effects of HSP70 can be partly attributed to suppression of apoptosis (51). Therefore, it is expected that exercise training suppress stress-induced apoptosis by increase of HSP70. In fact, Avula and Fernandes (2) suggested that voluntary wheel exercise decreased H2O2-induced apoptosis in immune cells. However, it is unknown whether chronic exercise training contributes to suppression of stress-induced apoptosis in liver.

The purpose of this study was to determine whether endurance exercise training could elevate the level of liver HSP70 compared with the resting level and provide protection against subsequent stresses. In addition, we determined whether exercise training could inhibit apoptosis induced by subsequent stresses.

MATERIALS AND METHODS

Experiment 1. Male ICR mice (Nippon Curea Industry, Tokyo, Japan), aged 10 wk, were used through this experiment. All experimentation and animal care procedures were approved by the Animal Care and Use Committee of Nippon Medical School. Mice were maintained in a controlled environment at 22°C and 50% humidity and with a day-night cycle of 12 h (e.g., lights on 0800 to 2000). Mice were randomly divided into two groups, one for sedentary and the other for training. The trained mice were familiarized with voluntary wheel running.
exercise on a motorized treadmill by running for 15 min/day, 3 days/wk at a treadmill speed of 20 m/min with 0% grade for 1 wk. They then performed 60 min of running, 5 days/wk for 4 wk, at a speed of 25 m/min with 0% grade. Exercise training was carried out at room temperature (20°C). Rectal temperature was measured by inserting a probe (Yellow Springs Instruments, Yellow Springs, OH) 1 cm into the rectum immediately after daily exercise training. Sedentary mice were fed without exercise training for 5 wk. During the period of the experiment, both groups of mice were fed a normal diet (Oriental Yeast, Tokyo, Japan) and water ad libitum. At 48 h after the last exercise bout, both sedentary and trained mice were killed by removal of blood via the abdominal aorta under ether anesthesia. The liver and soleus were immediately excised, rinsed with saline, frozen in liquid nitrogen, and stored at −80°C until analysis. Blood was centrifuged at 1,000 g for 30 min, and the resultant plasma was stored at −80°C. These tissue and plasma samples were used as a resting control for sedentary and trained mice.

**Experiment 2.** The same mice that were used in experiment 1 were divided into sedentary and trained groups. Trained mice performed the same exercise training as in experiment 1. Forty-eight hours after the last exercise bouts, sedentary and trained mice were further divided into five groups (7–8 mice/group) and exposed to five kinds of stresses as follows. 1) In the heat stress group, mice were anesthetized with pentobarbital sodium (35 mg/kg) by an intraperitoneal injection and placed in a environmental chamber at an ambient temperature of 42°C for 1 h. Before and after the heat stress, rectal temperature was measured. 2) In the cold stress group, mice were placed in an environmental chamber at an ambient temperature of 4°C for 3 h according to Matz et al. (30). Before and after the cold stress, rectal temperature was measured. 3) In the oxidative stress group, diethyl-dithiocarbamate (DDC) was intraperitoneally injected at a dose of 1.0 g/kg body wt into sedentary and trained mice. DDC is a cheating reagent for Cu²⁺ and leads to an inhibition of superoxide dismutase (SOD) activity, especially cytosolic SOD activity (10). Heiklila et al. (16) reported that intraperitoneal administration of DDC at a dose of 1.5 g/kg body wt decreased liver SOD activity, which was 71% lower compared with the initial level 3 h after administration. Abe et al. (1) indicated that the administration of DDC to human WISH cells increased HSP70 content twofold. 4) In the ethanol stress group, sedentary and trained mice were orally administered 40% ethanol at a dose of 15 g/kg body wt by using a flexible catheter, according to Tsuchimoto et al. (49). 5) In the exercise stress group, sedentary and trained mice were run on the treadmill (0% grade) according to the following protocol. After 3 min of running at a speed of 15 min/m, the mice ran to exhaustion with an increase in speed of 3 min/m.

Immediately after 1 h of heat stress, 3 h of cold stress, or 20–30 min of exhaustive exercise, or 3 h after DDC and ethanol administration, liver and blood were isolated and stored by the same method as in experiment 1.

**Polyacrylamide gel electrophoresis and immunobloting.** A portion of liver was homogenized in 10 vol of 15 mM Tris-HCl (pH 7.5) and 600 mM NaCl and centrifuged at 15,000 rpm for 30 min. The protein concentration of the supernatant was measured by using Coomassie Plus protein assay reagent kit (Pierce). The protein fraction from each supernatant (100 μg) was applied to a 1-mm-thick 10% SDS-polyacrylamide gel according to Laemmli (24). After electrophoresis, the proteins were blotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 60 V for 12 h in blotting buffer (25 mM Tris-HCl, 192 mM glycine, and 5% methanol) by using a Bio-Rad miniprotein gel system. After the membrane was blotted, it was washed twice in TBS containing Tween 20 [TBS-T; 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.9% Tween 20] and blocked in 10% fetal bovine serum in TBS-T at 37°C for 2 h. After two washes with TBS-T, the membrane was incubated with a monoclonal antibody specific for 12C or membrane was probed with a monoclonal antibody specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Biogenesis 4699-9556, 1:1,000) in TBS-T sup-
Resting level of liver HSP70. To investigate whether endurance exercise training elevates the resting level of liver HSP70 as shown in skeletal (44) and cardiac muscle (38), liver HSP70 contents were compared by Western blotting between sedentary and trained mice. A representative immunoblot for sedentary and trained mice. Heat stress led to an increase in the rectal temperature in sedentary and trained mice; however, no significant difference was observed between the groups. On the other hand, cold stress lowered the rectal temperature without any significant difference between sedentary and trained mice. DDC administration significantly suppressed liver SOD activity in both groups. The resting level of liver TBARS content did not differ between sedentary and trained mice. Administration of DDC or ethanol increased liver TBARS content in sedentary and trained mice with no significant difference between the groups. Because body temperature did not differ between the sedentary and trained mice on exposure to heat and cold stress, it should not contribute to the difference in the increase of HPS70 between the sedentary and trained mice. In addition, DDC and ethanol also led to a similar degree of oxidative stress in both groups, as judged from the liver TBARS content.

Liver HSP70 content of mice exposed to stress. It is known that a change of body temperature or the administration of oxidative reagents results in the induction of HSP70 expression. Therefore, we determined the liver HSP70 levels of the mice exposed to stress. When the trained mice were exposed, the levels of liver HSP70 increased compared with the resting control; however, a significant difference to the resting control of trained mice was found only with exposure to exercise stress (P < 0.05). On the other hand, when sedentary mice were exposed to the stresses, no increase in liver HSP70 was found compared with the resting control. However, when the liver HSP70 levels of mice exposed to the same stresses were compared, the levels in the trained mice were found to be increased 3.7-fold by heat, 12.4-fold by cold, 13.8-fold by DDC, 5.4-fold by ethanol, and 10.0-fold by exercise compared with the levels in sedentary mice. Differences between HSP70 amounts of the sedentary and trained mice were significant with exposure by various stresses except the heat stress.

Plasma GOT activity of the mice exposed to stress. To investigate whether the elevation of liver HSP70 induced by exercise training contributes to protecting liver tissue from stress-induced damage, we exposed sedentary and trained mice to several forms of stress and compared plasma GOT activity between sedentary and trained mice. The level of plasma GOT activity, which is used clinically as an indicator for liver disorders, in sedentary mice exposed to stress (heat, DDC, and ethanol) was significantly increased compared with the sedentary control (P < 0.05), whereas no significant increase was observed in trained mice exposed to any of the stresses (Fig. 3). Thus stress-induced liver damage was less severe in trained mice than in sedentary mice.

Liver caspase-3 activity. Heat or oxidative stresses are known to induce apoptosis. The molecular machinery that drives the apoptotic programs consists of caspases. Caspase-3

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Table 2. Rectal temperature, liver SOD activity, and liver TBARS of mice exposed to stresses

Values are means ± SE; n = 7–8 mice per each group. *Significantly different vs. sedentary rest (P < 0.05). †Significantly different vs. trained rest (P < 0.05). TBARS, thiobarbituric acid reactive substance.

![Fig. 1. Liver heat shock protein 70 (HSP70) content of resting animals and those killed immediately after exposure to stress. HSP70 content was assessed by Western blotting. Gels were loaded with 100 μg of liver homogenate. Data are means ± SE; n = 7–8 mice per group. A representative Western blot for each group is shown immediately below the histogram. DDC, diethylthiocarbamate. †Significant difference between sedentary and trained mice exposed to the same experimental condition (P < 0.05). *Significant difference vs. resting level of trained mice (P < 0.05).]
is a key enzyme downstream in the apoptotic processing, and activation of procaspase-3 leads to apoptosis. Therefore, to examine whether the stresses induced apoptosis in liver, liver caspase-3 activity was determined. The levels of liver caspase-3 activity in sedentary mice exposed to heat, DDC, and ethanol were significantly increased compared with the resting control \((P < 0.05)\), whereas no significant increase was observed in trained mice exposed to stress (Fig. 4). Stress-induced apoptosis seems more suppressed in trained than in sedentary mice.

**DISCUSSION**

**Overview of principal findings.** The principal finding of the present study was that endurance exercise training led to an elevation in the resting level of liver HSP70 content, as was previously reported in skeletal (12) and myocardial (38) muscle, and inhibited the increase in plasma GOT activity of the rats exposed to some stressors. This inhibition might be partly attributed to an inhibition of liver caspase-3 activity due to the increase in liver HSP70 caused by endurance exercise training.

**Factors that induce liver HSP70 expression on exercise training.** Previous studies indicated that endurance exercise training elevated the resting level of HSP70 in skeletal muscle and heart (20, 26, 44). The elevation of body temperature (5, 15, 45), formation of ROS (21, 46) and depletion of glycogen (8) are all regarded as factors that induce HSP70 expression in skeletal muscle during exercise. Our findings demonstrate that 4 wk of endurance exercise training also elevates the resting level of HSP70 in liver (Fig. 2).

With respect to the effect of an elevation in body temperature on the production of HSP70 in liver, mean rectal temperature was measured when daily exercise training was finished. Because the rectal temperature was not high (~38°C), it seems insufficient to induce production of HSP70. In addition, as reported by Skidmore et al. (45), even when body temperature was maintained during endurance exercise, HSP accumulated in skeletal muscle. Therefore, an elevation in body temperature could be excluded as a contributor to the increase of liver HSP70 in trained mice.

Second, it is considered that blood flow into the viscera is suppressed during exercise and is restored to the resting level after exercise. This situation resembles the case with ischemia-reperfusion and may lead to an increased formation of ROS. In the present study, the resting level of liver SOD activity was elevated in trained mice (Table 1). This finding agrees with a previous report (33) that endurance exercise on a treadmill enhanced liver SOD activity. The elevation of SOD activity is affected by an increase of oxidative stress (21). Therefore, the increased formation of ROS during exercise training may be a factor for the increase of liver SOD activity, which might induce HSP70 expression in trained mice. Further study would be required to elucidate the relationship between ROS formation and HSP70 expression in the liver of trained animals.

On the other hand, there is a possibility that treadmill running itself might be a stressor to induce HSP70 in vivo. Campisi et al. (4) reported that 8 wk of voluntary free wheel running did not increase the resting level of HSP70 in brain, liver, spleen, and heart, which was consistent with the findings of Noble et al. (34). These findings suggest the possibility that the treadmill running, as used in the present study, may stimulate stress response to induce HSP70. However, in the exercise training with free wheel, the rats ran only 3–5 km/day, whereas the mice ran 1.5 km/h in our study. Exercise intensity per unit time was probably higher in our study than in that of Campisi et al., so the threshold to evoke HSP70 induction may exist during exercise. However, this threshold is yet unclear. Further study is required to confirm this problem.

![Fig. 2. Plasma glutamic oxaloacetic transaminase (GOT) activity of resting animals and those killed immediately after exposure to stress. Plasma GOT activity was analyzed by using a commercial kinetic assay kit. Data are means ± SE; \(n = 7–8\) mice per group. ★Significant difference between sedentary and trained mice exposed to the same experimental condition \((P < 0.05)\). ♠ Significant difference vs. resting level of sedentary mice \((P < 0.05)\).](image-url)
Liver HSP70 induction on exposure to stress. The trained mice exposed to stress showed an accumulation of liver HSP70, whereas there was no accumulation in the sedentary mice even after exposure (Fig. 2). This finding corresponded to the observation that exercise-trained rats responded to stress with both greater and faster HSP72 responses compared with sedentary rats (4). It is not unreasonable that the sedentary mice showed no accumulation of liver HSP70 even after exposure to stress. In the present study, liver was isolated immediately after heat, cold, and exercise treatment. In addition, in DDC and ethanol stress, liver was isolated without eliminating the oxidative reagents from the body. Because the main purpose of this study was to investigate liver damage in the mice exposed to stress, we treated liver tissue immediately to detect any damage. On the other hand, previous studies demonstrated that HSP70 expression was delayed several hours after stress: 2 h for heat (13, 31), 1 h for cold (7), 2 h for oxidative reagent or ethanol (18), and 2 h for treadmill running (17). Because the translation of HSP70 mRNA gradually increases after stress (48), we may have isolated the liver before the induction of HSP, resulting in no accumulation of HSP70.

Theodorakis et al. (48) investigated the polysome localization of HSP70 mRNA in both naive and thermotolerant cells, which were pretreated with heat shock, after exposure to heat shock. Their finding suggests that the HSP70 mRNA of thermotolerant cells is always translated with higher efficiency than that of naive cells immediately after heat shock. In addition, the increases in liver and myocardial HSP70 accumulation in response to nonexertional heat stress are attenuated with senescence, which suggests that an aging organism has a reduced ability to properly maintain cellular function and integrity after a thermal challenge (23). Therefore, trained mice preconditioned through 4 wk of exercise training could translate HSP70 mRNA more efficiently than sedentary mice, which might lead to the increase in liver HSP70 even just after the stress. However, we only measured the HSP70 induction of rats exposed to stressors at one time point, so a more careful time course experiment of HSP70 induction in trained and sedentary mice is needed to make conclusions for this hypothesis.

Plasma GOT activity on exposure to stress. HSP70 production enhanced by heat stress leads to an improvement in stress resistance. In cell culture experiments, sublethal heat stress elevates cellular HSP70 and contributes to the inhibition of cell death after lethal heat stress (40). The cells with enhanced HSP70 expression on exposure to heat stress show elevated cellular chaperone activity (35). In animal experiments, production of myocardial HSP70 enhanced by heat stress was responsible for myocardial salvage after ischemia-reperfusion (19, 29). In addition, skeletal muscle HSP70 produced in response to heat stress before hindlimb unweighting can inhibit the protein degradation and reduce the rate of disuse muscle atrophy during hindlimb unweighting (32). With respect to an effect of exercise-induced HSP70 expression on stress resistance, myocardial HSP70 induced by exercise may be related to the enhanced recovery of myocardial function after ischemia (8, 38). In the present study, stress resistance was assessed by comparing the level of plasma GOT between sedentary and trained mice exposed to stress. Plasma GOT activity is generally known as a clinically useful marker for hepatic injury. On exposure to heat, DDC, and ethanol, the plasma GOT of sedentary mice was significantly elevated, whereas there was no elevation in trained mice (Fig. 4). Because the direct marker of liver damage was not measured and judging from results of plasma GOT activity, stress-induced damage of liver might be less severe in trained mice than in sedentary mice, and liver HSP70 enhanced by endurance exercise training may help to suppress liver damage on exposure to stress. These findings were consistent with those of a previous study (13), which showed similar findings between the age-related decrements in the expression of inducible HSP70 and the pathophysiological responses to heat stress.

Liver caspase-3 activity on exposure to stress. HSP70-overexpressed cells show inhibition of procaspase-3 activation and apoptosis (3, 25, 42). We determined liver caspase-3 activity to assess whether the stressors led to apoptotic processing. Liver caspase-3 activity in sedentary mice increased after heat, DDC, and ethanol stress (see Fig. 4), which suggested that apoptosis was induced in the liver of the mice exposed to these forms of stress. On the other hand, no elevation of liver caspase-3 activity was observed in the trained mice. Because the activation of liver caspase-3 was suppressed in the trained mice exposed to stress, the stress-induced apoptosis might be inhibited in the trained mice. This inhibition of caspase-3 activity in trained mice is probably due to liver HSP70 increased by exercise training. Because Xanthoudakis and Nicholson (51) have proposed that the survival-promoting effects of HSP70 can be partly attributed to suppression of apoptosis, the inhibition of caspase-3 activity might be attributed to an increase of plasma GOT activity, which was significantly lower in trained mice (Fig. 3).

This is the first paper to demonstrate that exercise training has a inhibitory effect on the increase of the markers for hepatic damage and apoptosis in the rats exposed to stressors.

In summary, endurance exercise training leads to an elevation in the resting level of liver HSP70. Exposure to heat, oxidative, or ethanol stress increased plasma GOT activity in sedentary mice; however, there was no elevation of the activity in trained mice. In addition, the elevation of liver caspase-3 activity observed in sedentary mice was inhibited compared with that in trained mice. Thus it is concluded that endurance exercise training elevates the resting level of liver HSP70 and that the resultant accumulation of HSP70 helps to protect stress-loaded cells from injury due to the elevation of chaperone activity and the suppression of apoptosis.

GRANTS
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REFERENCES
EXERCISE TRAINING AND LIVER HSP70 INDUCTION


