Aging reduces adaptive capacity and stress protein expression in the liver after heat stress

D. M. HALL,1 L. XU,1 V. J. DRAKE,1 L. W. OBERLEY,2 T. D. OBERLEY,2 P. L. MOSELEY,4 AND K. C. KREGEL1

1Department of Exercise Science, and 2Radiation Research Laboratory, The University of Iowa, Iowa City, Iowa 52242; 3Department of Pathology and Veterans Affairs Hospitals and Clinics, University of Wisconsin, Madison, Wisconsin 53705; and 4Department of Internal Medicine and Laboratory of Environmental Stress and Adaptation, University of New Mexico, Albuquerque, New Mexico 87131

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Although the mechanisms responsible for these outcomes are unclear, one area of focus is at the cellular level, where the accumulation of a highly conserved set of stress proteins, classified as heat shock proteins (HSPs), is associated with increased heat tolerance (15, 30). The 70-kDa HSP (HSP70) family of proteins is induced in a wide variety of cell types exposed to heat stress (6, 9, 19, 33) and includes a constitutive 73-kDa protein and a highly heat-inducible 72-kDa protein (36). The synthesis of HSPs appears to protect the cell from injury and to enhance cellular recovery from subsequent stresses (30). These findings suggest that the synthesis of HSP70 is an important component of the overall physiological response to stress. However, data from isolated cell systems (7, 37) and tissues extracted from intact animals (1, 19, 27) suggest that the expression of HSP70 and its potential protective role in cellular stress may decline with advancing age. Unfortunately, these studies have not addressed the effect of repeated heat exposures on stress protein regulation and the potential relationship between HSP regulation and reduced thermotolerance observed in aged organisms after this type of challenge.

Therefore, one aim of the present study was to determine the effects of repeated heat challenge on mortality rates and HSP70 accumulation in young and senescent Fischer 344 rats. In addition, because HSP70 may serve as a biomarker for tissues with the highest degree of cellular damage from exposure to stresses such as hyperthermia (9), experiments were designed to assess the relationship between HSP70 production and tissue injury in vivo. This approach allowed us to evaluate histological damage and HSP70 accumulation within the same tissues of young and senescent Fischer 344 rats. To determine the temporal pattern of HSP70 responses and cell injury in the liver after environmental stress, animals were assessed at multiple time points. In addition, immunohistochemical techniques were employed along with immunoblotting.

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tning to permit evaluation of the regional distribution of HSP70 accumulation and tissue injury within the liver. We chose to focus on the liver because it is a prime target of tissue injury in physiological challenges such as heat stroke (17, 26). The liver has also been demonstrated to be very sensitive to HSP70 accumulation during heat stress (9, 11, 18, 19). We hypothesized that senescent rats have a reduced ability to cope with heat stress because of attenuated HSP70 production and subsequent tissue injury.

**METHODS**

**Animals**

Two groups of male Fischer 344 rats (National Institute on Aging) were studied: a young, 6-mo-old group (300–400 g) and a senescent, 24-mo-old group (360–460 g). Each age group was composed of sham-heated control animals (n = 2) and experimental animals with various recovery periods after a heat stress protocol (n = 3 for 2, 12, and 24 h and n = 10 for 48 h). 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. All rats were handled daily and familiarized with a colon temperature (Tco) probe during the week before experimentation.

**Experimental Procedures**

On the day of an experiment, rats were fitted with a thermistor temperature probe (Yellow Springs Instruments) inserted 6–7 cm into the colon and then placed in plastic cages (45 × 25 × 20 cm), conscious and unrestrained. Tco was monitored continuously on a digital display and an analog output. A baseline Tco (37.0–38.0°C for both age groups) was established over a 30-min control period. Experimental animals were then heated as previously described (11, 20). An infrared lamp, positioned ~40 cm above the rats, was raised or lowered to obtain an ambient temperature of 38–40°C. Movement of the lamp permitted a constant heating rate or lowered to obtain an ambient temperature of 38–40°C. Heating was stopped when Tco reached 41°C; however, heating was resumed at appropriate times to maintain Tco 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. Experimental animals were subsequently subjected to a second heat stress 24 h after the first stress. Sham-heated control rats were handled identically to experimental rats, except ambient temperature was maintained at 22–24°C.

At 2, 12, 24, and 48 h after the second heat stress, rats were given an overdose of pentobarbital sodium (80 mg/kg ip) and perfused transcardially with 5% heparinized PBS (~250 ml/rat). Liver biopsies were collected and processed for protein immunobLOTS, immunohistochemical analysis, and histological evaluation of tissue injury. Tissue samples for immunobLOTS were collected from lobus caudatus dexter and lobus sinister, cleaned of connective tissue, frozen in liquid nitrogen, and stored at ~80°C. After collection of fresh tissues, animals were perfused with a 10% neutral-buffered Formalin (NBF) solution, and sections of each liver lobule were fixed for immunohistochemical quantitation of HSP70 expression and grading of tissue injury.

**Protein Immunoblots**

Tissues for immunobLOTS were minced and homogenized in 0.05 M phosphate buffer (pH 7.8). The homogenate was briefly spun (2 min, 16,000 g) at 4°C in a clinical centrifuge to remove connective tissue, and the supernatants were sonicated on ice (3 times for 30 s each). Total protein content was quantitated using the Bradford assay (Bio-Rad), and equal protein amounts (20 μg) of tissue samples were then separated on a one-dimensional 12.5% polyacrylamide gel under standard denaturing conditions according to the method of Laemmli (21). The separated proteins were then transferred onto nitrocellulose membranes (40 V, 4°C, 10 h). The membranes were blocked for 2 h at room temperature with 5% dry milk and 2% BSA in Tris-buffered saline (10 mM Tris and 150 mM NaCl, pH 8.0) and 0.05% Tween 20 (TBST). After extensive washing with TBST, membranes were probed for 2 h with a rat monoclonal antibody specific to the constitutive Hsp73 form (Hsc70) of HSP70 (1B5, 1:1,000 dilution) or a mouse monoclonal antibody specific to the inducible Hsp72 form (Hsp70) (C92, 1:1,000 dilution). The 1B5 and C92 antibodies were purchased from StressGen Biotechnologies (Victoria, BC, Canada). Nitrocellulose membranes were washed with TBST and incubated for 2 h with their respective antibodies. The 1B5 blots were probed with a rabbit anti-rat IgG peroxidase antibody (1:5,000 dilution; StressGen Biotechnologies), and C92 blots were probed with a goat anti-mouse IgG peroxidase antibody (1:1,000 dilution; Sigma Chemical). After the blots were washed thoroughly with TBST, they were developed using the enhanced chemiluminescence-Western blot detection kit (Amersham-Pharcma-Biotech) and BioMaxMR film (Kodak).

**Preparation of Nuclear Extracts**

Nuclear extracts from rat liver were prepared using a modification of the method of Hattori et al. (12). Livers were rapidly cleaned of blood and connective tissue in ice-cold PBS, blotted dry, and minced with a razor blade. All subsequent procedures were carried out at 4°C.

The minced tissue was weighed and placed in homogenizing buffer [0.3 M sucrose, 10 mM HEPES, pH 7.6, 10 mM KCl, 0.74 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1 complete protease inhibitor cocktail tablet (Boehringer Mannheim) per 50 ml of buffer]. The buffer-tissue mixture (2 ml of buffer per gram of tissue) was homogenized with a motor-driven Teflon pestle tissue homogenizer and then mixed with two volumes of cushion buffer (same components as the homogenizing buffer, except sucrose concentration was 2.2 M). The homogenate-cushion buffer solution (sucrose concentration ~1.57 M) was then layered over 5 ml of cushion buffer in 38.5-ml polyallomer tubes. Homogenates were centrifuged in a Beckman ultracentrifuge at 105,000 g (24,000 rpm, SW 28 rotor) for 150 min. The supernatant, containing the cytoplasmic fraction, was decanted, and buffer was removed from the walls of the tubes. Tubes were then inverted on ice for 10 min to drain excess buffer from the nuclear pellets. Each nuclear pellet was resuspended in 500 μl of nuclear lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and gently rotated for 40 min at 4°C. Nuclear membranes were pelleted by spinning the nuclear lysate in a Beckman ultracentrifuge at 35,730 g (14,000 rpm, SW 28 rotor) for 11 min. The resultant nuclear fractions were collected, and total protein content was determined for protein immunoblot analysis. Equal protein amounts (50 μg) of
the nuclear and cytoplasmic fractions were utilized, and immunoblots were performed as described above.

**Immunohistochemistry and Histology**

After transcardial perfusion with NBF, liver biopsies were collected, cut into 2-mm sections, and postfixed for 2 h in NBF. Tissue sections were subsequently processed through graded alcohols to paraffin blocks. Sections (4 μm) were cut from each block, mounted on Superfrost*-histology slides (Fisher Scientific), and deparaffinized in three changes of xylene (5 min each) followed by two changes in 100% ethanol. Endogenous peroxidase activity was blocked with a 30-min incubation in 3% H2O2-methanol. For Hsc70 antigen processing, sections were rehydrated and treated with Target Unmasking Fluid (Signet Laboratories, Dedham, MA) at 70°C for 20 min. After three changes of distilled water, sections were processed for quantitation of immunoreactive protein with a goat ABC staining kit (Santa Cruz Biotechnology) and goat anti-Hsc70 antibody (1:500 dilution; StressGen Biotechnologies). For Hsp70 antigen processing, a Tris-urea solution (1 M Tris and 5% urea, pH 9.5) was substituted for Target Unmasking Fluid. The Tris-urea solution was heated to a hard boil in a microwave oven, and then slides were inserted into the solution and incubated for 5 min. Slides were removed from the Tris-urea solution and placed in 100 mM Tris buffer at room temperature (22–24°C). This process was repeated three times. Sections were then treated with 10% normal goat serum for 40 min, washed, and incubated in anti-Hsp70 antibody solution (C92, 1:60 dilution; StressGen Biotechnologies) overnight at 4°C.

On the next day, both sets of tissue sections were rinsed with PBS three times (5 min each) and blocked with 10% normal goat serum for 40 min. Sections were then processed for quantitation of immunoreactive protein with an immunoenzymatic staining kit (DAKO, Carpinteria, CA) and Immunopure Metal Enhanced diaminobenzidine substrate (Pierce, Rockford, IL) as directed by the manufacturers. Histology sections were analyzed microscopically for evidence of injury. All sections were graded by the same reviewer, who was blinded as to their origin. Liver injury was graded using the follow scale adapted from Ryan et al. (31): 0, no hepatocellular necrosis or injury; 1, mild injury, with scattered cells showing coagulative changes; 2, moderate injury; 3, severe injury, with more than one-half of the lobule affected. Immunoreactive protein levels were graded using an intensity of color scale developed by Oberley et al. (28) ranging from 0 (negative) to 4+ (strongly positive).

**Data Analyses**

Values are means ± SE. Statistical comparisons between Tco values and heating rates were determined with an ANOVA for two factors (i.e., experimental intervention and treatment group) design. Post hoc comparisons were made with Duncan’s multiple range test. Differences were considered significant at P < 0.05.

**RESULTS**

**Heating Rates and Outcomes**

Baseline Tco before heating was similar in the young and senescent groups on day 1 (37.8 ± 0.14 vs. 37.5 ± 0.18°C) and day 2 (37.8 ± 0.17 vs. 37.5 ± 0.17°C). Experiments were designed to minimize differences in heating rates between age groups and within a group between heating trials. This was achieved as demonstrated by the similar heating rates obtained on day 1 (0.060 ± 0.0033 vs. 0.066 ± 0.0034°C/min) and day 2 (0.057 ± 0.0031 vs. 0.063 ± 0.0039°C/min) for the young and old groups, respectively.

All rats in the young group that underwent the 2-day heating protocol reached their designated time point (i.e., 2, 12, 24, or 48 h after the 2nd heating trial). Similarly, older rats in the 2-, 12-, and 24-h groups survived both heat exposures. However, 40% (4 of 10) of the senescent rats in the 48-h group did not survive the protocol. All four of these rats died between 24 and 48 h of the recovery period. Because of the variability in the time of death in these senescent rats, only the adaptive responses of animals in the 48-h group that survived the entire protocol are presented.

**HSP70 Immunoblots**

Representative immunoblots of liver samples from young and senescent animals probed with monoclonal antibodies specific for the constitutive (Hsc70) or inducible (Hsp70) form of HSP70 are presented in Fig. 1. For Hsc70, low-to-moderate protein accumulation was observed in the young animals at all time points, with peak protein expression at 12–24 h after the heat stress. In contrast, the Hsc70 response in senescent animals was characterized by a progressive increase in hepatic protein expression throughout the recovery period compared with the control condition, with peak expression at 48 h. The Hsc70 response was also higher in the old than in the young animals at all time points, including the control condition.

An immunoblot probed with a monoclonal antibody specific for only the inducible Hsp70 is also presented in Fig. 1. There was no Hsp70 accumulation in liver...
samples from the young or senescent control rats. In the young rats that underwent the heat stress protocol, there was strong accumulation of Hsp70 at 2, 12, 24, and 48 h after heating, with the peak expression at 48 h. In contrast, a bimodal pattern of Hsp70 expression was observed in liver samples from the senescent group. This response was characterized by strong Hsp70 expression at 2 h of recovery but an attenuated pattern of accumulation at 12 h. At 24 h, the Hsp70 response was again elevated, although not to the level observed at 2 h. Interestingly, there was very little Hsp70 protein induced at 48 h of recovery in the senescent liver samples, which was in striking contrast to the 48-h response in the young group.

Immunohistochemistry

Hsc70 expression. Liver sections stained immunohistochemically for Hsc70 protein are presented in Fig. 2. Under control conditions, both age groups showed ev-

Fig. 2. Cellular localization of immunoreactive Hsc70 protein in the liver after heat stress. Representative photomicrographs of liver biopsies stained with a monoclonal antibody specific for Hsc70 are presented. Samples were collected from young (A, C, and E) and senescent (B, D, and F) Fischer 344 rats. Individual micrographs represent euthermic control conditions (A and B) and 12 h (C and D) and 48 h (E and F) after heat stress. There was low-level cytoplasmic expression of immunoreactive Hsc70 in hepatocytes from young control rats (A), whereas senescent controls (B) exhibited moderate levels of protein expression (arrows). At 12 h of recovery, cytoplasmic Hsc70 expression was mildly increased above control levels in isolated zone 2 and 3 hepatocytes (arrows) of young rats (C). In contrast, Hsc70 expression was moderately elevated throughout zone 2 and 3 regions (arrows) in older rats (D). By 48 h of recovery, Hsc70 expression was at or below control levels in young rats (E). Conversely, high Hsc70 expression was present in the cytoplasm of zone 2 and 3 hepatocytes in senescent rats at 48 h (F). Magnification: ×80.
Table 1. Nuclear and cytoplasmic expression of Hsc70 and Hsp70 in different regions of the liver at selected time points after heat stress

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Immunoreactive protein levels were graded using an intensity of staining scale ranging from 0 (negative) to 4 (strong); tr+, trace. Hsc70, constitutive form of Hsp70; Hsp70, inducible form of Hsp70; 6 mo, 6-mo-old (young) rats; 24 mo, 24-mo-old (senescent) rats; C, cytoplasmic staining; N, nuclear staining. Zone 1 hepatocytes, cells located adjacent to terminal branches of hepatic arterioles or portal venules; zone 2 hepatocytes, cells located between hepatic arterioles or portal venules and central hepatic veins; zone 3 hepatocytes, cells located adjacent to hepatic veins.

idence of gradients of Hsc70 expression, with protein levels being elevated in zone 3 regions. However, senescent animals showed markedly higher immunoreactive Hsc70 expression than their young counterparts in hepatocytes adjacent to central hepatic veins (zone 3) and hepatocytes located between hepatic arterioles or portal venules and central hepatic veins (zone 2; Fig. 2, A vs. B; Table 1). Hepatocytes located adjacent to terminal branches of hepatic arterioles or portal venules (zone 1) were negative (0) in the young cohort and marginally positive (trace +) in the senescent group.

Hsc70 expression increased in hepatocytes from all liver zones in the senescent group at 2 h after heat stress. Hsc70 levels were elevated further at 12 h after heat stress (Fig. 2D) and remained high through 48 h of recovery (Fig. 2F). In contrast to the old group, young animals showed only marginally increased Hsc70 expression in zone 3 hepatocytes at 12 h of recovery (Fig. 2C) and in zone 2 and 3 cells at 24 h after heat stress. By 48 h of recovery (Fig. 2E), hepatocellular Hsc70 expression had returned to euthermic control levels in all liver zones. Hsc70 staining was diffuse and cytoplasmic at all time points for both age groups.

Hsp70 expression. There was no evidence of the inducible Hsp70 protein in the livers of euthermic controls from either age group (Table 1, Fig. 3, A and B). However, consistent with the immunoblot results, heat stress increased liver Hsp70 expression in the 6- and 24-mo-old animals. Strong gradients of Hsp70 expression were observed in both age groups; however, the stress-induced Hsp70 response was markedly reduced in the senescent group (Fig. 3). In young animals, cytoplasmic and nuclear Hsp70 levels were elevated at 2 and 12 h of recovery, peaked at 24 h, and remained high through 48 h (Fig. 3, C and E). In contrast, cytoplasmic and nuclear Hsp70 expression were elevated at 2 h but only marginally present at 12 h in the senescent group. Also, although protein levels increased above control at 24 and 48 h of recovery, the magnitude of Hsp70 expression was still much lower in the senescent rats (Fig. 3, C vs. D and E vs. F).

In addition to quantitative differences in protein expression between the two age groups, there were distinct differences in the zonal distribution of Hsp70 (Fig. 4). Young rats showed consistent evidence of robust (4+) Hsp70 expression in zone 3 hepatocytes, moderate levels (2+) in zone 2 cells, and much lower levels of immunoreactive protein (1+) in the more well-perfused zone 1 areas. In contrast, Hsp70 distribution was extremely heterogenous in the senescent animals, with isolated areas of increased protein that were widely scattered and distributed among all liver zones. However, comparing the zonal distribution of Hsp70 between young and old rats demonstrated that age dramatically reduced Hsp70 expression in all liver zones (Table 1, Fig. 4).

To confirm the nuclear localization of Hsp70 observed in the immunohistochemistry experiments, nuclei were isolated from fresh liver of young and old rats after the heat stress protocol by cell fractionation techniques. A representative immunoblot of nuclear and cytoplasmic extracts from young and senescent rats is presented in Fig. 5 for qualitative assessment purposes. In both age groups, immunoreactive Hsp70 expression was present in the nuclear fraction of hepatocytes at 12 and 24 h after heating, which corresponds with immunohistochemical findings presented in Fig. 4.

Histology

Liver morphology was normal (grade 0 injury) in young and senescent euthermic control animals (Fig. 6,
A and E); however, senescent rats showed evidence of alterations in hepatocyte morphology, including fatty hepatic infiltrates with mild cytoplasmic vacuolization of zone 2 and 3 hepatocytes.

At 2 h after heat stress, grade 1 injury, including moderate cytoplasmic vacuolization of zone 2 and 3 hepatocytes with mild-to-moderate sinusoidal congestion (Fig. 6B), was widespread in young animals. In contrast, senescent animals showed evidence of grade 1-to-grade 2 injury (Fig. 6F). Moderate-to-severe cytoplasmic vacuolization of zone 2 and 3 hepatocytes was present, along with severe sinusoidal congestion, monocyte infiltration, and clustered masses of macrophages and neutrophils located within sinusoids and around portal veins.

At 12 h after heat stress, young animals still showed evidence of grade 1 injury, but hepatocyte vacuolization and sinusoidal congestion were markedly reduced from the 2-h time point. Sinusoidal congestion was also reduced in the senescent cohort. However, the senescent animals showed evidence of grade 2 injury that was expressed as severe hepatocyte vacuolization in
zone 2 and 3 areas, with pyknotic nuclei and focal hepatocellular necrosis in periportal areas.

At 24 h after heat stress, young rats showed moderate hepatocellular vacuolization with little evidence of sinusoidal congestion (Fig. 6C). In contrast, senescent animals showed evidence of sinusoidal congestion with grade 2-to-grade 3 injury that included severe hepatocellular vacuolization with multifocal or centrilobular hepatocyte necrosis in livers with grade 2 injury and hepatocyte necrosis that covered all zones in livers rated as severe (Fig. 6G).

At 48 h of recovery, young rats showed normal liver morphology (Fig. 6D). In contrast, senescent animals showed grade 2 injury with widespread cytoplasmic vacuolization and necrosis in zones 2 and 3 and lymphocyte infiltration around portal triads and bile ducts (Fig. 6H).

DISCUSSION

The primary purpose of the present investigation was to test the hypothesis that aging reduces heat stress-inducible Hsp70 expression and increases stress-related morbidity and mortality. We focused on the liver, because previous work from our group has demonstrated that the liver is a sentinel organ for thermal stress (9, 11, 19). In addition, data from mod-
Fig. 6. Hematoxylin and eosin staining of liver sections. Representative photomicrographs of liver biopsies were obtained under euthermic control conditions and at 2, 12, 24, and 48 h after consecutive heat exposures. Samples were collected from young (A–D) and senescent (E–H) Fischer 344 rats. Individual photomicrographs represent euthermic control conditions (A and E) and 2 h (B and F), 24 h (C and G), and 48 h (D and H) after heat stress. Tissue morphology was normal in euthermic control sections from both age groups (A and E). At 2 h of recovery, there was mild sinusoidal congestion in young animals (B, arrows), whereas senescent rats showed evidence of extensive monocyte infiltration (F, arrows). At 24 h of recovery, which was the time at which both age groups displayed maximal liver injury, young rats (C) displayed evidence of moderate hepatocellular vacuolization in zone 3 (arrows) and zone 2 cells, with little evidence of sinusoidal congestion. In contrast, senescent animals (G) presented with severe zone 2 and 3 hepatocyte vacuolization (arrows) and centrilobular hepatocyte necrosis. At 48 h of recovery, normal morphology was observed in young rats (D), whereas their senescent counterparts (H) showed cytoplasmic hepatocellular vacuolization and lymphocyte infiltration (arrows). Magnification: ×80.
els of heat stress (11) and endotoxin shock (31) support the critical role of the liver in the response to thermal and endotoxin challenge. Our results were supportive of the hypothesis and yielded three primary findings that suggest a functional link between age-related decrements in Hsp70 expression and pathophysiological responses to heat stress. First, immunoblot and immunohistochemistry results clearly demonstrated that the magnitude of the Hsp70 response was significantly reduced with age. Senescent rats showed moderately increased Hsp70 expression at 2 and 24 h of recovery, but by 48 h after heating Hsp70 expression was dramatically lower. In contrast, a strong Hsp70 response was observed throughout the 48-h recovery period in the young cohort. Strikingly, immunohistochemistry results in the senescent group also demonstrated that stress protein expression was extremely low in acinus zone 2 and zone 3, which are regions of the liver containing reduced blood flow and lower oxygen tensions.

Second, the older animals showed extensive zone 2 and 3 liver injury, which corresponded to the diminished Hsp70 response in these regions, and a significantly reduced ability to survive consecutive heat stresses. Only 60% of the senescent group survived the heating protocol. In contrast, liver injury was markedly lower in the young cohort, with all these animals surviving the heating protocol.

Finally, a comparison of the stress-induced patterns of Hsc/Hsp70 expression showed distinct differences in the two age groups. Young rats responded to heat stress with robust nuclear and cytoplasmic Hsp70 expression that was maintained for 48 h in hepatocytes located in central vein regions. Furthermore, the magnitude of Hsc70 expression was lower and of shorter duration in the young cohort. With aging, nuclear and cytoplasmic Hsp70 expression was delayed and reduced, and older animals appeared to compensate for a lack of Hsp70 protein with increased cytoplasmic Hsc70 expression in the vicinity of the central vein, which is a putative hypoxic region. Taken together, these results suggest that the blunted Hsp70 response in the older animals may have functional consequences that correlate with the increased cellular injury and reduced stress tolerance that is associated with advancing age.

Increased Hepatic Injury With Aging

The damage noted at several time points after heating in the older rats indicates that the aged liver cannot tolerate repeated heat stress. Also, there are several reasons to believe that the older rats' inability to generate a sufficient Hsp70 response is a primary factor contributing to this outcome. For example, data indicate that HSPs are the first proteins produced by the liver in response to hemorrhagic shock (4). In addition, we recently observed that long-term caloric restriction reduces cellular injury and improves heat tolerance of old animals by lowering reactive oxygen species production and preserving cellular ability to adapt to stress through antioxidant enzyme induction (11). These data strongly suggest that the aged liver is more susceptible to the oxidative stress and damage that may accompany heat stress.

The senescent animals appeared unable to mount an appropriate adaptive response after heat stress, as evidenced by the blunted Hsp70 response and widespread liver damage. Importantly, there was significant mortality in the older animals with repeated heat stress, whereas all the young animals, which mounted a robust Hsp70 response, survived the heating protocol. The striking pathology and mortality findings in the senescent group suggest that the maintenance of liver function is a critical determinant of an organism's survival. Several reports, ranging from clinical observations in humans (17) to a variety of animal studies (2, 10, 11), support this contention. For example, data in humans indicate that death in heat stroke patients is associated with liver dysfunction and disseminated intravascular coagulation (17).

Temporal Pattern of HSP70 Family Expression

A number of studies have shown that the aged eukaryotic organism has a reduced ability to generate HSPs in response to thermal challenge (1, 7, 19). These findings suggest a potential explanation for the reduced thermotolerance observed in older populations (13, 32). Although the lack of a stress protein response could be interpreted as an age-associated inability to produce and accumulate HSPs, our laboratory has recently shown that, when exercise is combined with environmental heating as a stimulus, senescent rats generate the same magnitude of HSP70 expression as young rats (18). This observation indicates that aging reduces but does not eliminate cellular ability to express HSPs in response to environmental stress and, furthermore, suggests that aging results in important alterations in the type or intensity of stress needed for HSP accumulation. Results from the present study confirm and extend this concept. The blunted Hsp70 response to heating in the older rats was not the result of a complete inability to produce this stress protein, because these animals had a robust response to thermal challenge at 2 h. However, Hsp70 expression was substantially reduced at 12 and 48 h after heating. This phasic expression of Hsp70 in response to heat stress in the older animals was in sharp contrast to the progressively increased induction observed in the young rats, suggesting that there are changes in key regulatory elements with aging that contribute to reduced Hsp70 protein expression. For example, Liu et al. (23) proposed that the mechanism of the attenuated Hsp70 response with aging can be attributed to a dysfunction in transcriptional regulation. However, several other possible explanations are viable.

Accumulation of the inducible form of HSP70, which has been associated with tolerance to a variety of stresses, including hyperthermia (22), oxidative stress (29), ischemia (24), and exposure to cytokines such as tumor necrosis factor-α (TNF-α) (14), is widely de-
scribed to have a reduced rate of induction with aging (1, 7, 19, 37). The constitutively expressed Hsc70 is responsive to a variety of cellular signals, such as serum factors and viral activation, and serves as a chaperone in protein folding and translocation across membranes (36). However, very few studies have directly examined the pattern of regulation and production of the constitutively expressed Hsc70 in aged animals in basal or stress conditions. In aged cells, there is an increased accumulation of misfolded and oxidized proteins, along with advanced glycosylation end products (25, 35). On the basis of these observations, it is tenable to postulate that the expression of Hsc70, at rest or in conditions of cellular stress, might be altered in the aged organism by the accumulation of these modified and misfolded proteins.

The pattern of Hsc70 expression in the present study supports this postulate. Hsc70 was present in sham control livers of young and senescent rats, with higher levels in the older group. This pattern of elevated Hsc70 levels with aging was also consistent throughout the time course of recovery after heat stress. Furthermore, the pattern of expression of the constitutive Hsc70 was distinctly different from that of the inducible Hsp70, in terms of the time course of expression and the magnitude of expression when the young and old animals were compared. Young rats had a progressive increase in Hsp70 in the liver, whereas senescent rats had a diminished response. Interestingly, the older animals had a progressive increase in Hsc70 expression in the face of a decrease in Hsp70 levels, suggesting an attempt by hepatocytes to increase their constitutive Hsc70 levels at a time when the expression of inducible Hsp70 is markedly blunted. Moreover, since the expression of Hsc70 occurs at a lower threshold than does expression of Hsp70 during stresses such as hyperthermia (25) and ischemia (16), Hsc70 may play a role in protecting cells during conditions such as mild stress or reduced cellular Hsp70 expression.

One interesting possibility raised by these data is that the early increase in Hsp70 noted in the senescent animals at 2 h represents an unsuccessful attempt at self-preservation by individual hepatocytes. Because of the decreased ability of the aged cell to generate a sustained Hsp70 response similar to that observed in the young animal, cell injury and even cell death may occur. Recent data demonstrate that HSP70, when released into the extracellular environment, drives a vigorous cytotoxic immune response with associated local production of cytokines such as TNF-α, interleukin (IL)-2, IL-12, and interferon-γ (5, 34). In this scenario, the HSP70 proteins may also activate macrophages and cytotoxic T lymphocytes (3). Thus, one unfortunate outcome of an insufficient cellular HSP response could be death of the stressed cell, immune activation, and a localized inflammatory response with associated tissue injury. Paradoxically, stressed cells in younger animals, which are able to sustain an HSP response, do not undergo a similar degree of cell death. This not only results in cellular tolerance to the initiating stress, but it protects the organism through cellular tolerance to cytokines such as TNF-α and IL-1 and through the downregulation of production of these same cytokines by macrophages that have accumulated HSPs. The net result in the younger animals would be less cellular damage from the thermal challenge and a diminished local inflammatory response in the organism. Although results from the present project do not directly address this concept, dramatic increases in liver injury and inflammatory cell infiltration were observed in the older animals (Fig. 6).

Conclusions. We have shown that liver Hsp70 expression is blunted over a prolonged time course of recovery from heat stress in aged compared with young rats. In addition, extensive liver injury and significant mortality rates were noted in the older animals, whereas young rats tolerated the stress very well. These data highlight the regional nature of stress-induced injury and HSP70 expression in the liver and the impact of aging on these responses. Furthermore, the results suggest a functional link between the age-related decrements in the expression of the inducible Hsp70 and the pathophysiological responses to heat stress.

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