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Chronic Antioxidant Enzyme Mimetic Treatment Differentially Modulates Hyperthermia-Induced Liver Hsp70 Expression with Aging

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ABSTRACT

One postulated mechanism for the reduction in stress tolerance with aging is a decline in the regulation of stress-responsive genes such as inducible heat shock protein 72 (Hsp70). Increased levels of oxidative stress are also associated with aging, but it is unclear what impact a pro-oxidant environment might have on Hsp70 gene expression. This study utilized a superoxide dismutase (SOD)/catalase mimetic (EUK-189) to evaluate the impact of a change in redox environment on age-related Hsp70 responses to a physiologically relevant heat challenge. Results demonstrate that liver Hsp70 mRNA and protein levels are reduced in old compared to young rats at selected time points over a 48-h recovery period following a heat stress protocol. While chronic systemic administration of EUK-189 suppressed hyperthermia-induced liver Hsp70 mRNA expression in both age groups, Hsp70 protein accumulation was blunted in old rats but not on their young counterparts. These data suggest that a decline in Hsp70 mRNA levels may be responsible for the reduction in Hsp70 protein observed in old animals after heat stress. Further, improvements in redox status were associated with reduced Hsp70 mRNA levels in both young and old rats, but differential effects were manifested on protein expression, suggesting that Hsp70 induction is differentially regulated with aging. These findings highlight the integrated mechanisms of stress protein regulation in eukaryotic organisms responding to environmental stress, which likely involve interactions between a wide range of cellular signals.

Key Words: heat shock protein, EUK-189, oxidative stress, reactive oxygen species, ROS, heat stress

INTRODUCTION

The progression of an organism toward old age is associated with a reduction in physiological function that is manifested at molecular, cellular and organ-system levels. This decline in function is usually accompanied by a diminished ability to cope with environmental stress (1, 5, 6, 10, 20, 24, 25, 32). While the mechanisms underlying these age-related alterations in stress tolerance are complex and not well-defined, it has been postulated that a decreased ability to regulate the expression of acute response genes could contribute to an older organism's diminished capacity to cope with stress.

One primary area of focus has been the family of highly conserved stress proteins known as heat shock proteins (HSPs). Some of the most well-studied HSPs in mammals are those with molecular masses of ~70 kDa, and these ubiquitous proteins include the highly heat-inducible 72 kDa protein (Hsp70). There is substantial evidence showing that Hsp70 plays a critical role in providing cellular protection against the adverse effects of a wide array of stress and toxic conditions (24). Interestingly, investigators have also shown that the ability to induce Hsp70 under a variety of physiologically-relevant conditions, including hyperthermic challenge, is reduced with aging (7, 19, 25, 36). This decrease in Hsp70 induction, observed in a wide range of tissues, is associated with reduced tolerance to heat stress in aged animals, as evidenced by increases in heat-related cellular injury and elevated rates of morbidity and mortality (15, 30).

Hsp70 appears to be regulated at both transcriptional and translational levels (8, 37). One known Hsp70 activator is a family of heat shock factor (HSF) proteins, and these HSFs bind to the promoter region of the Hsp70 gene during heat stress to induce Hsp70 gene transcription. However, it is not clear whether additional factors besides HSF are also involved in the regulation of Hsp70 gene expression. We have previously demonstrated that increases in

reactive oxygen species (ROS) and oxidative stress are associated with substantial cellular damage, decreased organ function and reduced thermotolerance in old animals in response to an environmental challenge (44). In subsequent studies in which animals have received chronic low-dose administration of an antioxidant enzyme mimetic as a means to reduce ROS generation and oxidative injury, we have shown that old rats can be protected from the damaging effects of heat stress by improving *in vivo* redox status and decreasing oxidative damage to macromolecules (42). While it is unclear whether a stress-mediated increase in ROS impacts on *in vivo* Hsp70 regulation, evidence from *in vitro* studies suggests that ROS can induce Hsp70 gene expression (29, 38). Based on these observations, it is reasonable to postulate that a change in redox status in an aged system could modulate Hsp70 responses.

Therefore, one aim of the present study was to determine the temporal pattern of liver Hsp70 transcription and translation in heat-stressed young and old rats. We chose to focus on the liver because it shows age-dependent evidence of increased cellular ROS production (41, 44) and is a prime target of tissue injury in physiological challenges such as heat stress (15, 22, 44) and ischemia-reperfusion (14). Animals were stressed on two consecutive days and then allowed to recover for a period up to 48 h after the second challenge. This design was based on our observations that older rats are markedly less thermotolerant and have a much greater degree of cellular injury than their younger counterparts following repeated heat challenge (15, 16, 26), thereby providing an excellent *in vivo* model for the high morbidity and mortality rates observed in older humans with stress (28, 34). A second aim was to determine whether modulation of *in vivo* redox status would impact on stress-induced Hsp70 expression. Studies were conducted in which low-dose Eukarion-189 (EUK-189), an antioxidant enzyme mimetic that exhibits both superoxide dismutase (SOD) and catalase activities (2, 4, 12), was chronically delivered to young

and old rats prior to hyperthermic challenge. It was postulated that synthetic SOD/catalase mimetic treatment, which we have previously shown to be effective in reducing ROS levels and oxidative damage in the liver of heat stressed old rats (42), would also be effective in diminishing stress-induced Hsp70 expression in these animals.

MATERIALS AND METHODS

Animals

Young (6-mo-old; 300-400 g) and old (24-mo-old; 350-450 g) male Fischer 344 rats (National Institute on Aging) were used in these experiments. Rats were housed in The University of Iowa Animal Care Facility and all experimental procedures conformed to institutional animal care guidelines. Animals were maintained at 22-24°C on a 12:12 h light:dark cycle and provided food (standard rat chow) and water *ad libitum*.

Separate sets of young and old rats were utilized in two different experimental protocols. The first protocol involved a time course study of Hsp70 expression in which rats were placed into one of six experimental conditions (n=3/group at each age): a control (sham-heat) group and heat-stressed groups that were euthanized at 0 (i.e., immediately after heating), 2, 6, 12, 24 and 48 h after a heat stress protocol. In the second protocol, rats were chronically treated with the antioxidant enzyme mimetic EUK-189 to study the effects of *in vivo* redox modulation on Hsp70 expression. Rats were randomly placed into four experimental groups (5-9 rats per group): i) vehicle-treated and sham-heated (control); ii) vehicle-treated and heat-stressed (V/H); iii) EUK-189-treated and heat-stressed (E/H); and iv) EUK-189-treated and sham-heated (E/NH). A small number of animals in the control and V/H groups were not implanted with pumps; however, because responses in these animals were similar to those in animals receiving the vehicle treatment, the data were combined.

Eukarion 189 supplement

As part of the second experimental protocol, young and old rats were implanted with an Alzet mini-osmotic pump (Alza Corporation, Palo Alto, CA; model 2004) for the chronic

delivery of EUK-189 four weeks before testing. Animals were anesthetized with methohexital sodium (50 mg/kg ip) and the skin was shaved at the nape of the neck. After a 2.5-cm incision was made in the neck region, a pump was inserted into the subcutaneous space and the incision was then closed using sutures.

The mini-osmotic pumps contained 240 μl of either EUK-189 (15 mM dissolved in distilled water) or vehicle (distilled water only). EUK-189 was synthesized and assayed for SOD and catalase activity as described by Baker et al. (2). Each pump released a total of ~6 μl of solution (~45 μg EUK-189) per day over the 30-day period. At the end of this period, pumps were retrieved from animals and the volume of solution remaining in each pump was determined. The total volume of solution released (an average of 189 μl per rat) was similar between the two age groups and treatments. In the drug-treated groups, this was equivalent to an average of 3.8 mg EUK-189/kg body mass.

Heat stress protocol

All rats were handled daily and familiarized with a colonic temperature (T_{co}) probe during the week prior to the heat stress protocol. On the day of the first heat exposure, each rat was fitted with a thermistor temperature probe inserted 6-7 cm into the colon and then placed in a plastic cage (45 x 25 x 20 cm), conscious and unrestrained. T_{co} was continuously monitored on a digital display. A baseline T_{co} (37.0-38.0°C for both age groups) was established over a 30-min control period for each rat, followed by a heating protocol that has previously been described (16, 44). An infrared lamp was positioned ~40 cm above each rat and either raised or lowered to obtain an ambient temperature of 38-40°C. Movement of the lamp permitted a constant heating rate (~0.06°C/min) to be attained. Heating was terminated when T_{co} reached

41°C, but was then commenced at an appropriate time to maintain T_{co} at 41°C for 30 min. At the end of this period, the thermistor probe was removed and rats were allowed to passively cool in a cage at room temperature. Animals were subsequently subjected to the same heating protocol 24 h after the first stress. Sham-heated control rats were handled identically to experimental rats, with the exception that ambient temperature was maintained at 22-24°C.

At the designated time points of 0, 2, 6, 12, 24 and 48 h after the second heating (first protocol) or 2 h after the second heating, (second protocol), rats were administered an overdose of sodium pentobarbital (80 mg/kg ip). Livers were collected and rinsed twice in phosphate-buffered saline, then immediately frozen in liquid nitrogen for subsequent assays. Frozen liver samples were homogenized, centrifuged, and sonicated in phosphate buffered saline for antioxidant enzyme activity assays and for western blot and RT-PCR analyses (16, 44).

Antioxidant enzyme activity measurements

SOD activity was measured by the modified nitroblue tetrazolium (NBT) method (35, 43), which is an indirect assay based on a competition reaction between SOD and the superoxide indicator molecule, NBT. The rate of change in the absorption at 560 nm over a 5-min period indicates the reduction of NBT by superoxide. The competitive inhibition of this reaction by SOD is an indicator of total SOD activity. In this assay, the xanthine/xanthine oxidase system was used to generate superoxide. An excess amount of catalase was present in the system to remove hydrogen peroxide (H_2O_2). Varying amounts of total protein from a tissue homogenate were added to the reaction until maximal inhibition was obtained as determined by spectrophotometry. Total SOD activity was determined by the amount of protein necessary for

half-maximal inhibition of the NBT reaction. One unit of activity was defined as the concentration of SOD that reduced the NBT reaction to one-half of the maximum.

Catalase activity was measured by following the disappearance of H_2O_2 at 240 nm as described by Claiborne (9). Tissue homogenates were mixed with 50 mM potassium phosphate buffer (pH7.8) containing 10 mM H_2O_2 immediately before the recording of absorbance. Catalase activity was expressed in k/mg protein.

Glutathione peroxidase (GPx) activity was determined as described by Lawrence and Burk (27). Tissue homogenates were incubated with a phosphate-buffered solution containing glutathione, glutathione reductase, and NaN_3 for 10 min before addition of NADPH. The oxidation of NADPH was measured immediately after addition of t-butyl peroxide at 340 nm. One unit of GPx activity was defined as the amount of protein required to oxidize 1 μmol NADPH per min.

Protein immunoblots

Twenty μg of total protein for each sample was separated on a one-dimensional, 7.5% polyacrylamide gel under standard denaturing conditions and then transferred onto nitrocellulose membranes. The nitrocellulose membranes were then incubated with specific primary antibody to rat Hsp70 (1:1000, Stressgen) and a secondary antibody conjugated to horseradish peroxidase (1:10,000 dilution). Blots were stained by the chemiluminescent ECL method (Amersham Life Sciences, Piscataway, NJ) and visualized by exposure to X-ray films. Immunoblot images were scanned and analyzed by a computerized image software program (Gel-Pro Analyzer).

Total RNA isolation

Total liver RNA was isolated using RNeasy kits from Qiagen. Frozen liver samples were ground in liquid nitrogen. Thirty mg of ground liver tissue was homogenized in a denaturing buffer containing guanidine isothiocyanate. Subsequent total RNA isolation steps were carried out according to manufacturer protocols. RNA concentrations were quantified spectrophotometrically at 260 nm.

Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was performed for Hsp70 mRNA using a Qiagen one-step RT-PCR kit. One μg of total RNA was used to synthesize a 1060-bp DNA fragment corresponding to rat Hsp70 cDNA using specific primers. The primer sequences utilized were: sense, 5'-GCCAAGAAAACAGCGATCGG-3'; anti-sense, 5'-TGAAGAAGTCCTGCAGCAGC-3'. Produced DNA fragments were stored at 4°C until they were separated on an argarose gel. The separated DNA fragments were visualized under an ultraviolet light with ethidium bromide. Images were taken using an AlphaImager (Alpha-Innotech, San Leandro, CA). The density of each band was examined with a computerized digital imaging software program. RT-PCR was repeated 3 to 4 times per sample. Primers for rat GAPDH or β-actin cDNA were used simultaneously with Hsp70 primers. The synthesis of GAPDH or β-actin cDNA fragments was used as a loading control for Hsp70 mRNA.

Data analysis

Data are presented as mean values ± SEM. ANOVA-Tukey's multiple comparison test was used to determine the statistical significance of the data at a level of $P<0.05$.

RESULTS

Heating rates

T_{co} values before heating in both protocol 1 (time course) and protocol 2 (EUK-189 treatment) were similar in the young and old groups on both day 1 and day 2 of testing (37-38°C). These experiments were designed to minimize differences in heating rates between the two age groups and within an age group between heating trials (i.e., day 1 vs. day 2). For both protocols, similar heating rates were obtained on the two testing days for the young and old age groups (~0.06°C/min).

Reduced Hsp70 protein in old animals responding to heat stress

To evaluate the effects of aging on stress-induced Hsp70 expression, liver Hsp70 protein levels in young and old animals were examined by western blot analysis at several time points after heating. A representative western blot of liver samples from young and old rats is presented in Figure 1 along with densitometry values (n=3 rats/time point) at the designated time points after heating. As expected, there was no expression of the inducible Hsp70 in liver samples in the control sham-heated condition.

In young rats that underwent hyperthermic challenge, there was strong and relatively stable Hsp70 protein accumulation immediately after heating (0 h) at 1.5, 6, 12, 24 and 48 h of recovery from heat stress. Liver Hsp70 responses were similar in the old rats during the early time points of recovery. However, there was a progressive decline in Hsp70 levels starting at 12 h post-heating, and by 48 h Hsp70 protein was significantly reduced in old compared to young rats (statistical comparisons were not made for control versus post-heating values within an age group because no Hsp70 protein was present in control conditions).

Reduced Hsp70 mRNA in old rats in response to heat stress

To evaluate whether aging affects stress-induced Hsp70 expression at the transcriptional level, Hsp70 mRNA was assessed by semi-quantitative RT-PCR in young and old liver samples at several time points after the heat stress. Representative RT-PCR results from one young and one old rat at each time point are presented in Figure 2a. Hsp70 mRNA was present at the 0 and 2 h time points after heating in both age groups, but was undetectable at all other time points. Densitometry analysis at these time points indicated that liver Hsp70 mRNA levels were similar in the two age groups immediately after heating (0 h). However, at the 2-h time point, Hsp70 mRNA levels were significantly reduced in old rats compared to young rats, as well as compared to old rats at 0 h (Fig. 2b). The mRNA level of a house-keeping gene, GAPDH, was analyzed simultaneously with Hsp70 mRNA as an assay control.

Effects of EUK-189 treatment on antioxidant enzyme activities

In a second protocol, young and old rats were chronically treated with EUK-189 to assess whether modulation of intracellular redox status impacts on stress-induced Hsp70 accumulation. For these experiments, only the control condition and a 2-h recovery time point (a time point where we have consistently noted high liver Hsp70 protein levels in both young and old rats after heat stress) were assessed.

SOD, catalase and GPx activities were measured in those animals treated with EUK-189 and compared to values obtained in vehicle-treated animals at 2 h. Figure 3a presents the averaged total SOD activities for livers harvested at 2 h post-heating from four different treatment conditions. Total SOD activities were similar between young and old animals under all conditions. Control animals had the lowest SOD activity among the four groups, while rats

supplemented with EUK-189 and sham-heated (E/NH) had significantly higher SOD activity levels than the rats in other groups. Rats that were heat-stressed with EUK-189 (E/H) or without EUK-189 treatment (V/H) had similar SOD activities. However, these rats had higher SOD activities than controls and lower SOD activities than the non-heated EUK-189 treated (E/NH) rats.

Catalase activity was also measured in these liver samples. Overall, young rats had higher catalase activity than old rats in all four groups regardless of EUK-189 treatment or heat stress (Figure 3b). However, no differences were found across any of the treatments within an age group. In addition, GPx activity was not affected by age, heat stress, or EUK-189 treatment (data not shown).

EUK-189 differentially modulates Hsp70 protein levels in young and old rats

Hsp70 protein accumulation was also measured in young and old liver samples from the four groups in protocol 2 to determine if chronic antioxidant enzyme mimetic treatment would modulate the levels of Hsp70. Sham-heated young and old rats had no detectable Hsp70 protein as measured by western blot analysis, regardless of whether animals were treated with vehicle or EUK-189 (data not shown). In Figure 4a, representative immunoblots highlight the effects of EUK-189 on Hsp70 protein accumulation at 2 hours post-heating for heat-stressed old and young rats. Each band represents a different rat in each age and treatment group (n=6 each), and densitometry values for each group are presented in Figure 4b. Hsp70 protein accumulation was not affected by EUK-189 treatment in young heated rats, as its levels were similar in the V/H and E/H groups. However, EUK-189 markedly suppressed heat-induced liver Hsp70 protein levels in old rats when compared to vehicle-treated rats that underwent the same heating protocol (E/H

vs. V/H). The levels of liver Hsp70 protein were also lower in old compared to young E/H rats when samples from both age groups were analyzed on the same western blot (data not shown).

EUK-189 reduced levels of Hsp70 mRNA in both young and old rats

Levels of Hsp70 mRNA were also measured by semi-quantitative RT-PCR in all the liver samples from protocol 2 to determine if the differential modulation of Hsp70 protein accumulation by EUK-189 was present at the transcription level. Similar to the protein levels, Hsp70 mRNA was not detected in sham-heated animals regardless of treatment or ages (data not shown). However, as illustrated in the top panel of Figure 5, in which one band represents each individual animal, levels of Hsp70 mRNA at the 2-h time point after heat stress were significantly reduced in both young and old rats by EUK-189 treatment when compared to the age-matched vehicle-treated groups. Densitometry analysis also indicated that young rats had higher Hsp70 mRNA than their old counterparts in both vehicle-treated (V/H) and EUK-189-supplemented groups.

DISCUSSION

Our previous work has demonstrated that aging is associated with reduced stress tolerance and extensive hepatic injury after hyperthermic challenge. Specifically, biochemical and histological analysis has revealed that heat stress produces an increase in ROS levels and oxidative damage to hepatocellular macromolecules, along with alterations in intracellular redox status, aberrant activation of stress-response transcription factors, and an elevation in serum levels of alanine aminotransferase (ALT), which is a clinical indicator of liver injury. These data, along with reports from other laboratories (14, 18, 40), suggest that the liver is a prime target of tissue injury with environmental challenges such as heat stress.

The purpose of the current study was to investigate the impact of aging on *in vivo* Hsp70 regulation in response to an acute stress. We focused on Hsp70 because it is a critical stress response protein that is induced by hyperthermic challenge. Moreover, an age-related decline in the Hsp70 response to heat stress has been reported by many researchers in different tissues and cell types (7, 19, 25, 36). Consistent with previous findings from our laboratory, the current results demonstrate that both young and old animals are capable of inducing liver Hsp70 protein to similar levels in the early phases of recovery after a heat challenge. However, at later time points of recovery, old animals failed to maintain the high Hsp70 protein levels that were noted in their young counterparts.

More importantly, using semi-quantitative RT-PCR, our results indicate that both young and old animals are capable of up-regulating Hsp70 mRNA transcription to a similar level immediately following heat stress, while levels of Hsp70 mRNA in old rats decreased significantly at 2 hours after heat stress. The decrease in mRNA levels could potentially result from either lowered transcriptional abilities or accelerated mRNA degradation in old rats.

Therefore, further studies aimed at elucidating the pathway for Hsp70 mRNA regulation are certainly warranted, and should provide important insight into key aspects of the effects of aging on stress protein regulation and stress tolerance at the molecular level. Nevertheless, we speculate that the decreased Hsp70 mRNA levels in old rats may be responsible for the reduced Hsp70 protein following heat stress.

The process of aging has been associated with increased oxidative damage to macromolecules such as lipids, proteins and DNA in a wide array of tissue types in many eukaryotic species. Recent experiments from our laboratory have demonstrated that decreased tolerance to heat stress in old rats is associated with increased ROS generation, substantial oxidative-mediated injury, and an imbalance in intracellular redox status (44). In a subsequent study, chronic administration of an antioxidant enzyme mimetic was shown to have protective effects, as both oxidative stress and cellular injury in the liver were dramatically reduced in old rats treated with EUK-189 (42).

In the present set of experiments, an analysis of liver antioxidant enzyme profiles after SOD/catalase mimetic administration (Fig. 4) yielded several interesting observations. First, aging does not appear to have an influence on total SOD activity in the liver, while heat stress may be associated with an increase in total SOD levels. The induction of SOD activity by hyperthermic challenge has also been observed by other investigators (17, 39). Second, chronic EUK-189 supplementation led to a significant increase in total SOD activity compared with vehicle-treated controls in both age groups. Since endogenous CuZnSOD and MnSOD protein levels were not affected by EUK-189 as measured by western blot analysis (data not shown), this increase in total SOD activity was most likely contributed by EUK-189. However, catalase activity was not increased with EUK-189 treatment. Instead, a reduction in catalase in the range

of 20% was detected in old animals in all the treatment groups, which could be one factor contributing to the decrease in stress tolerance that accompanies aging. Third, no change in hepatic GPx activity was observed in any of the groups. It should be noted that similar observations regarding changes in SOD and catalase activities with EUK-189 supplementation have previously been reported (21). A potential explanation for this observation could be that while intact EUK-189 compound has both SOD and catalase activities, its breakdown metabolite contains only SOD activity. This is consistent with the structure-activity relationship analyses showing that catalase activity, but not SOD activity, can be structurally modulated in this class of compounds (13). Overall, combined with a recent study from our laboratory showing increased hepatic glutathione (GSH) to glutathione disulfide (GSSG) ratios (indicative of a more reduced hepatic environment) in old rats following a month of EUK-189 treatment (42), these results strongly support the view that chronic low-dose supplementation with an SOD/catalase mimetic enhances antioxidant capacity and improves redox status in old animals.

Alterations in redox status that lead to increased ROS levels and oxidative stress are also known to induce Hsp70 expression in isolated cell culture preparations (3, 31). Our current results showing that EUK-189 differentially regulates heat-induced Hsp70 mRNA and protein accumulations between young and old animals suggest a complicated, yet direct causal relationship between the regulation of Hsp70 gene expression and intracellular redox status. It has been reported that redox-sensitive signal transduction pathways that involve the activation of two transcription factors, JNK and AP-1, are also involved in the activation of HSF, a key regulatory factor for Hsp70 gene expression (11, 23). We have previously observed that SOD/catalase mimetic treatment abolishes the activation of AP-1 in response to acute heat stress in both young and old animals (42). Therefore, it is tenable to postulate that redox-sensitive

signal transduction pathways, such as those involving AP-1, may participate in the regulation of Hsp70 gene expression in both young and old animals. A more detailed time-course study would be insightful to assist in more fully delineating the effects of redox balance on the kinetics of Hsp70 gene expression at both mRNA and protein levels. Overall, the observations in the present study strongly suggest that a connection exists between redox regulation of signal transduction pathways and oxidant-mediated stress gene modulation.

In summary, the present findings highlight the highly integrated and complex responses of eukaryotic organisms to environmental stress. Understanding these interactions between different cellular signaling pathways, such as those involved in stress protein responses, redox modulation, and transcription factor activation, will be critical in developing a better understanding of the mechanisms contributing to the decline in stress tolerance that accompanies aging. This type of inquiry will also provide a basis for developing effective therapies related to oxidative-stress related diseases and aging-associated pathophysiological conditions.

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FIGURE LEGENDS

Figure 1. Reduced liver Hsp70 protein expression in old rats. Young and old rats were heat-stressed on two consecutive days (see Methods). Liver samples were obtained from sham-heated controls (C) or at 0, 1.5, 6, 12, 24 and 48 h after the second heat stress. Proteins were probed with a monoclonal antibody specific for the inducible form of HSP70 (Hsp70).

A) Representative Hsp70 immunoblots for young and old rats during a time course of recovery from a heat stress protocol. Each lane represents a liver sample from a different rat with 20 μg of protein loaded per lane. GAPDH is shown as a loading control for each sample.

B) Densitometry values for immunoblot results. Five rats were analyzed per age group at each time point, and immunoblotting was performed twice for each sample. The band density of Hsp70 was normalized to the corresponding GAPDH band density. Data are presented as mean ± SEM. *, $P<0.05$ vs. young group for a specific time point.

Figure 2. Decreased liver Hsp70 mRNA levels in old rats. Young and old rats were heat-stressed on two consecutive days. Liver samples were obtained from sham-heated controls (C) or at 0, 2, 6, 12, and 24 h after the second heat stress. Total RNA was isolated from liver samples and then the levels of Hsp70 mRNA were measured by semi-quantitative RT-PCR. For each sample, RT-PCR was repeated at least 3 times. **A)** A representative RT-PCR image for young and old rats during a time course of recovery from a heat stress protocol. Each lane represents a liver sample from a different rat. GAPDH is shown as a loading control for each sample. **B)** Densitometry values for RT-PCR. The band density of Hsp70 mRNA was normalized to its corresponding GAPDH band density. Data are presented as mean ± SEM. *, $P<0.05$ vs. young group at the 2-h time point. †, $P<0.05$ vs. old group at the 0-h time point.

Figure 3. Effects of chronic EUK-189 treatment, heat stress, and aging on liver antioxidant enzyme activities. Young and old rats were treated with EUK-189 or vehicle (control) via osmotic pumps for four weeks prior to a heat stress protocol. Rats were then heat-stressed or sham-heated. Livers were collected 2 h after heating on the second day. Antioxidant enzyme activities of total superoxide dismutase (SOD), catalase and GPx were then measured in the liver samples. (A) NBT activity assay demonstrates a significant increase in total SOD activity after EUK-189 treatment and after heat stress in both young and old rats. E/NH, EUK-189-treated and sham-heated; V/H, vehicle-treated and heat-stressed; E/H, EUK-189-treated and heat-stressed. *, $P<0.01$ vs. control, V/H, and E/H within an age group; † $P<0.005$ vs. control within an age group. (B) Catalase activity in the liver decreases with aging. Catalase activity assays were repeated three times for each rat (n=5-9 per age group at each treatment). *, $P<0.01$ vs young rats within a treatment group. Heat stress and EUK-189 treatment had no effect on catalase activity. GPx activity was not influenced by heat, age, or EUK-189 supplement (data not shown).

Figure 4. Liver Hsp70 expression is suppressed in old but not young rats following EUK-189 treatment. Rats of both age groups were treated with either EUK-189 or vehicle via osmotic pumps for four weeks prior to a heat stress protocol. These animals were then heat-stressed or sham-heated. Livers were collected 2 h after heating on the second day. Liver Hsp70 levels were determined by immunoblot analysis. Proteins were probed with a monoclonal antibody specific for the inducible form of HSP70 (Hsp70). **A)** Representative Hsp70 immunoblots for young and old rats at the 2-h recovery time point after a heat stress protocol. Each lane

represents a liver sample from a different rat at that time point with 20 μg of protein loaded per lane. β-actin is shown as a loading control for young animals. E/H, EUK-189-treated and heat-stressed; V/H, vehicle-treated and heat-stressed. **B)** Densitometry values for immunoblot results. Six rats were analyzed per age group at the 2-h time point, and immunoblotting was performed twice for each sample. The expression of Hsp70 in young rats was not effected by EUK-189 treatment. However, EUK-189 reduced Hsp70 levels in the older animals. Data are presented as mean ± SEM. *, $P<0.01$ vs. young E/H group; †, $P<0.01$ vs. V/H old group.

Figure 5. Liver Hsp70 mRNA levels are lowered in both young and old rats following EUK-189 treatment. Total RNA was isolated from liver samples of young and old rats treated with the same conditions as described in Figure 4. The levels of Hsp70 mRNA were measured by semi-quantitative RT-PCR. **A)** A representative RT-PCR image for young and old rats. Each lane represents a liver sample from a different rat. β-actin is shown as a loading control for each sample. E/H, EUK-189-treated and heat-stressed; V/H, vehicle-treated and heat-stressed. **B)** Densitometry values for RT-PCR. The band density of Hsp70 mRNA was normalized to its corresponding β-actin band density. Data are presented as mean ± SEM. *, $P<0.05$ vs. young within a treatment group; †, $P<0.05$ vs. V/H treatment within an age group.

Figure 1

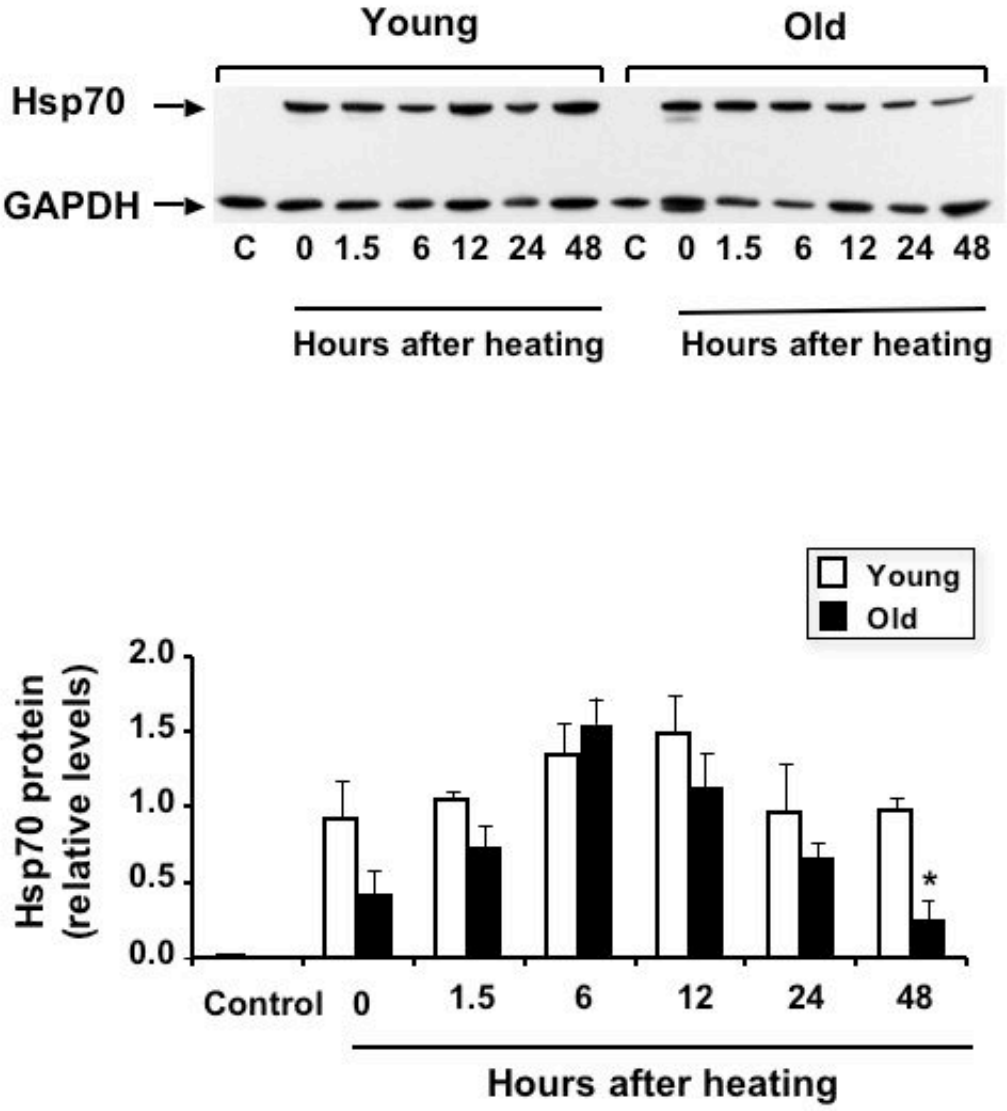
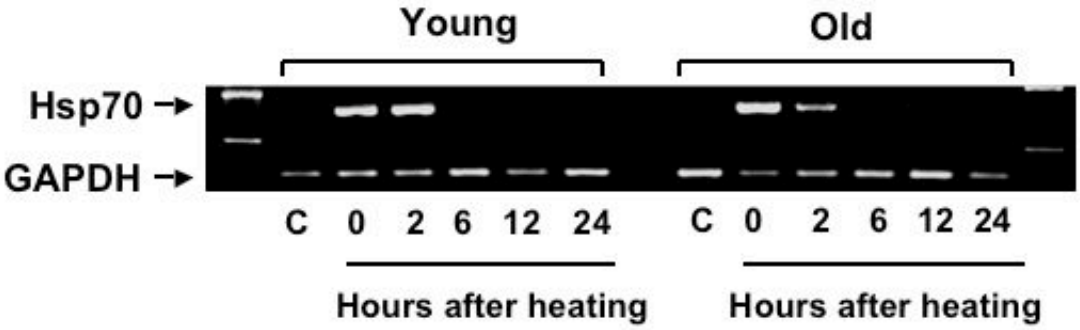

Young
Old
Hsp70
GAPDH
C 0 1.5 6 12 24 48 C 0 1.5 6 12 24 48
Hours after heating
Hours after heating
Young
Old
Hsp70 protein (relative levels)
2.0
1.5
1.0
0.5
0.0
*
Control 0 1.5 6 12 24 48
Hours after heating

Figure 2


Young
Old
Hsp70
GAPDH
C 0 2 6 12 24
C 0 2 6 12 24
Hours after heating
Hours after heating

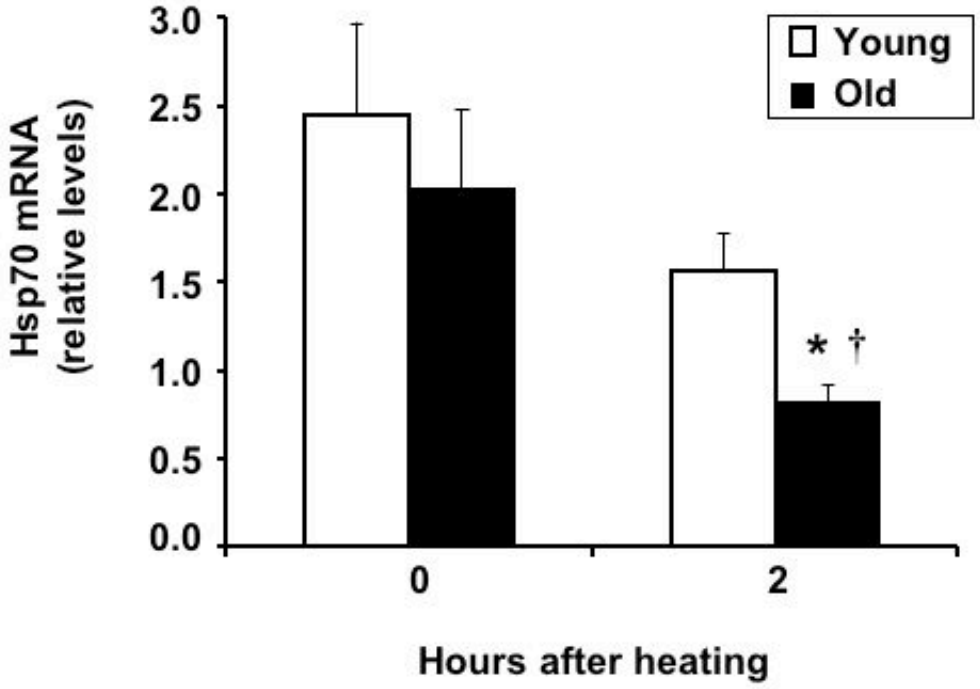

Hsp70 mRNA (relative levels)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Young
Old
* †
0
2
Hours after heating

Figure 3

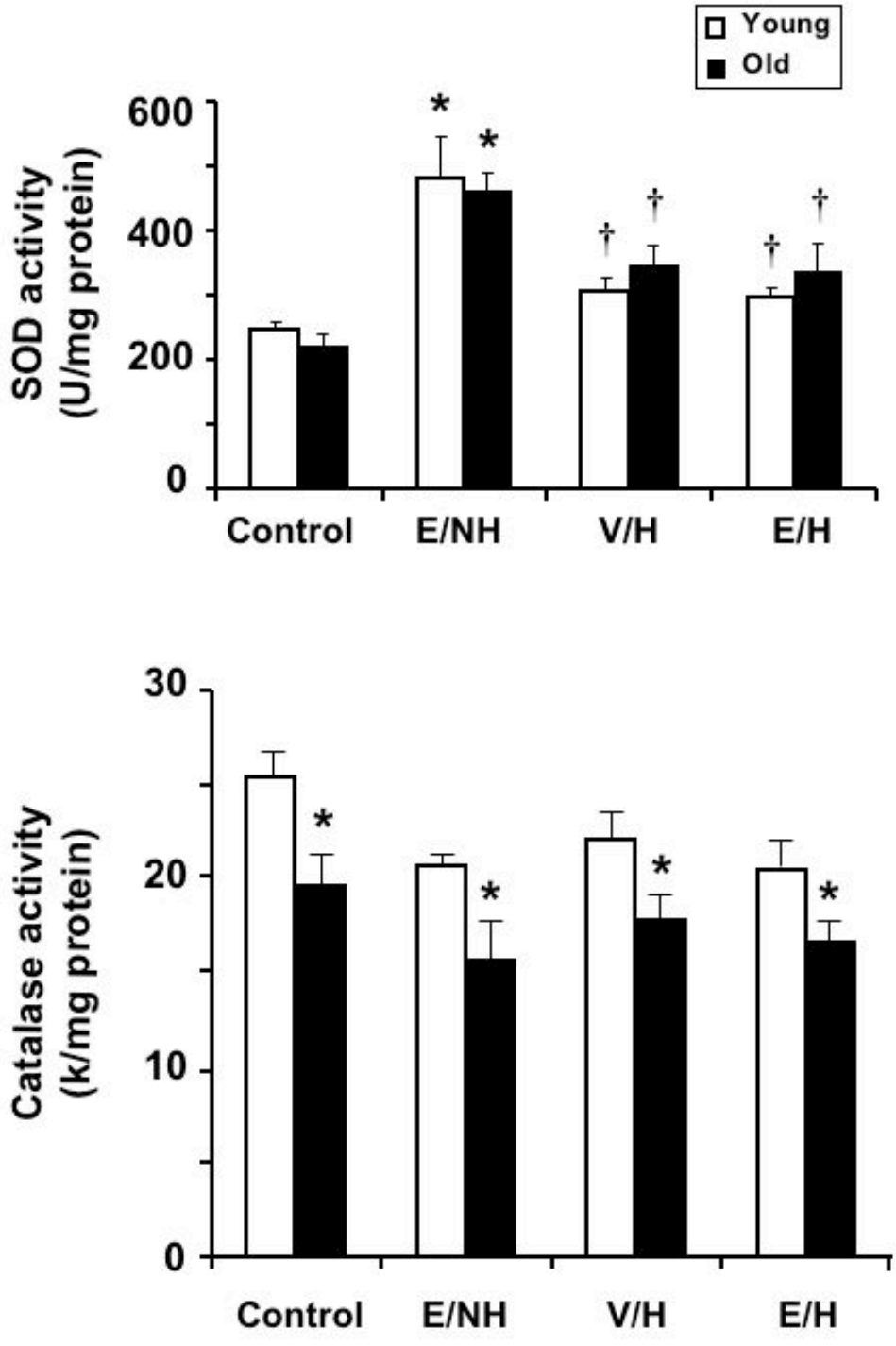

Young
Old
SOD activity (U/mg protein)
600
400
200
0
*
*
†
†
†
†
Control
E/NH
V/H
E/H
Catalase activity (k/mg protein)
30
20
10
0
*
*
*
*
Control
E/NH
V/H
E/H

Figure 4

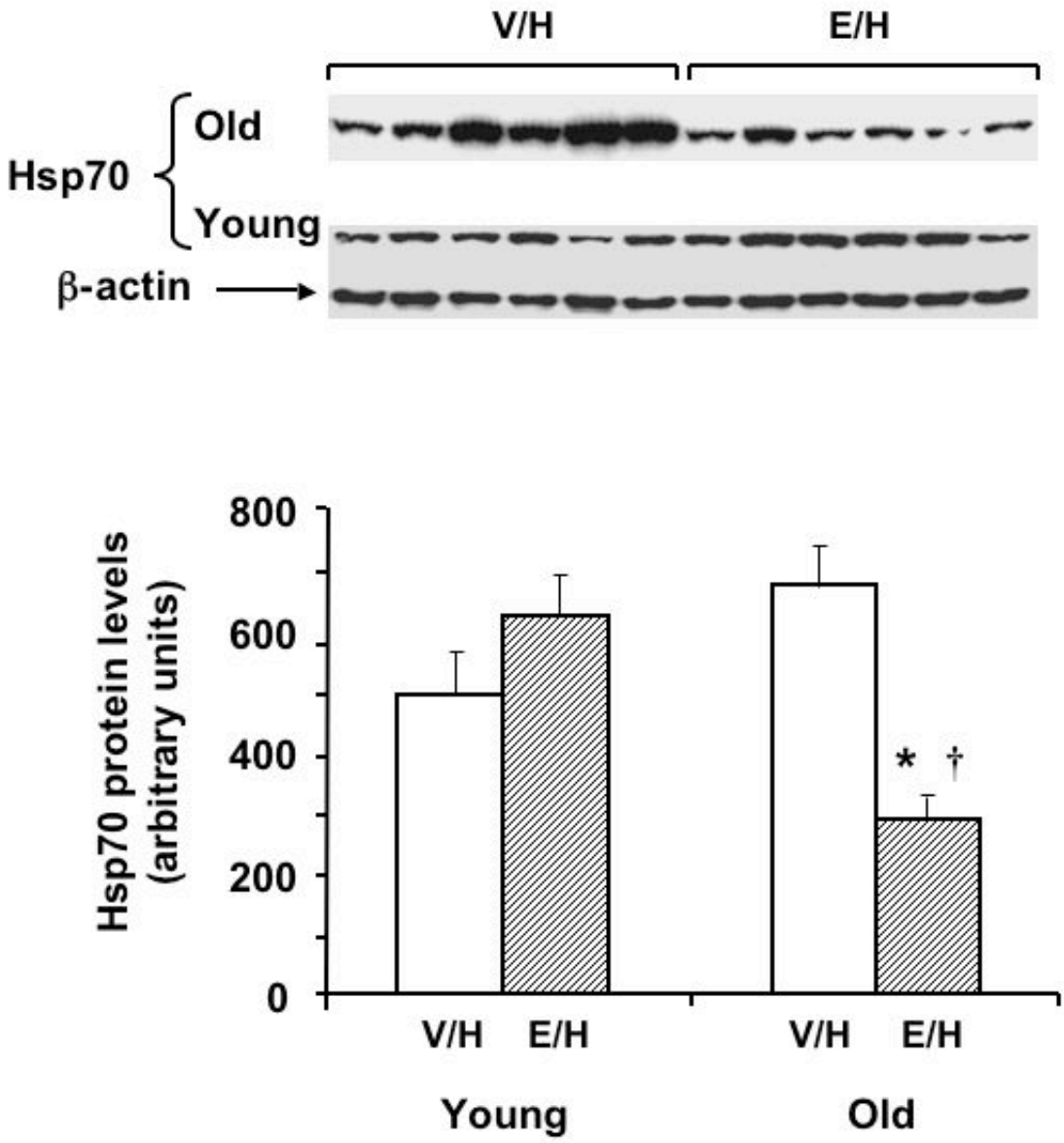
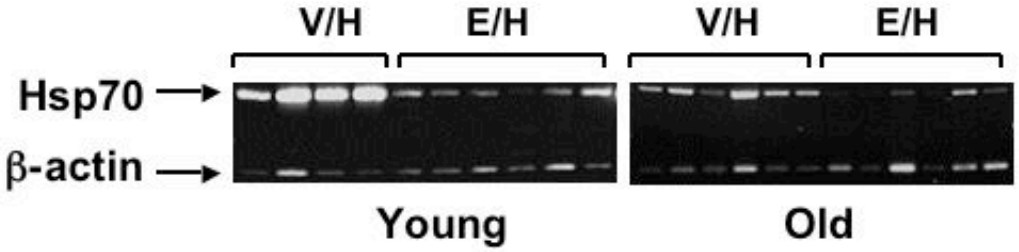
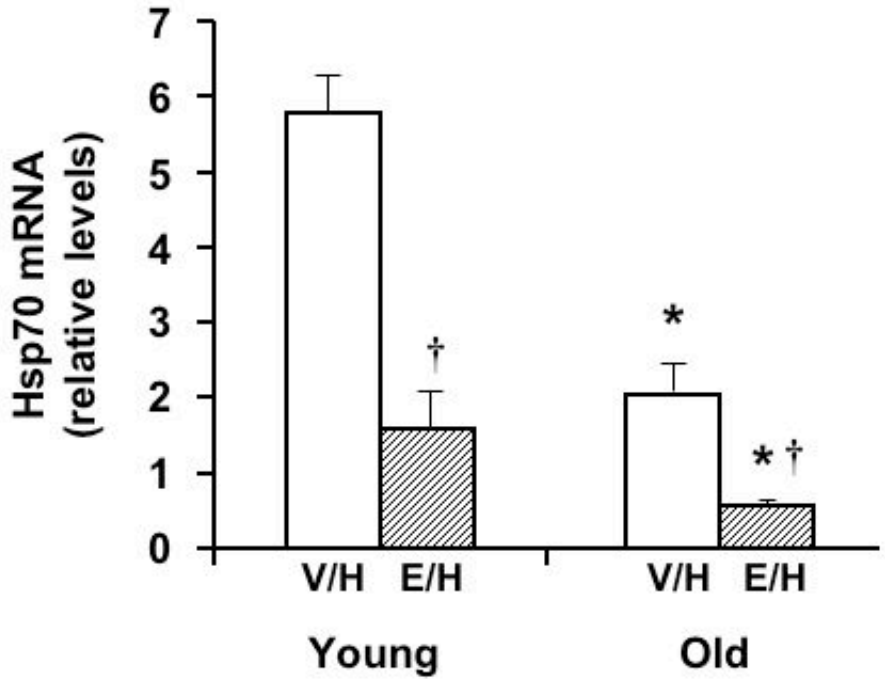

V/H
E/H
Hsp70
Old
Young
β-actin
Hsp70 protein levels (arbitrary units)
800
600
400
200
0
*
†
V/H
E/H
V/H
E/H
Young
Old

Figure 5


V/H
E/H
V/H
E/H
Hsp70
β-actin
Young
Old


Hsp70 mRNA
(relative levels)
7
6
5
4
3
2
1
0
†
*
* †
V/H
E/H
V/H
E/H
Young
Old