Caloric restriction improves thermotolerance and reduces hyperthermia-induced cellular damage in old rats

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ABSTRACT **Adult-onset, long-term caloric restriction (CR) prolongs maximum life span in laboratory rodents. However, the effect of this intervention on an organism's ability to cope with a physical challenge has not been explored. We investigated the influence of CR and aging on stress tolerance in old rats exposed to an environmental heating protocol on two consecutive days. We hypothesized that CR would increase heat tolerance by reducing cellular stress and subsequent accrual of oxidative injury. All calorically restricted rats survived both heat exposures compared with only 50% of their control-fed counterparts. CR also decreased heat-induced radical generation, stress protein accumulation, and cellular injury in the liver. In addition, heat stress stimulated marked induction of the antioxidant enzymes manganese-containing superoxide dismutase and catalase, along with strong nuclear catalase expression in liver samples from rats subjected to CR. In contrast, stress-related induction of antioxidant enzymes was blunted, and nuclear catalase expression was unchanged from euthermic conditions in the control-fed group. These data suggest that CR reduces cellular injury and improves heat tolerance of old animals by lowering radical production and preserving cellular ability to adapt to stress through antioxidant enzyme induction and translocation of these proteins to the nucleus.—Hall, D. M., Oberley, T. D., Moseley, P. M., Buettner, G. R., Oberley, L. W., Weindruch, R., Kregel, K. C. Caloric restriction improves thermotolerance and reduces hyperthermia-induced cellular damage in old rats. *FASEB J.* 14, 78–86 (2000)**

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AGING IS ASSOCIATED with a loss of ability at the molecular, cellular, and whole organism levels to cope with environmental stressors such as heating (1–4). The mechanisms underlying these alterations with senescence are not well defined but likely include modifications in transcriptional regulation of stress-inducible genes (4), inappropriate production of inflammatory cytokines (5), blunted synthesis of protective acute-phase proteins such as heat shock proteins (1, 3, 6) and, at a more integrated level, multi-organ system failure (2, 4). One clinically important expression of this age-associated loss of function is the increase in heat-related morbidity and mortality rates reported during periodic heat waves in urban areas (7–9).

Although mechanisms underlying age-related alterations in stress responses are not well defined, accumulating evidence supports the validity of the oxidative stress hypothesis of aging, which suggests that cellular accumulation of oxidative injury contributes to the lowered functional capacity in aged organisms (10, 11). One proposed mechanism for these observations is that the increase in oxidative stress and subsequent biomolecular damage associated with aging are the result of an increased rate of reactive oxygen species (ROS) generation and a greater susceptibility of tissues to oxidative injury. However, it is not known whether the increased generation of radicals typically associated with the application of a stress (12–14) is exaggerated with advancing age and whether these radicals play a role in the reduced thermotolerance and increased mortality observed with senescence.

One intervention that has been demonstrated to offset the age-associated accrual of oxidative injury is caloric restriction (CR) (11, 15). CR, which involves a reduction in calorie intake while maintaining ade-

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quate nutrition, preserves the activities of antioxidant enzymes in post-mitotic tissues, maintains organ function, opposes the development of spontaneous diseases, and prolongs maximum life span in laboratory rodents (11, 16–18). It has been proposed that reductions in ROS production and cellular oxidative injury are central to the positive effects of CR. Although extensive data support this tenet, these experiments were largely conducted in isolated tissues from nonstressed animals such that little information exists regarding the ability of CR animals to respond to an acute stress or adapt to repeated challenges.

We have been investigating the impact of aging on physiological control mechanisms using stressors such as environmental heating and have found that the ability to generate protective intracellular stress proteins is blunted in old compared with young rats (3). Consistent with this observation, we have discovered that with repeated heat challenge, older rats are markedly less thermotolerant and have significantly higher mortality rates than their younger counterparts (2), thereby providing an excellent *in vivo* model for the high morbidity and mortality rates observed in older humans (7–9). Also, we have established that heat stress increases radical generation and radical-mediated tissue injury in the splanchnic region of rats (19). Low concentrations of ROS can induce antioxidant enzyme gene expression while high levels can inactivate antioxidant enzymes and subsequently induce stress protein gene expression (20). The degree to which antioxidant enzyme and stress protein systems can withstand and respond to stress is an important determinant of stress tolerance (21). Therefore, the aim of the present study was to determine the effects of heat stress on mortality rates, radical generation, antioxidant enzyme responsiveness, and stress protein accumulation in old rats subjected to CR. Experiments were focused on the liver because it shows age-dependent evidence of increased cellular ROS production (22) and is a prime target of tissue injury during physiological challenges such as heat stress (23) and ischemia-reperfusion (13). We hypothesized that long-term intervention with CR would improve heat tolerance in older animals by reducing cellular oxidative stress in a critical splanchnic tissue such as the liver.

MATERIALS AND METHODS

Animals and diets

Experiments were performed in male Lobund-Wistar rats, 22–24 months old. Weanling rats were obtained from the Lobund Laboratories (Notre Dame University, South Bend, Ind.). After receipt in Madison, the rats were housed singly in the AALAC-approved Shared Aging Rodent Facility at the Madison VA Geriatric Research, Education and Clinical Center and were provided a nonpurified diet (PLI 5001, Purina Labs, St. Louis, Mo.) and acidified water, both *ad libitum*. The temperature-controlled facility provided a 12:12 light–dark cycle. Animals were fed the nonpurified diet until 8 months of age, then randomly assigned to either a control-fed (CON) or CR group and maintained on separate diets throughout the project.

Semi-purified diets were obtained from Teklad (Madison, Wis.). Compared with the diet fed to CON rats, the diet fed the CR rats was enriched in content of protein (casein), vitamins, and minerals in order to provide adequate amounts of these nutrients. The compositions of these diets were previously reported (24). The powdered diets were suspended (1:1) in a 1% agar solution, mixed thoroughly, and allowed to gel. Initial feedings were 65 kcal/day to CON rats and 51.4 kcal/day to CR rats (24). Over the next 1.5 months, the diets were gradually reduced to 45.5 kcal/day (CON rats) and to 30.4 kcal/day (CR rats) such that the CR rats ate 33% fewer calories than the CON rats. Thereafter, the diets were fed on Tuesdays and Thursdays; each feeding provided 91.0 kcal/day (CON) or 60.8 kcal/day (CR). On Saturday, each rat was given either 136.0 kcal (CON) or 91.2 kcal (CR).

At the age of 22–24 months, rats were moved from Madison, Wis., to Iowa City, Iowa, and housed in The University of Iowa Animal Care facility for a minimum of 7 days before experimentation. Rats from each cohort, CON and CR, were fed as in Madison and randomly assigned to heat stress (n=8) or sham (n=5) groups. An additional cohort of normally-fed Lobund-Wistar rats (4 months old, n=5) was used as young, euthermic controls. Animals were handled daily and familiarized with a colonic temperature (T_{co}) probe before testing. All procedures and experiments were performed in accordance with institutional animal care guidelines.

Experimental procedures

Experiments were performed at midday between 1000 and 1400, 48 h after the last feeding. Rats were fitted with a thermistor temperature probe (Yellow Springs Instruments, Yellow Springs, Ohio) inserted 6–7 cm into the colon and then placed in plastic cages (45×25×20 cm), conscious and unrestrained. T_{co} was monitored continuously on a digital display and an analog output. After a control period of 30 min, in which baseline T_{co} was similar between the groups (37–38°C), CON and CR rats were heat stressed as described previously (6). An infrared lamp was placed ~40 cm above the animal and raised or lowered to achieve a constant rate of rise in T_{co}. Ambient temperature (T_a) ranged from 38 to 44°C inside the cage. The infrared lamp was switched off when T_{co} reached 41°C. T_{co} was then maintained at 41°C for 30 min by periodically resuming heating. At the end of this period heat exposure was terminated and the rat was placed in a clean cage (T_a of 25°C) for recovery. Rats subsequently underwent a second identical heat exposure and recovery protocol 24 h later. During the heating protocol, heating rates (~0.04°C/min) and time to reach 41°C (~80 min) were matched for each heating trial and between heating trials on day 1 vs. day 2 within each group. Weight-matched euthermic controls (shams) from the CR and CON groups were handled identically to their heat-stressed counterparts except that the sham rats were maintained at a T_a of 24–26°C during the experimental periods. The young normally fed euthermic control group underwent an equivalent 2-day sham protocol.

Forty-eight hours after the second heat exposure (i.e., day 4), mortality was recorded and surviving rats were administered an overdose of sodium pentobarbital (100 mg/kg i.p.) followed by transcardial perfusion with cold phosphate buff-

ered saline (PBS). Liver biopsies (lobi caudatus) were then collected and immediately placed in ice-cold PBS. Portions of each tissue were processed for quantitation of the 70 kDa heat shock protein (HSP70), assessment of radical species via low temperature electron paramagnetic resonance (EPR) spectroscopy, light microscopy characterization and grading of injury severity, and immunohistochemical quantitation of immunoreactive antioxidant enzyme expression and subcellular localization. The same process was followed on death of a rat that did not survive the 4-day experimental protocol. These rats were closely monitored throughout the protocol and tissue samples were obtained before death.

HSP70 protein immunoblots

Liver and intestinal samples were assayed for constitutive plus inducible HSP70 protein as described previously (3). Proteins were quantitated by the Bradford method, then serial dilutions of the proteins were separated by 1-dimensional gel electrophoresis, transferred to nitrocellulose, and probed with a monoclonal antibody specific to both the constitutive (HSP73) and inducible (HSP72) form of HSP70 (N27, Stressgen, Victoria, BC, Canada). Protein immunoblots were scanned with a flatbed scanner and analyzed with commercially available software (NIMH Image 1.35). To ensure uniformity, density determinations were only made among bands on the same immunoblot.

EPR spectroscopy

Biopsies from the liver were cleaned of blood, placed in a 2.5 mm length of Teflon™ tubing (3 mm i.d.), and stored in liquid nitrogen. To evaluate radical content, a sample was removed from the tubing and placed in a Dewar of liquid nitrogen in the spectrometer cavity. EPR spectra were recorded with a Bruker ESP 300 EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with an ER-036M gaussmeter, ER4111VT variable temperature unit, and EIP-625A microwave frequency counter as described previously (19). Signal averaging (multiple scans of the same sample) was used to improve the signal-to-noise ratio. Sample volume and geometry were kept constant to allow direct comparisons of relative radical concentration among samples. All spectra were collected at 77 K with data reported as the normalized average of 20 scans. EPR settings included a receiver gain of 5.00×10^5, a modulation frequency of 100 kHz, a modulation amplitude of 4.0 G, a microwave frequency of 9.43 GHz, a microwave power of 10 mW, and a scan rate of 6.2 G.5.

Immunohistochemistry and histology

Liver and intestine samples were prepared for immunohistochemical and histological evaluation as described previously (25). Triplicate slides were prepared for each stain. Normal rabbit serum or preimmune serum controls were run for each antibody tested. These controls were uniformly negative.

Histology sections were analyzed microscopically for evidence of injury. In addition, sections were incubated with antibodies specific for manganese-containing superoxide dismutase (MnSOD), copper-zinc SOD (CuZnSOD), and catalase to assess immunoreactive protein levels. The specificity of these antioxidant enzyme antibodies has been previously described (25). All sections were graded by the same reviewer, who was blinded to their origin. Liver injury was graded using scales modified from Chui et al. (26, 27). Immunoreactive protein levels were graded using an intensity of staining scale developed by Oberley et al. (28) ranging from 0 (negative) to 4+ (strongly positive).

Data analyses

Results were presented as means ± SE. Statistical comparisons between T_{co} values, heating rates, and liver HSP70 levels were determined with an analysis of variance for two factors (i.e., experimental intervention and treatment group) design. *Post hoc* comparisons were made with Duncan's multiple range test. The effect of treatment (CR vs. CON) on survival was tested statistically by performing multiple linear regression with a computer spreadsheet program (Microsoft Excel). Dummy variables were used as independent variables to encode the treatment effect (diet) and to account for inter-individual variability among rats (29). Differences were considered significant at the $P < 0.05$ level.

RESULTS

Survival

CR improved thermotolerance as evidenced by the survival of all eight of the heat-stressed CR rats, while only 50% (4 out of 8) of heat-stressed CON rats ($P<0.05$) survived both heat exposures. In the CON group, one rat died following the first stress, another rat died during the second heating, and two other rats died within the 48 h recovery period after the second heat exposure. As expected, all 10 sham rats (5 CON and 5 CR) and 5 young euthermic control rats survived the protocol. Because of the wide variability in the time of death post-heat stress in the CON rats that did not survive the protocol, we present only the adaptive responses of animals that survived the entire protocol.

HSP accumulation

Densitometric analyses of immunoblots from tissues probed for constitutive plus inducible HSP70 demonstrated that HSP70 accumulation in the liver of heat-stressed animals was markedly influenced by the CR intervention (**Fig. 1**). In the CON group, heat stress stimulated a 142% increase in liver HSP70 levels vs. sham controls (123±6 vs. 51±6 units, $P<0.001$). In contrast, there was no change in liver HSP70 accumulation in heat-stressed compared with sham CR rats (71±5 vs. 59±3 units). Comparing both heat-stressed groups, HSP70 levels were 74% higher in CON vs. CR rats ($P<0.005$). HSP70 levels were similar in the CON and CR sham groups.

EPR

Low temperature (77 K) EPR spectra collected from liver of rats from all groups showed evidence of multiple species, including both transition metal and organic radicals (**Fig. 2**). A major component of

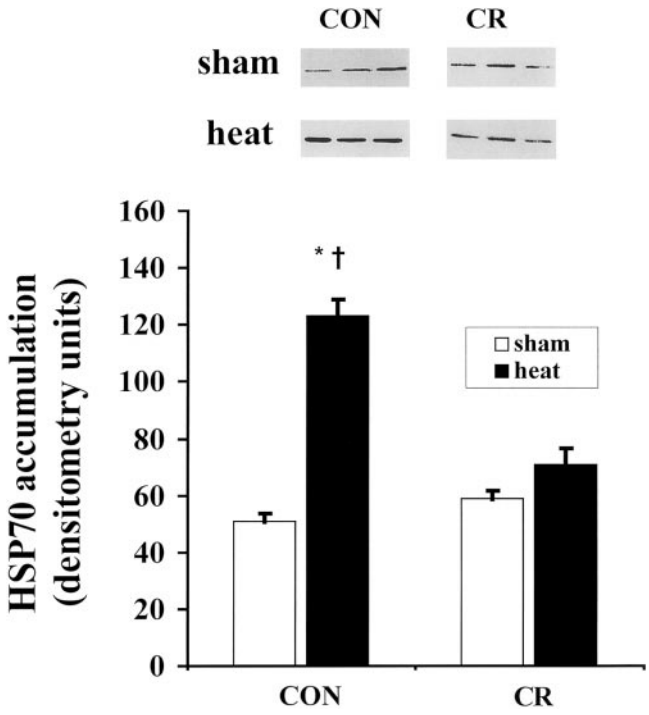

Figure 1. Effects of heat stress on liver HSP70 accumulation. Representative protein immunoblots (top) and HSP70 accumulation values (bottom) from liver sections of control-fed (CON) and calorically restricted (CR) old rats that underwent either a euthermic sham protocol or a heating protocol (see Methods). Tissues were probed with a monoclonal antibody specific for both the constitutive (HSP73) and inducible (HSP72) forms of HSP70. Heat stress produced a significant increase in HSP70 levels in the CON group. In contrast, there was no difference in HSP70 levels in sham and heat stressed CR rats. *$P < 0.05$ vs. CON sham rats; †$P < 0.05$ vs. CR heat-stressed rats.

these spectra is a strong six-line feature centered at $g = 2.03$ with a hyperfine splitting constant of ≈90 G that is consistent with immobilized manganese(II). For comparison, we show a protein-manganese(II) spectrum collected from *E.coli* MnSOD treated with sodium hydrodisulfite, a reducing agent (Fig. 2*F*). Note the similarities between spectrum F and spectra A–D; however, superimposed on the manganese(II) spectrum are additional features at $g = 2.25$, 2.005, and 1.935. The relatively narrow line at $g = 2.005$ (ΔHpp ≈10–15 G) is highly responsive to microwave power; the intensity of this line decreases relative to all other spectral lines with increasing microwave power (data not shown). This response, coupled with the g value and line width, is consistent with the $g = 2.005$ species being an organic radical, such as a semiquinone radical (30). The broad features at $g = 2.25$ and 1.935 are indicative of iron(III), possibly from mitochondrial succinate dehydrogenase (31).

Contrasting the EPR signals from liver (Fig. 2) of young (*A*), CR (*B*), and CON (*D*) groups under nonstress conditions showed remarkable similarity between the young and CR animals (*A* vs. *B*) but elevated radical levels, both metal and organic, in the old CON cohort (*B* vs. *D*). Heat stress marginally increased the concentration of semiquinone radical in liver from old CON rats and did not alter transition metal levels. In contrast, heat stress and recovery markedly reduced the level of both metal and organic radicals in liver from CR animals.

Histology and immunoreactive antioxidant enzyme expression in the liver: euthermic controls

Under euthermic conditions, hepatocellular antioxidant enzyme expression and injury profiles were similar for young and CR rats. In contrast, CON animals showed clear evidence of injury and increased antioxidant enzyme expression prior to heat stress. Hematoxylin and eosin stained liver sections from young sham and old CR sham rats showed normal morphology. Similar samples from old sham CON rats contained extensive evidence of diffuse hepatic fatty changes and mild cell injury that was expressed as pyknotic nuclei and cytoplasmic vacuolization (grade 1; data not shown).

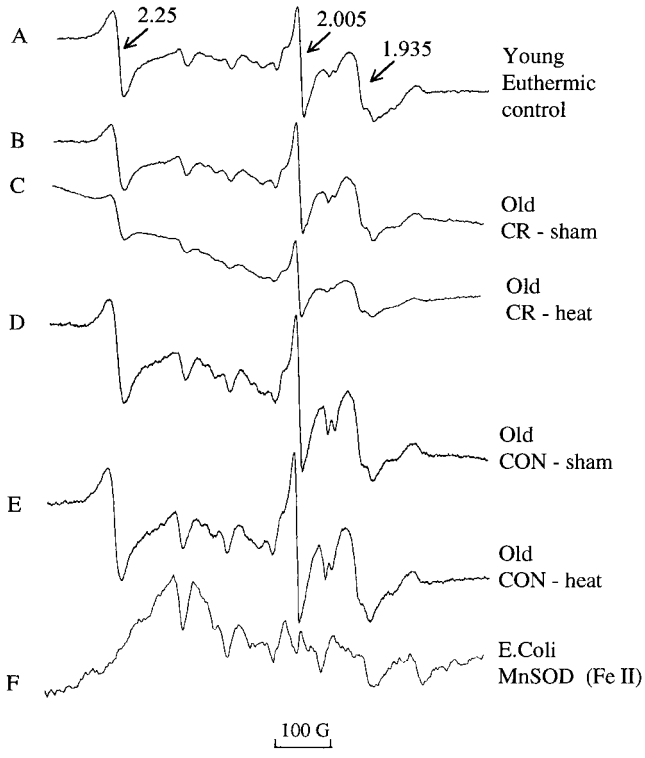

Figure 2. Caloric restriction lowers liver radical content both before and after heat stress. EPR spectra (77 K) of liver biopsies collected from (*A*) young euthermic control, (*B*) old CR sham-stressed control, (*C*) old CR heat-stressed, (*D*) old CON sham-stressed control, (*E*) old CON heat-stressed, and (*F*) *E. coli* MnSOD showing an immobilized manganese(II) EPR spectrum. The spectra show at least three species: Mn(II), a six-line feature centered at $g = 2.03$ hyperfine splitting ≈90 G; features consistent with Fe(III) at $g = 2.25$ and 1.935; and a likely semiquinone radical at $g = 2.005$, ΔHpp ≈10–15 G. Heat stress marginally elevated metal and semiquinone radical content in the CON group but lowered concentrations of these species in CR animals.

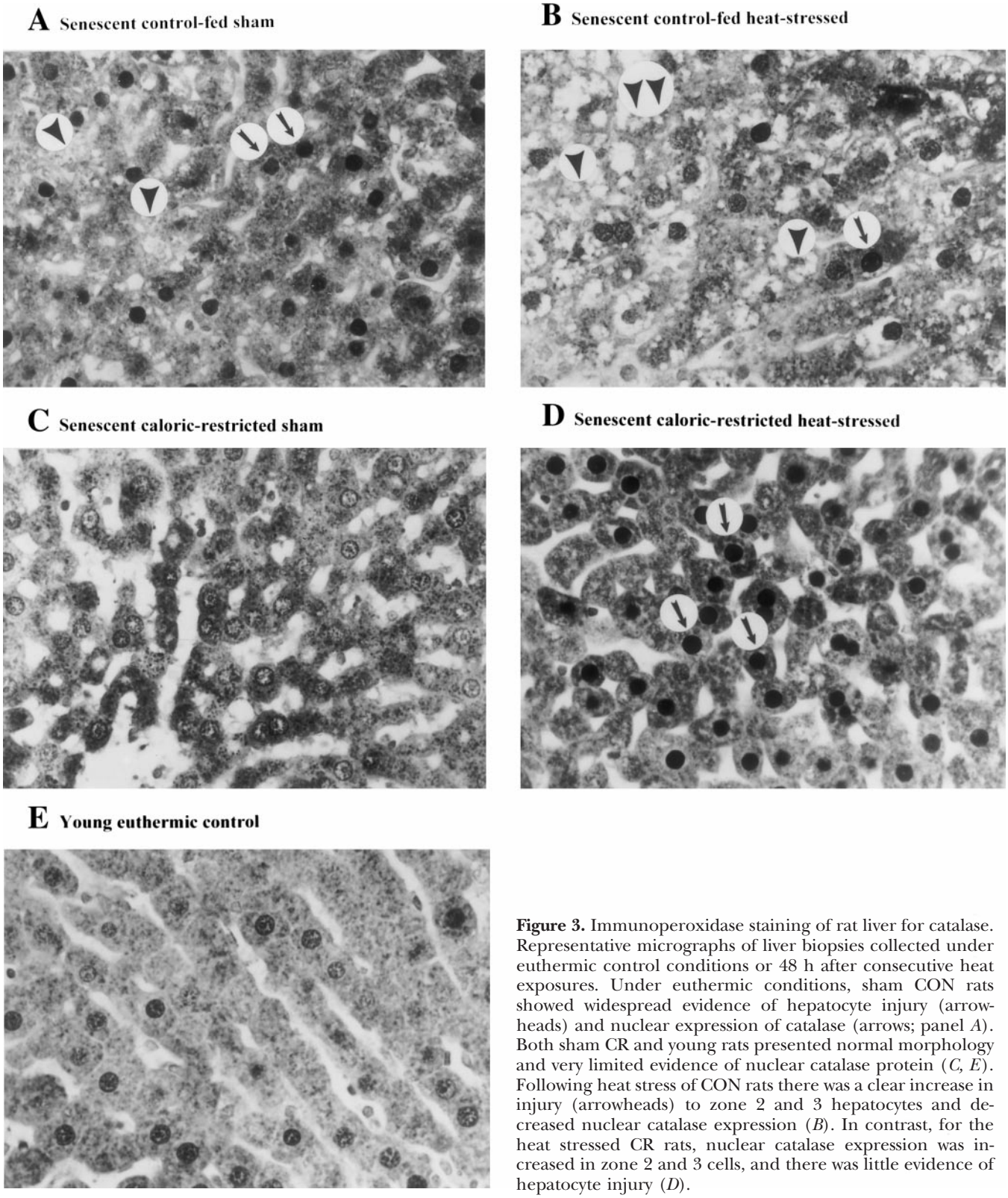

Figure 3. Immunoperoxidase staining of rat liver for catalase. Representative micrographs of liver biopsies collected under euthermic control conditions or 48 h after consecutive heat exposures. Under euthermic conditions, sham CON rats showed widespread evidence of hepatocyte injury (arrowheads) and nuclear expression of catalase (arrows; panel *A*). Both sham CR and young rats presented normal morphology and very limited evidence of nuclear catalase protein (*C, E*). Following heat stress of CON rats there was a clear increase in injury (arrowheads) to zone 2 and 3 hepatocytes and decreased nuclear catalase expression (*B*). In contrast, for the heat stressed CR rats, nuclear catalase expression was increased in zone 2 and 3 cells, and there was little evidence of hepatocyte injury (*D*).

Figure 3 depicts immunoperoxidase staining for catalase of liver sections from representative rats. Immunohistochemical determination of hepatocellular MnSOD, CuZnSOD, and catalase protein expression and subcellular localization in euthermic control animals (Fig. 3*E*) showed striking evidence of gradients of antioxidant enzyme expression within hepatic acini of rats from all groups. The intensities of staining for immunoreactive MnSOD, CuZnSOD, and catalase were high (3+) in central vein regions (zone 3 hepatocytes) and lower in centrilobular areas (1+/2+) in zone 1 and zone 2 cells (**Table 1**).

CuZnSOD and MnSOD levels and cellular distributions were similar in young control and CR rats,

TABLE 1. *Influences of age, diet, and heat stress on hepatocyte antioxidant enzyme protein expression*[a]

Protein	Cell type/ location	4-month-old normally-fed		Old CR sham		Old CON sham		Old CR heated		Old CON heated	
		C	N	C	N	C	N	C	N	C	N
Catalase	Hepatocyte										
	Zone 1	1+	0	1+	0	2+	2+	1+	1+	2+	1+
	Zone 2	2+	1+	2+	1+	2+	2+	3+	4+	2+	2+
	Zone 3	3+	1+	3+	1+	3+	3+	4+	3+	3+	3+
MnSOD	Hepatocyte										
	Zone 1	1+	0	1+	0	2+	0	1+	0	2+	0
	Zone 2	1+	0	1+	0	2+	0	2+	0	2+	0
	Zone 3	2+	0	2+	0	2+	0	3+	0	3+	0
CuZnSOD	Hepatocyte										
	Zone 1	1+	0	1+	0	2+	0	1+	0	2+	0
	Zone 2	1+	0	1+	0	2+	0	2+	0	3+	2+
	Zone 3	2+	0	2+	0	3+	2+	2+	0	4+	3+

[a] CR, calorically restricted old group; CON, control-fed old group; C, cytoplasmic staining; N, nuclear staining; % value indicates the percent of nuclei per 200 μM^2 area as defined by an ocular graticule. 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.

but MnSOD and CuZnSOD expression was increased in acinus zone 2 and zone 3 hepatocytes of CON rats (Table 1). In addition, moderate (2+/3+) nuclear expression of both CuZnSOD and catalase was observed in zone 2 and 3 hepatocytes in the CON group (Table 1, Fig. 3*A*), while nuclear CuZnSOD and catalase expression was not seen in the young group and only rarely observed in zone 3 hepatocytes from CR rats.

Histology and immunoreactive antioxidant enzyme expression in the liver: heat stress

Histological evaluation of sections from heat-stressed CR rats revealed normal to mild hepatocyte damage that was expressed as mild hepatocyte vacuolization (grade 1). In contrast, moderate to severe injury (grade 3) was observed in samples from heat-stressed CON rats that included widespread cytoplasmic hepatocyte vacuolization with pyknotic nuclei, evidence of membrane loss, and sinusoidal congestion (data not shown).

Both CR and CON rats showed evidence of hepatocellular MnSOD, CuZnSOD, and catalase induction in zone 2 and zone 3 hepatocytes during recovery from heat stress (Table 1). However, induction of protein was heterogenous in CON tissues and cells showed no consistent change from euthermic control levels. In contrast, uniform induction of antioxidant proteins combined with striking evidence of nuclear catalase expression was observed in zone 2 and zone 3 hepatocytes of the CR group (Fig. 3*D*; Table 1). Nuclear catalase staining intensity was 4+ in all 8 CR animals after heat stress, suggesting a consistently large increase in protein concentration. In contrast, there was no heating-induced increase in nuclear catalase protein above euthermic sham conditions for CON rats.

Cytoplasmic CuZnSOD levels were elevated in zone 3 hepatocytes and nuclear CuZnSOD expression was prominent in zone 2 and 3 cells of heat-stressed CON animals. Conversely, cytoplasmic levels of CuZnSOD were increased only in zone 3 cells, and nuclear expression of CuZnSOD was not observed in CR rats (Table 1).

DISCUSSION

The objective of this study was to determine whether long-term reductions in caloric intake would improve the ability of old animals to tolerate a natural physiological challenge such as environmental heat stress. We hypothesized that CR would enhance heat tolerance by reducing the magnitude of cellular oxidative stress and subsequent accrual of injury in hepatic tissues. The study yielded three primary findings in support of this hypothesis. First, there was an impressive reduction in heat stress-induced mortality in the CR group. All of the CR rats survived consecutive nonlethal heat exposures compared with only 50% of the CON cohort. Second, CR markedly decreased hepatic injury and stress protein accumulation. These responses were closely associated with induction of primary antioxidant enzymes and reduced hepatic radical generation. Finally, there were distinct contrasts in the pattern of adaptive hepatocellular antioxidant enzyme induction between the CR rats and their CON counterparts. For example, the magnitude of stress-related antioxidant enzyme induction was greater in the CR group. In addition, CR rats showed striking evidence of

nuclear translocation of catalase in central vein and centrilobular regions of the liver following heat stress. In contrast, CON rats demonstrated no increase in either the number of hepatocytes expressing nuclear catalase or the intensity of catalase expression in the heat stressed compared with the euthermic condition. Taken together, these data suggest that CR improves heat tolerance in old rats by preserving cellular ability to adapt to stress through induction of antioxidant enzyme gene expression and translocation of catalase to the nucleus in response to cellular radical production. The results also suggest that hepatocellular radical production is blunted and activation of low molecular weight transition metals is decreased during recovery from heat stress in CR rats, which would likely contribute to the reduction in cellular oxidative stress and injury observed in this group.

CR slows the rate of progression of senescence and alters the phenotypic changes of aging such that oxidative injury is reduced, suggesting that lowered cellular oxidative stress may cause the positive effects of CR (11, 15). Consistent with this concept, liver morphology and cellular antioxidant enzyme profiles of sham (i.e., euthermic) CR rats in the present study were remarkably similar to that of young animals. There was consistent evidence of uniform gradients of antioxidant enzyme protein expression within hepatic acini in both groups. In addition, we observed only limited evidence of nuclear catalase expression in the CR group, and neither young nor CR rats showed significant evidence of hepatocellular injury or stress under sham conditions. In contrast, liver biopsies from CON rats showed widespread centrilobular necrosis and hepatocellular injury. Moreover, gradients of antioxidant enzyme proteins were much less evident as centrilobular antioxidant enzyme expression was higher in CON than CR rats. We also observed widespread centrilobular nuclear CuZnSOD and catalase expression in hepatocytes adjacent to injured tissue. Taken together, these observations establish that long-term CR protects against age-related liver pathology and suggest that chronic cellular oxidative stress may be an important mediator of hepatocellular injury.

After heat stress, MnSOD and catalase were strongly induced in central vein and centrilobular regions of CR rats. Extremely high levels of nuclear catalase were also observed in a large majority of hepatocytes in these areas. Not surprisingly, liver radical content was lower in the CR group. In contrast, the magnitude of zone 2 and zone 3 cellular antioxidant enzyme induction was reduced in CON rats and there was no increase in nuclear catalase expression above sham levels. Radical levels were increased in liver samples of CON rats in recovery from heat stress. We interpret these data as evidence that CR blunted cellular stress and injury by preserving the capacity to respond to heat challenge with appropriate antioxidant enzyme expression. Although we cannot discount a CR-mediated decrease in ROS production, the fact that we observed such a strong induction of antioxidant enzymes suggests that levels of their respective ROS substrates are also increased.

We have previously reported that heat stress stimulates the generation of both ROS and reactive nitrogen species in intact animals (19). In the present study, EPR spectra from liver showed the presence of iron(III), manganese(II), and semiquinone. Heat stress marginally increased semiquinone radical levels in the CON group but reduced the concentration of EPR-detected transition metals and semiquinone radical in the CR rats. The Mn^{2+} oxidation state is the most prevalent for manganese in biological systems (32). The concentration of Mn^{2+} in rat hepatocytes ranges from 0.25 to 0.70 μM (33), and hepatic tissues have high levels of the manganese-containing metalloenzymes MnSOD, arginase, and serine/threonine protein phosphatase-1 (34). Thus, the strong Mn^{2+} EPR signal obtained in liver samples in the current study was not unexpected. However, the observation that heat stress reduced metal and organic radical production in CR animals is a provocative result worthy of further study. One explanation is that metal-catalyzed oxidative stress is important in the hepatic pathology that we observed after stress (12). Alternatively, the CR group may have responded to the stress of hyperthermia earlier in the post-heating period such that this adaptation was not measurable at the 48 h point.

Another interesting result from this project is the observation that heat stress produced strong nuclear catalase expression in the CR rats but stimulated no additional increase above euthermic levels in the CON animals. It has previously been reported that hyperthermia induces CuZnSOD activity *in vitro* and *in vivo* in rat lung (35). In addition, induction of MnSOD activity has been observed in *E. coli* B exposed to heat shock (36). However, our current results are the first demonstration in a eukaryotic system of increased total cellular catalase levels following a hyperthermic challenge as well as the first evidence in any system of stress-induced nuclear catalase translocation. These data are of interest in light of the proposal that the age-related increase in cellular oxidative stress and injury could be in part a result of declining antioxidant enzyme activities (10). Though some studies do not support this idea (37), the experimental approaches that have been used to date have not examined compartmental-

ized alterations in cellular antioxidant status with aging, nor have they investigated stress-inducible antioxidant enzyme expression. The present data show clear age-related and diet-dependent differences in hepatic antioxidant enzyme expression and subcellular compartmentalization. For example, in the CR group, catalase expression was prominent in a majority of hepatocyte nuclei, including both the centrilobular and periportal regions, 48 h after the second heat exposure. Concomitantly, liver damage in these same animals was negligible. In contrast, the CON group appeared unable to mount an appropriate adaptive antioxidant enzyme response after heat stress as evidenced by the unchanged nuclear catalase expression from euthermic sham conditions at the 48 h time point. Massive, widespread liver injury and significant mortality were also observed in CON rats. We postulate that this pattern of antioxidant enzyme expression and translocation plays a critical role in determining survival of senescent animals during a chronic physical challenge.

In support of our findings that the increases in cell stress produced with heating in the CON group were sharply reduced in CR animals, HSP70 accumulation in the liver also differed between these two groups. Data from several laboratories, including our own (3, 6), show that HSP70 levels increase in response to heating, suggesting that this protein may serve as an index of the magnitude of cellular stress. Consistent with past results, the current data support the concept that the hyperthermic challenge used in these experiments produced less cellular stress in rats that had undergone CR than their CON counterparts. Thus, mortality, ROS, injury, antioxidant enzyme, and stress protein outcomes provide strong evidence at the whole organism and cellular levels supporting the conclusion that CR attenuates the age-related decline in heat tolerance.

In summary, the present data show that long-term CR in rats protects the liver from the pathogenesis of normal age-related injury, as well as providing significant protection from the cytotoxic effects of heat stress. Increased thermotolerance of CR animals was associated with robust stress-inducible primary antioxidant enzyme expression and nuclear translocation of catalase, suggesting that the ability to synthesize antioxidant enzymes in response to stress in an important adaptive response to an environmental challenge such as heat stress. FJ

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