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Expression of Heat Shock Protein 70 Is Altered by Age and Diet at the Level of Transcription

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Because heat shock proteins have been shown to play a critical role in protecting cells from hyperthermia and other types of physiological stresses, it was of interest to determine what effect age and caloric restriction have on the ability of cells to regulate the expression of heat shock protein 70 (hsp70), the most prominent and most evolutionarily conserved of the heat shock proteins. Caloric restriction is the only experimental manipulation known to retard aging and increase survival of mammals. The ability of hepatocytes isolated from young/adult (4- to 7-month-old) and old (22- to 28-month-old) male Fischer F344 rats fed ad libitum or a caloric restriction diet (60% of the content of the ad libitum diet) to express hsp70 was determined after a mild heat shock (42.5°C for 30 min). We found that the induction of hsp70 synthesis and mRNA levels by heat shock was 40 to 50% lower in hepatocytes isolated from old rats than in hepatocytes isolated from young rats. Using in situ hybridization, we found that essentially all hepatocytes from the young/adult and old rats expressed hsp70 in response to heat shock; therefore, the age-related decrease in the induction of hsp70 expression was not due to an age-related accumulation of cells that do not respond to heat shock. Measurements of hsp70 mRNA stability and hsp70 transcription demonstrated that the age-related decline in hsp70 expression arose from a decline in hsp70 transcription. Interestingly, the age-related decline in the induction of hsp70 expression was reversed by caloric restriction; e.g., the induction of hsp70 synthesis, mRNA levels, and nuclear transcription were significantly higher in hepatocytes isolated from old rats fed the caloric restricted diet than in hepatocytes isolated from old rats fed ad libitum. The levels of the heat shock transcription factor in nuclear extracts isolated from heat-shocked hepatocytes were measured in a gel shift assay. Binding of the heat shock transcription factor to the heat shock element decreased with age and was significantly higher in hepatocyte extracts isolated from old rats fed the caloric restriction diet than in those from old rats fed ad libitum. Thus, our study demonstrates that the ability of hepatocytes to respond to hyperthermia and express hsp70 decreases significantly with age and that this decrease occurs at the transcriptional level. In addition, caloric restriction, which retards aging, reversed the age-related decline in the induction of hsp70 transcription in hepatocytes.

Organisms ranging from bacteria to humans and plants respond to hyperthermia by synthesizing a group of proteins known as heat shock proteins (hsps). Because the synthesis of hsps is induced not only by hyperthermia but also by a variety of other stresses (ethanol, amino acid analogs, heavy metals, free radicals, etc.), hsps also have been referred to as stress-induced proteins (26). The number of hsps varies from organism to organism and even between cell types in an organism (9). However, all organisms (e.g., archaebacteria and eubacteria as well as plants and animals) express hsps that have a molecular size of 65 to 78 kDa and belong to the HSP70 family (26). At the present time, it appears that at least four groups of genes make up the HSP70 family: hsp70, which is expressed in very low levels in nonstressed cells and is dramatically induced by hyperthermia; hsc70 (p72 or hsc73), which is expressed constitutively and only slightly induced by heat; grp78, which is induced by glucose starvation but not by hyperthermia; and grp75, which was found in mitochondria (38).

The most prominent member of the HSP70 family expressed after heat shock is hsp70, which also is the most conserved hsp throughout the evolution of prokaryotes and eukaryotes (26). hsp70 appears to play a critical role in protecting cells against the adverse effects of hyperthermia because several studies have shown that the thermosensitivity of cells is altered if the expression of hsp70 is enhanced or reduced. For example, Riabowol et al. (44) showed that the microinjection of a monoclonal antibody against hsp70 rendered fibroblasts thermosensitive, and Johnson and Kucey (20) reported that the competitive inhibition of hsp70 transcription had a similar effect. Furthermore, Angelidis et al. (1) and Li et al. (25) showed that the transfection of cells with expression vectors that constitutively expressed hsp70 made cells thermotolerant. The gene coding for hsp70 has been cloned from a variety of eukaryotes, and its regulation by hyperthermia has been studied extensively. hsp70 expression is regulated primarily at the level of transcription (22, 42, 52, 55), and this regulation is evolutionarily conserved; e.g., *Drosophila* hsp70 genes are expressed in a heat-regulated fashion in frog, mouse, and monkey cells (37, 42). Research over the past 5 years has shown that the induction of hsp70 expression in response to heat shock is mediated by the binding of a transcriptional activator, heat shock factor (HSF), to a highly conserved DNA sequence known as the heat shock element (HSE), which is found in the 5′-flanking sequence of all genes coding for hsps (17, 51, 54). An increase in temperature in mammalian cells results in the conversion of HSF from an inactive form that does not bind DNA to an active form that binds the HSE (22, 39, 52, 55).

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The binding of HSF to the HSE on the promoter of the hsp70 gene is followed by the phosphorylation of the HSF to create a complex with high transcriptional activity (24, 51).

The regulation of hsp70 expression is an excellent example of a cellular mechanism that has evolved to protect all living organisms from hyperthermia and other types of stress. Therefore, changes in this system could seriously compromise the capacity of an organism to respond to changes in its environment. Changes in hsp70 expression might be important in aging because the most characteristic feature of senescent organisms is their reduced ability to respond to stimuli or stress and to maintain homeostasis (50). Therefore, we have compared the ability of hepatocytes isolated from young and old rats to express hsp70 in response to increased temperature. We found that the induction of hsp70 expression is significantly reduced in cells from old rats and that the reduced expression is due to a deficit in the transcription of the hsp70 gene. In addition, we have shown that caloric restriction (CR), which is the only experimental manipulation known to retard aging in mammals, reverses the effect of aging on hsp70 transcription. Thus, it appears that changes in the induction of hsp70 transcription are closely associated with the aging process in rats.

MATERIALS AND METHODS

Animals and isolation of hepatocytes. Male specific-pathogen-free Fischer 344 rats used in this study were obtained at 3 weeks of age from Harlan/Sprague Dawley, Inc. (Indianapolis, Ind.). These rats were maintained on two dietary regimens: one group was fed ad libitum, and the other group was placed on a CR diet; the latter group received 60% of the diet consumed by the rats fed ad libitum as described previously in detail by Armbrecht et al. (2). CR was initiated when rats were 6 weeks old. The median and maximum survival of the CR rats was approximately 30% greater than that of the rats fed ad libitum (2). Hepatocytes were obtained by the in situ collagenase perfusion of liver as described by Engelmann et al. (11), with modifications (4). The number of hepatocytes was determined by counting with a hemacytometer. A hepatocyte preparation with high (95 to 98%) viability was obtained by using Percoll (Pharmacia) gradient centrifugation as described by Kreamer et al. (23). Hepatocytes (3 million cells per ml) were suspended in Eagle's minimal essential medium (Sigma Chemical Co.) supplemented with 1% bovine serum albumin (BSA) and incubated with shaking under an atmosphere of oxygen-carbon dioxide (95:5) at either 37 or 42.5°C for 30 min (heat shock).

2-D PAGE. The synthesis of hsp70 was measured as the amount of radioactivity incorporated into hsp70 by using two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE). Hepatocytes were incubated for 2 h at 37°C after the initial heat shock in Eagle's minimal essential medium containing L-[^{35}S]methionine (20 μCi/ml; 1,000 Ci/mmol). Hepatocyte viability was determined every 30 min during the course of the incubation. The viability of the cells was consistently greater than 85% after 2 h of incubation, and the viability was similar for hepatocytes isolated from young and old rats fed ad libitum or the CR diet. The reaction was terminated by placing the cells on ice and collecting the cells by low-speed centrifugation. In all cases, only samples that were labeled with radioactivity under identical conditions were compared. Experiments were conducted in triplicate for each animal. The hepatocyte samples were prepared according to the method of Garrels (16). 2-D PAGE was performed as described by Butler et al. (4), and the gels containing ^{35}S-labeled proteins were analyzed by fluorography (16). As a practical consideration, all gels of a group to be compared with each other were loaded with samples prepared from the same number of cells, and each group of fluorographs was exposed for the same length of time to Kodak XAR film at −70°C with an intensifying screen. The spots representing hsp70 on the autoradiographs were quantified with a Bio-Image densitometer, and the data were expressed as units of optical density × square millimeter (integrated intensity).

Northern (RNA) blot analysis. Total cellular RNA was isolated from hepatocytes by using guanidinium thiocyanate as described by Chirgwin et al. (7). For Northern blot analysis, the total cellular RNA (20 μg) was fractionated on a 1.2% agarose–formaldehyde gel and was transferred to a nylon membrane, using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described previously (15). The relative levels of hsp70 mRNA were measured by cRNA-RNA hybridization, using a [^{32}P]cRNA probe to hsp70 as described by Melton et al. (35). The entire hsp70 cDNA insert (1.6 kbp) isolated from plasmid pM3 (14) was inserted into the *Pst*I site of pGEM3 (Promega) and was used as template in the synthesis of the hsp70 cRNA probe. The levels of hsp70 were determined from autoradiograms of Northern blots by using a Bio-Image densitometer. RNA internal calibration system was used to quantify mRNA levels. A known amount of a small fragment (1.2 kb) of the hsp70 sense riboprobe was added to each RNA sample. The intensity of the [^{32}P]cRNA probe that hybridize to the hsp70 sense riboprobe served as an internal standard and allowed us to correct for variations in transfer, hybridization, and autoradiography. The data from various samples were normalized on the basis of the amount of total RNA (micrograms) analyzed in each assay. In addition, we measured the levels of albumin mRNA in the RNA samples by using a cDNA probe to albumin (48).

Nuclear transcription assay. The nuclear transcription of hsp70 was measured by nuclear runoff assay as described by Celano et al. (6). Nuclei from whole liver were isolated as described by Gorski et al. (18). Nuclei were isolated from hepatocytes by the following procedure, with all manipulation being performed at 4°C. Hepatocytes (20×10^6) were suspended in 5 ml of homogenization buffer (15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.6], 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 1% Nonidet P-40) and were homogenized in an all-glass Dounce homogenizer until more than 95% of the cells were disrupted (10 to 15 strokes). The homogenate was centrifuged at 650 × *g* for 10 min, and the resulting pellet, which contain nuclei, was suspended in 5 ml of homogenization buffer without Nonidet P-40. The suspension of nuclei was layered over 5 ml of the homogenization buffer with 2 M sucrose and centrifuged at 35,000 rpm for 60 min in an SW41 rotor (Beckman). The nuclei were suspended in 100 μl of storage buffer (60% [vol/vol] glycerol, 20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 160 mM KCl, 0.2 mM EDTA, 2 mM dithiothreitol [DTT]), frozen in liquid nitrogen, and stored at −80°C until used for transcription as described by Richardson et al. (46). The isolated nuclei were suspended in 200 μl of transcription buffer (30% [vol/vol] glycerol, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA, 1 mM DTT, 4 mM ATP, 4 mM GTP, 4 mM CTP, 100 μCi of [α-^{32}P]UTP [3,000 Ci/nmol]) and were incubated at 26°C. Transcription was allowed to occur for 15 min. Nuclear RNA then was isolated from the reaction mixture, and the amount of radioactivity

incorporated into hsp70 RNA was determined by hybridizing the nuclear RNA to the hsp70 gene immobilized on the nylon membrane as described by McKnight and Palmiter (34), using plasmid pGEM3 as a control. The autoradiograms obtained were analyzed with a Bio-Image densitometer, and the amount of radioactive nuclear RNA that hybridized to the probes was expressed as the integrated intensity of each spot per 20 × 10^6 nuclei. In the latter studies, the amount of radioactive nuclear RNA was measured with a Molecular Dynamics PhosphorImager 400B, and the data were expressed as machine counts per 20 × 10^6 nuclei.

In situ hybridization. The procedure for in situ hybridization was similar to that described by Lum (30). Hepatocytes, which were suspended in phosphate-buffered saline (PBS; pH 7.4), were deposited on poly-L-lysine (0.1% [wt/vol] in deionized water)-coated glass slides (approximately 20,000 cells per slide) with a cytocentrifuge (Shandon-Elliot) by centrifuging the cells at 1,200 rpm for 7 min. The cells then were fixed in freshly prepared paraformaldehyde solution (4% [wt/vol] PBS [pH 7.4]) for 3 to 5 min and were stored in a solution of 70% ethanol at 4°C. The slides were rinsed twice in 2× SSC and were incubated for 15 to 30 min at 37°C in a proteinase K solution (1 μg of proteinase K per ml in 2 mM $CaCl_2$–20 mM Tris-HCl [pH 7.4]). The slides were washed twice with RNase-free water and then neutralized by incubation at room temperature for 10 min in an acetic anhydride solution (0.25% [vol/vol] acetic anhydride in 0.1 M triethanolamine [pH 8.0]). The slides were rinsed in 2× SSC and immersed in 100 mM Tris-HCl–100 mM glycine (pH 7.0) for 30 min prior to hybridization.

The cell preparation was hybridized to a [^{35}S]cRNA probe to hsp70 at 50°C for 12 h in a solution of 14 μl of formamide containing 20% (wt/vol) dextran sulfate, 2 μl of sheared salmon sperm DNA (10 mg/ml), 1 μl of radioactive labeled cRNA (1 × 10^6 to 10 × 10^6 cpm/μl), 2 μl of BSA (20 mg/ml), and 1 μl of 200 mM DTT. After hybridization, the slides were rinsed with 2× SSC and washed three times with 50% (vol/vol) formamide in 2× SSC at 60°C for 30 min with gentle agitation. The slides then were incubated in an RNase T_1 solution (10 μg/ml in 500 mM NaCl–10 mM Tris-HCl [pH 8.0]–10 mM DTT) at 37°C for 30 min, rinsed twice in 2× SSC, and washed twice in 0.1× SSC at 60°C for 30 min with gentle agitation. The slides then were dehydrated successively in 60, 70, 80, and 90% (vol/vol) ethanol and air dried. The slides were coated with Ilford K.5D nuclear track liquid emulsion (Polysciences, Inc.), and the emulsion-coated slides were stored in a light-tight box in the presence of desiccant at 4°C for 7 to 14 days. The slides were developed and treated for 2 to 5 min with 10% (wt/vol) Wright's Giemsa stain solution to stain the cells on the slides. The background radioactivity that hybridized to the slides was determined by using an hsp70 sense RNA probe, and the number of grains per cell was quantified with a BioQuant image analyzer (R&M Biometrics, Inc.). Cells expressing hsp70 were scored as positive if the number of grains was at least three times the background level.

Gel shift assay. Nuclear extracts were prepared from hepatocytes by a procedure similar to that described by Dignam et al. (10). The cells were washed twice in cold PBS and centrifuged at 100 × *g* for 5 min. Cell pellets (10 × 10^6 cells) were suspended in buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF), and all manipulations were carried out on ice. Hepatocytes were disrupted by 10 to 15 strokes of a Dounce homogenizer. The homogenate was centrifuged at 650 × *g* for 15 min, and the resulting pellet, which contains the nuclei, was washed twice in buffer A. The nuclei were suspended in buffer B (20 mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF), and the resulting suspension was incubated on ice for 20 min with occasional gentle shacking. Nuclear extracts were obtained by centrifuging the lysate for 10 min at 100,000 × *g*. The protein concentration of the nuclear extract was determined by using the Bio-Rad DC protein assay kit with BSA as the standard, and aliquots (200 μl) of the cell extract were stored at −80°C.

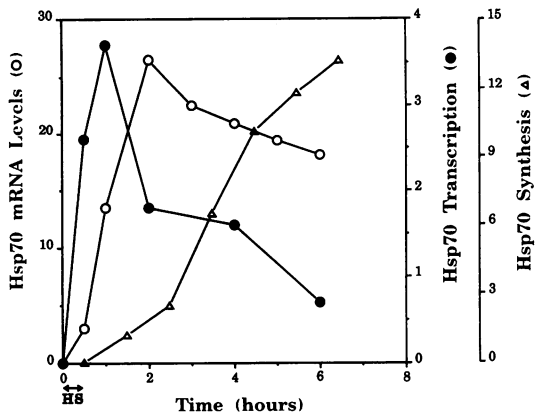

FIG. 1. Time course for the induction of hsp70 expression by heat shock (HS) in isolated hepatocytes. Hepatocytes isolated from young rats fed ad libitum were incubated at 42.5°C for 30 min and were returned to 37°C. The synthesis, mRNA levels, and nuclear transcription of hsp70 were measured as described in Materials and Methods.

The gel shift assay was performed with the nuclear extracts as described by Mosser et al. (39). Binding reactions were performed by incubating 50 pg of 5'-end-labeled, double-stranded HSE oligonucleotide (5'-CTAGAAGCT TCTAGAAGCTTCTAG-3') or SP-1 oligonucleotide (5'-AT CGGGGCGGGGATCGGGGCGGGGATCGGGGCGGG GATC-3') with 2 to 5 μg of the nuclear extracts for 20 min at 25°C in a total volume of 20 μl that contained 10 mM Tris (pH 7.9), 1 mM EDTA, 5% (vol/vol) glycerol, 100 mM NaCl, 1 mM DTT, 20 μg of BSA, and 2 μg of poly(dI-dC) · poly(dI-dC). For competition experiments, probe and competitor (5- to 100-fold molar excess of nonradioactive HSE or SP-1 oligonucleotide) were mixed together before addition of the binding solution (39). The samples were loaded onto a nondenaturing 4% polyacrylamide gel buffered with 0.25× TBE (22.5 mM Tris-borate and 0.5 mM EDTA) and electrophoresed at 130 V for 2.5 h at room temperature. Gels were dried, and the radioactivity in shifted bands was measured with a PhosphorImager. The data were expressed as machine counts per microgram of protein applied to the gel.

RESULTS

The time course for the induction of hsp70 synthesis, mRNA levels, and nuclear transcription by heat shock in suspensions of isolated hepatocytes is shown in Fig. 1. Neither the synthesis, mRNA levels, nor transcription of hsp70 was detectable in either freshly isolated hepatocytes or hepatocytes incubated at 37°C (data not shown). Therefore, hsp70 is not induced by the physical process of isolating hepatocytes. However, Fig. 1 shows that a mild heat shock of 42.5°C for 30 min, which has no significant effect on the viability of the hepatocytes (data not shown),

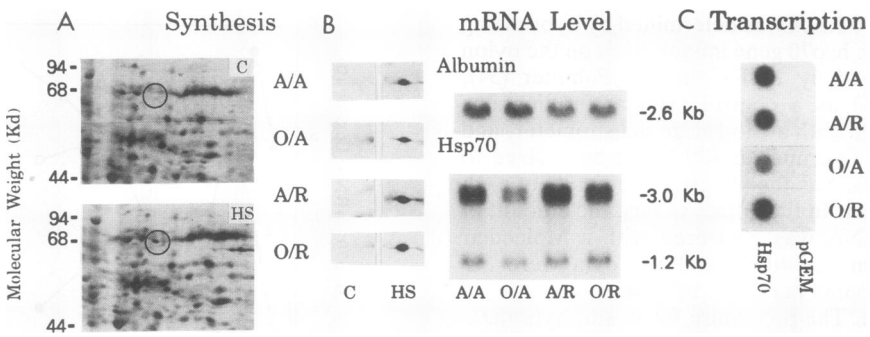

FIG. 2. Effects of age and CR on the induction of hsp70 expression by heat shock. Hepatocytes were isolated from young/adult (4- to 6-month-old) rats fed ad libitum (A/A) or a CR diet (A/R) and old (26- to 28-month-old) rats fed ad libitum (O/A) or a CR diet (O/R). The data shown were obtained from hepatocytes pooled from three animals for each age group. Hepatocytes were heat shocked for 30 min at 42.5°C, and hsp70 expression was measured at times described in the footnote to Table 1. (A) Portions of fluorographs of 2-D gels with a molecular size range of 40 to 100 kDa and pI range of 5.5 to 7.4. The circle indicates the position of hsp70, which has an apparent molecular size of 68 kDa and a pI of 6.3. The portions of the 2-D gels where hsp70 migrates are shown for hepatocytes before (C) or after (HS) heat shock for different age groups. (B) Autoradiographs of Northern blots hybridized to either the riboprobe for hsp70 or the cDNA probe for albumin to total RNA isolated from heat-shocked hepatocytes. (C) Autoradiograph of the hybridization of radiolabeled transcripts isolated from the nuclei of heat-shocked hepatocytes to a plasmid containing the hsp70 gene or plasmid pGEM as a control.

dramatically induces the expression of hsp70. Nuclear transcription of hsp70 was maximum 30 min after the 30-min heat shock, and the hsp70 mRNA levels were maximum 90 to 120 min after the heat shock. The kinetics of the induction of hsp70 expression were similar for hepatocytes isolated from young and old rats (data not shown).

Levels of induction of hsp70 expression by heat shock in hepatocytes isolated from young/adult and old rats are compared in Fig. 2 and Table 1. Figure 2 shows that the synthesis of hsp70 was undetectable in hepatocytes isolated from either young/adult or old rats. Neither hsp70 mRNA transcripts nor nuclear transcription of hsp70 was detectable in hepatocytes isolated from young/adult and old rats (data not shown). hsp70 synthesis was induced in hepatocytes isolated from both young/adult and old rats; however, the data in Table 1 show that the synthesis of hsp70 by hepatocytes isolated from old rats was approximately one-half that observed for hepatocytes isolated from young/adult rats. Table 1 also shows that the level of hsp70 mRNA induced by heat shock was significantly lower for hepatocytes isolated from old rats. As a control, the levels of albumin mRNA in the RNA preparations isolated from the hepatocytes of young/adult and old rats were measured, and Fig. 2 shows that the levels of albumin mRNA were similar for the two groups. The data in Fig. 3 show the stability of hsp70 mRNA in hepatocytes isolated from young/adult and old rats after the heat shock. The half-life of hsp70 mRNA was approximately 1 h for hepatocytes isolated from young/adult rats. This half-life is similar to the half-life of 30 to 60 min reported for *Drosophila* hsp70 mRNA (43, 49). The data in Fig. 3 show that the half-life of hsp70 mRNA was approximately twofold greater for hepatocytes isolated from old rats. Therefore, the age-related decrease in the induction of hsp70 mRNA was not due to an increased degradation of the hsp70 mRNA in hepatocytes isolated from old rats. Table 1 also shows that the nuclear transcription of hsp70 by hepatocytes isolated from old rats was significantly lower than hsp70 transcription by hepatocytes isolated from young/adult rats. In summary, the data presented in Fig. 2 and 3 and Table 1 illustrate that the induction of hsp70 expression by heat shock decreases with age in hepatocytes and that this decrease appears to be due primarily to a decrease in the transcription of the hsp70 gene.

The age-related decrease in the induction of hsp70 expression in hepatocytes could arise at the cellular level through at least two mechanisms: a decrease in the proportion of the hepatocytes that are able to respond to hyperthermia and express hsp70, or a decrease in the level of hsp70 expression by individual hepatocytes. To determine whether there is an accumulation of hepatocytes with age that are unable to mount a heat shock response and express hsp70, we used in situ hybridization to measure the ability of individual hepatocytes to express hsp70 in response to heat shock. Figure 4 shows that by using in situ hybridization, one can easily distinguish hepatocytes that respond to hyperthermia and express hsp70 (hsp70 positive) from control hepatocytes that do not express hsp70. When we compared the percentage of hsp70-positive cells in hepatocyte populations isolated from

TABLE 1. Effects of age and CR on the induction of hsp70 expression[a]

Age (mo)	Protein synthesis		mRNA levels		Nuclear transcription	
	Ad libitum	Restricted	Ad libitum	Restricted	Ad libitum	Restricted
4–6	2.15 ± 0.38[b]	3.05 ± 0.27[c]	37.6 ± 3.8[b]	47.6 ± 5.5[c]	6.2 ± 0.7[b]	6.8 ± 0.2[b]
26–28	1.17 ± 0.33[d]	2.38 ± 0.32[b,c]	22.7 ± 4.1[d]	35.3 ± 2.2[b]	4.3 ± 0.6[c]	6.4 ± 1.0[b]

[a] Suspensions of isolated hepatocytes were heat shocked at 42.5°C for 30 min, and hsp70 expression was measured as described in Materials and Methods. Synthesis of hsp70 was measured as the incorporation of [^{35}S]methionine into hsp70 during the 2-h incubation at 37°C following the heat shock and is expressed as the integrated intensity per 3 million cells. Levels of hsp70 mRNA were measured in RNA isolated from hepatocytes 90 min after heat shock and are expressed as the integrated intensity per 20 μg of total RNA. Transcription of hsp70 was measured in nuclei isolated from hepatocytes 30 min after the heat shock and is expressed as the integrated intensity per 20 million nuclei. The data are the means ± standard errors of the means for four to six animals for each group. Values with different superscript letters are significantly different from each other at the $P \leq 0.01$ level as determined by Fisher's least-significant-difference test.

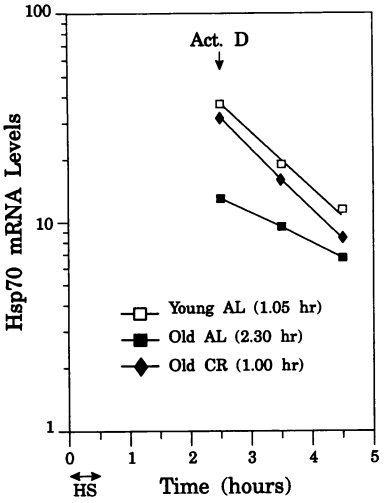

FIG. 3. Effects of age and CR on the stability of hsp70 mRNA. Hepatocytes isolated from young (4-month-old) rats fed ad libitum and old (26-month-old) rats fed ad libitum (AL) or a CR diet (CR) were pooled from three animals for each age group and heat shocked for 30 min at 42.5°C. The cells then were incubated at 37°C, and actinomycin D (0.5 μg/ml) was added to the medium 120 min after the heat shock (HS). This concentration of actinomycin D has been shown to inhibit RNA synthesis by hepatocytes over 95% (5). Cells were collected at the time intervals indicated, and RNA was extracted from the cells. The relative levels of hsp70 mRNA in the total RNA were determined by Northern blot analysis. The half-life of the hsp70 mRNA for each group of animals was determined as described by Harrold et al. (19) and is given in parentheses.

6- and 26-month-old rats, we found that essentially all hepatocytes were hsp70 positive (see the legend to Fig. 4). Thus, the age-related decrease in the induction of hsp70 expression was not due to an accumulation of cells that do not respond to heat shock.

The effects of CR on the induction of hsp70 expression in hepatocytes isolated from young/adult and old rats are shown in Fig. 2 and Table 1. hsp70 synthesis by hepatocytes isolated from old rats fed the CR diet was approximately twofold higher than that observed for hepatocytes isolated from old rats fed ad libitum. The data in Table 1 also show that the induction of hsp70 mRNA levels was significantly higher for either young/adult or old rats fed the CR diet than for young/adult or old rats fed ad libitum. In contrast, CR did not increase the levels of albumin mRNA (Fig. 2). Interestingly, the half-life of hsp70 mRNA for hepatocytes isolated from old rats fed the CR diet was similar to the half-life of hsp70 mRNA of hepatocytes isolated from young/adult rats fed ad libitum and shorter than the half-life of hsp70 mRNA of hepatocytes isolated from old rats fed ad libitum (Fig. 3). The data in Table 1 also show that transcription of the hsp70 gene was significantly higher in nuclei isolated from old rats fed the CR diet than in nuclei from old rats fed ad libitum. Thus, CR, which increases life span and retards aging, increases the induction of hsp70 expression by increasing the transcription of the hsp70 gene.

We were surprised to find that CR also altered hsp70 expression even in the young/adult rats, i.e., at 4 to 6 months of age after only a few months of CR. The induction of hsp70 synthesis and mRNA levels by heat shock were significantly higher for hepatocytes isolated from young/adult rats fed the CR diet than for hepatocytes isolated from young/adult rats

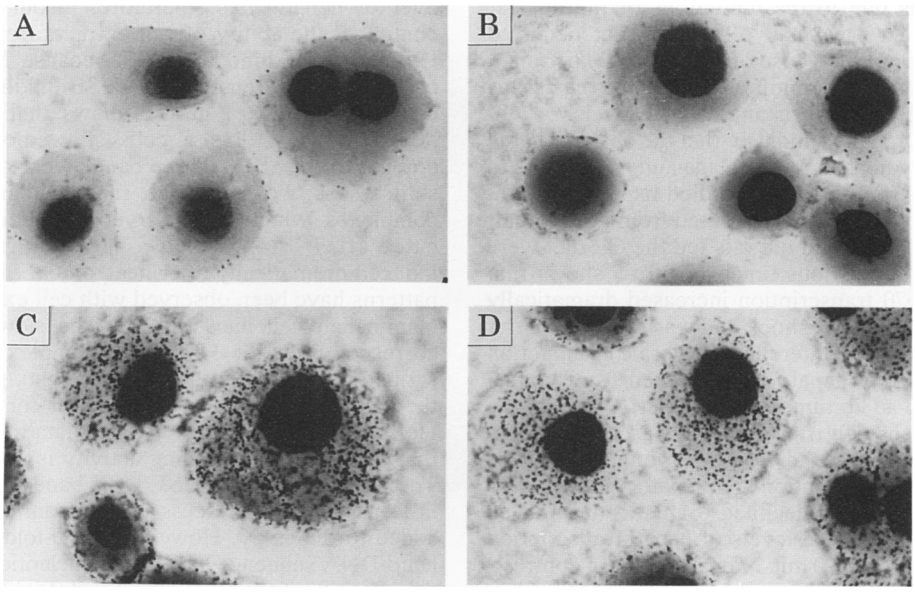

FIG. 4. In situ analysis of hsp70 expression in individual hepatocytes. Hepatocytes isolated from 6- and 26-month-old rats were incubated at 42.5°C (heat shock) or 37°C (control) for 30 min and then incubated for an additional 120 min at 37°C. The cells were fixed on microscope slides and hybridized to the hsp70 riboprobe, and the slides were exposed to emulsion and developed as described in Materials and Methods. The photomicrographs of young control (A), old control (B), young heat-shocked (C), and old heat-shocked (D) hepatocytes are shown. The expression of hsp70 by individual hepatocytes isolated from 6- and 26-month-old rats was measured by in situ hybridization, using 200 hepatocytes from each rat. Hepatocytes were identified as hsp70 positive (i.e., expressing hsp70) if the number of grains per cell was more than three times the background level. The background level, which ranged from 15 to 20 grains per cell, was the number of grains observed in control hepatocytes (incubated at 37°C for 60 min) or heat-shocked hepatocytes hybridized to a sense hsp70 riboprobe. The percentages of hepatocytes that were hsp70 positive were 99 ± 1 and 97 ± 4 (mean ± standard deviation) for hepatocytes isolated from four 6-month-old and four 26-month-old rats, respectively.

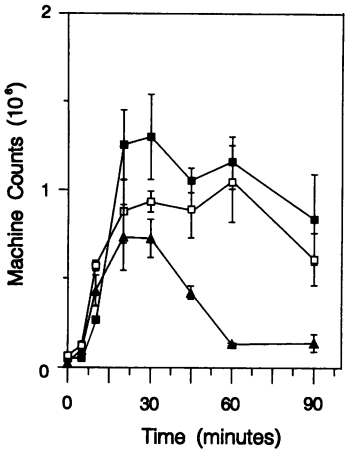

FIG. 5. Effects of age and CR on the induction of hsp70 transcription by heat shock. Hepatocytes isolated from young/adult rats (■) and old rats (▲) fed ad libitum and old rats fed the CR diet (□) were incubated at 42.5°C for various times. The nuclei were isolated from the hepatocytes, and hsp70 transcription was determined as described in Materials and Methods. The transcription of hsp70 was quantified with a PhosphorImager; the amount of radioactivity that bound to the hsp70 gene (machine counts) per 20 × 10^6 nuclei is given. Each point represents mean ± standard error of the mean for data obtained from three separate experiments in which hepatocytes were pooled from two rats for each experiment.

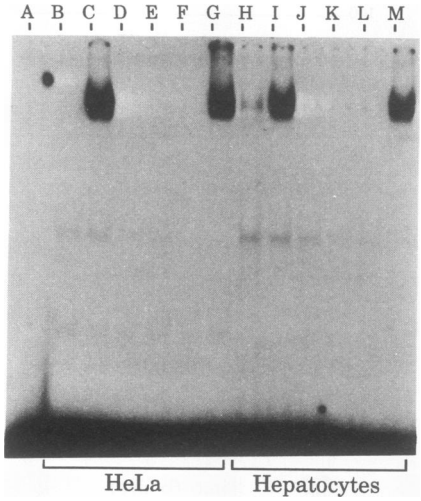

FIG. 6. HSF binding to the HSE in cell extracts from HeLa cells and hepatocytes. HSF binding to the HSE was determined in nuclear extracts from control (lanes B and H) and heat-shocked (lanes C to G and I to M) cells in a gel shift assay as described in Materials and Methods. The specificity of HSE binding was determined by incubating the cell extracts from heat-shocked cells in the presence of 5-fold (lanes D and J), 20-fold (lanes E and K), and 50-fold (lanes F and L) molar excesses of unlabeled HSE or in the presence of a 100-fold molar excess of unlabeled SP-1 oligonucleotide (lanes G and M). Lane A is a negative control and does not contain any cellular extract.

fed ad libitum even though the induction of hsp70 transcription was similar for hepatocytes isolated from the two groups of young/adult rats. We believe that the inability to detect a difference in hsp70 transcription between the two groups of young/adult rats was due to the limitations of the nuclear runoff assay to detect the small difference that occurred between these two groups because of the greater experimental variability in the nuclear runoff assay than in the assays used to measure hsp70 synthesis and mRNA levels.

Because the data in Table 1 indicate that the changes in the induction of hsp70 by heat shock with age and CR are due to changes in hsp70 transcription, we studied hsp70 transcription in greater detail. Hepatocytes isolated from young/adult and old rats fed ad libitum and old rats fed the CR diet were incubated at 42.5°C for various times. Figure 5 shows that the induction of hsp70 transcription increased dramatically during the first 20 min of heat shock and was maximum after 20 to 30 min. There was no difference in the rate of induction of hsp70 transcription in hepatocytes isolated from young/adult and old rats fed ad libitum or the CR diet; however, the maximum level of hsp70 transcription was significantly higher for hepatocytes isolated from the young/adult rats. Interestingly, hsp70 transcription by hepatocytes isolated from old rats fed ad libitum declined dramatically after 30 min of heat shock; only basal levels of hsp70 transcription were observed after 60 and 90 min of heat shock. In contrast, hsp70 transcription by hepatocytes isolated from the young/adult rats remained high and relatively constant when heat shocked for 20 to 90 min. Figure 5 also shows that the induction of hsp70 transcription in hepatocytes isolated from the old rats fed the CR diet was more similar to that in hepatocytes isolated from young/adult rats than to that in hepatocytes isolated from old rats fed ad libitum.

Because HSF plays a critical role in regulating the transcription of heat shock genes, especially the hsp70 gene, it was of interest to determine how aging and CR affected the activation of HSF. Using a gel shift assay, we measured the ability of cell extracts isolated from the nuclei of hepatocytes to bind the HSE. There are no previous studies on HSF of liver or hepatocytes; therefore, in our initial studies, we compared the ability of cell extracts from hepatocytes and HeLa cells to bind the HSE because Mosser et al. have characterized extensively the HSE-binding activity of HeLa cell extracts (39). Figure 6 shows that cell extracts from hepatocytes and HeLa cells showed similar patterns of binding to the HSE. Three bands were observed by the gel shift assay: two very faint, rapidly migrating HSE-protein complexes, which are observed in extracts from cells incubated at 37 or 42°C, and a slowly migrating band that is induced dramatically after heat shock. Similar HSE-binding patterns have been observed with cell extracts from various mammalian cell lines (8, 39). The slowly migrating band corresponds to the HSE-HSF complex that has been described by Mosser et al. (39). Interestingly, the mobility of the HSE-HSF complex is identical for cell extracts from HeLa cells and hepatocytes. The data in Figure 6 also show that the HSE-HSF binding activity is specific for the HSE. The slowly migrating HSE-HSF band disappeared when as little as a fivefold excess of the nonradioactive HSE was added to the assay. However, a 100-fold excess of an unrelated DNA sequence (SP-1 oligonucleotide) had no effect on the intensity of the HSE-HSF band (Fig. 6). In this assay, the amount of the HSE-HSF complex formed (i.e., the radioactivity present in shifted bands) is directly proportional to the amount of nuclear extract (i.e., HSF) added to the assay under the conditions described in Materials and Methods (data not shown). Therefore, the gel shift assay can be used to measure the relative amounts of HSE-binding activity in hepatocyte extracts after heat shock by measuring the radioactivity associated with the slowly migrating HSF-HSE band.

The effects of age and CR on HSF binding to the HSE are

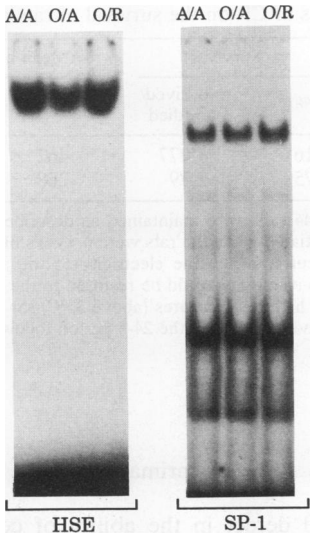

FIG. 7. Binding of cell extracts from rat hepatocytes to the HSE and SP-1 oligonucleotides. Hepatocytes isolated from young/adult (4- to 6-month-old) rats fed ad libitum (A/A) and old (26- to 28-month-old) rats fed ad libitum (O/A) or a CR diet (O/R) were heat shocked for 30 min at 42.5°C. Nuclear extracts were obtained from the hepatocytes and were analyzed (2 μg of protein per assay) by the gel shift assay, using the radiolabeled HSE or SP-1 oligonucleotide. The autoradiographs shown were obtained from cell extracts pooled from six rats for each age group.

shown in Fig. 7 and Table 2. Cell extracts isolated from the nuclei of heat-shocked hepatocytes from young/adult and old rats fed ad libitum and the CR diet showed similar patterns of binding to the HSE in the gel shift assay (Fig. 7). However, it is clear from the data presented in Fig. 7 and Table 2 that the binding of HSF to the HSE was significantly lower in hepatocyte extracts obtained from old rats than in hepatocyte extracts isolated from young/adult rats fed ad libitum or old rats fed the CR diet. Thus, there appears to be an age-related decrease in HSE-binding activity in cell extracts from heat-shocked hepatocytes, and this decrease is reversed by CR. To ensure that the change in HSF binding to the HSE in the hepatocyte extracts was not an artifact of the preparation of the extract or the assay conditions, we measured the ability of the same hepatocyte extracts to bind another *cis*-acting element, the SP-1 oligonucleotide. Figure 7 shows that hepatocyte extracts gave at least three distinct bands with the SP-1 oligonucleotide. These bands appear to represent specific protein complexes with the SP-1 oligonucleotide because they disappeared when fivefold unlabeled SP-1 oligonucleotide was added to the assay (data not shown). The autoradiogram in Fig. 7 shows there is very little difference in the intensities of the three bands in hepatocyte extracts from the three groups of rats. When the actual amount of radioactive SP-1 oligonucleotide associated with the three bands was measured in hepatocyte extracts isolated from young/adult and old rats fed ad libitum and the CR diet, it was found that the cell extracts from the three groups of animals bound a similar amount of radioactive SP-1 oligonucleotide (Table 2).

TABLE 2. Effects of age and CR on HSF and SP-1-binding activity[a]

Oligonucleotide	Binding activity (mean ± SEM)		
	Young/adult, ad libitum	Old	
		Ad libitum	Restricted
HSF	10,583 ± 607	6,083 ± 926	11,333 ± 205
SP-1	1,116 ± 53	1,058 ± 48	1,113 ± 52

[a] Nuclear extracts from young/adult- (4- to 6-month-old) rats fed ad libitum and old (26- to 28-month-old) rats fed ad libitum or a CR diet (restricted) were analyzed by the gel shift assay, using the radiolabeled HSE or SP-1 oligonucleotide as shown in Fig. 6. The dried gel was scanned, and the relative amounts of radiolabeled HSE bound to HSF and radiolabeled SP-1 oligonucleotide bound to the three bands shown in Fig. 6 were quantified for the hepatocyte extracts by using a PhosphorImager. The data are expressed as the amount of radioactivity bound (machine counts) per microgram of nuclear extract. The data were obtained from three separate experiments in which cell extracts were pooled from two rats for each experiment. The amount of HSF binding to the HSE by cell extracts isolated from old rats fed ad libitum was significantly different from the amount of HSF binding to the HSE by cell extracts isolated from young/adult rats fed ad libitum or old rats fed the CR diet at the $P \leq 0.01$ level as determined by Fisher's multiple-comparison test.

DISCUSSION

In this study, we characterized in detail the effect of aging and CR on the induction of hsp70 expression by hepatocytes after a mild heat shock (42°C for 30 min). The induction of hsp70 synthesis and mRNA levels by heat shock were significantly lower in hepatocytes isolated from old rats than in hepatocytes isolated from young/adult rats. Previous studies from Holbrook's laboratory also showed that the induction of hsp70 expression by heat shock was reduced with increasing age in rats. For example, the induction of hsp70 synthesis and mRNA levels by hyperthermia was lower in primary cultures of fibroblasts isolated from either skin or lung of old male Wistar rats compared with young rats (12), and the induction of hsp70 mRNA in vivo decreased with age in lung and skin, as well as brain, when rats were exposed to elevated temperatures (3). Pardue et al. (41) also showed a significant age-related decrease in induction of hsp70 mRNA in the hippocampus of male Fischer rats when the rats were exposed to hyperthermia in vivo. Thus, it appears that cells from old animals show a significant decrease in the induction of hsp70 synthesis and mRNA levels by hyperthermia. Our in situ experiments show that the decrease in the induction of hsp70 expression with age was not due to an inability of cells from old rats to respond to hyperthermia (Fig. 4). This observation is important from an aging perspective because previous studies have suggested that age-related deficits in the response of cells to signals may be due to an accumulation of cells that cannot respond to the signal (13, 36). This phenomenon does not occur in the heat shock response in hepatocytes; essentially all hepatocytes isolated from either young/adult or old rats responded to heat shock and expressed hsp70 mRNA.

Our study extends the previous studies (3, 12, 41) by showing that the decrease in the induction of hsp70 expression occurred at the level of transcription. The nuclear transcription of hsp70 was significantly lower for hepatocytes isolated from old rats than for hepatocytes isolated from young/adult rats (Fig. 5 and Table 1). Because HSF plays a central role in the regulation of hsp70 expression through its binding to the HSE, we conducted a series of preliminary experiments in which the levels of HSF that bound to the HSE were measured in nuclear extracts isolated from hepatocytes by using a gel shift assay. We found that the induction of HSF binding to the HSE by heat shock

was significantly lower in hepatocyte extracts isolated from old rats than in hepatocyte extracts isolated from young/adult rats. Thus, the age-related decrease in hsp70 transcription appears to arise from a decrease in the induction of HSF binding to the HSE by heat shock. The age-related change in HSF binding to the HSE does not appear to be due to a general age-related change in other transcription factors because the level of the transcription factor Sp1 in the hepatocyte extracts, as measured by the gel shift assay, did not change with age. This observation is in agreement with the report by Spindler et al. (53), in which it was reported that the levels of Sp1 mRNA did not change significantly with age in the liver of female $C3B10RF_1$ mice.

A decline in the induction of hsp70 has also been reported for cultured cells when they reach the end of their replicative life span and are unable to proliferate, a phenomenon termed cell senescence. Liu et al. (28) showed that the induction of hsp70 expression by heat shock or the amino acid analog canavanine was significantly lower in late-passage IMR-90 human diploid fibroblasts than in early-passage fibroblasts. They showed that the decrease in the induction of hsp70 expression was due to a decrease in the transcription of hsp70. Luce and Cristofalo (29) also showed that the induction of hsp70 mRNA levels by either hyperthermia or sodium arsenite was lower in late-passage WI-38 fibroblasts than in early-passage fibroblasts. More recently, Liu et al. (27) showed that the decrease in hsp70 transcription, which occurs during cell senescence, is correlated with HSF-binding activity. Cell extracts from late-passage fibroblasts that were heat shocked showed a decrease in the binding of HSF to HSE in a gel shift assay compared with cell extracts from early-passage fibroblasts. Thus, the change that we have observed in the induction of hsp70 expression with age in hepatocytes is similar to that reported for human fibroblasts as they senesce in culture and are unable to proliferate; i.e., the transcription of hsp70 and the binding of HSF to HSE are reduced. These observations are interesting because they suggest that similar changes in hsp70 expression might occur when cells age either in vivo or in vitro in culture.

A major observation of this study was that CR reversed the age-related decline in the induction of hsp70 expression by hyperthermia. CR is the only experimental manipulation that has been shown consistently to prolong the longevity of mammals such as rodents (31, 45). Because CR reduces the incidence of almost all age-related diseases and increases the maximum survival of rodents, it is generally accepted that CR increases the longevity of rodents by altering the aging process (32, 33). Thus, CR is a powerful tool for studying the biological mechanism underlying aging. We found that the induction of hsp70 synthesis, mRNA levels, and transcription by heat shock were significantly higher for hepatocytes isolated from old rats fed the CR diet than for hepatocytes isolated from old rats fed ad libitum. Our preliminary experiments indicate that hepatocyte extracts isolated from old rats fed the CR diet had a significantly higher binding of HSF to the HSE than extracts of hepatocytes isolated from rats fed ad libitum (Fig. 7 and Table 2). Interestingly, hepatocytes isolated from old rats fed the CR diet responded to heat shock as well as did hepatocytes isolated from the young/adult rats fed ad libitum; e.g., the induction of hsp70 expression, hsp70 mRNA stability, and the induction of HSF-binding activity were similar for hepatocytes isolated from old rats fed the CR diet and young/adult rats fed ad libitum. Thus, CR reversed the age-related deficit in the ability of the cells to respond to hyperthermia, and this reversal appears to occur primarily at the transcriptional level.

TABLE 3. Effects of CR on the survival of rats after heat stress[a]

Diet	Survival		Mean body wt (g) ± SEM	
	%	No. lived/ no. died	Survivors	Dead
Ad libitum	16	15/77	462 ± 3	452 ± 2
Restricted	75	27/9	268 ± 4	273 ± 3

[a] Male Fischer F344 rats were maintained as described in Materials and Methods. CR was initiated when the rats were 6 weeks old. At 20 months of age, an accident occurred when the electricity to the animal facility was disrupted. Before the electricity could be restored to the animal facility, the rats were exposed to high temperatures (above 33°C) for several hours. The number of rats that lived or died in the 24-h period following the accident is shown.

An age-related defect in the ability of cells to express hsp70 in response to hyperthermia could make senescent organisms more vulnerable to hyperthermia because it has been shown that cells become more thermosensitive when the expression of hsp70 is inhibited (20, 44). Recently, Luce and Cristofalo (29) reported that late-passage human diploid fibroblasts showed increased thermosensitivity to acute hyperthermic exposure compared with early-passage cells. Thus, the decline in hsp70 expression that occurs during cell senescence in vitro is associated with a decrease in the ability of the cells to withstand hyperthermic stress. Currently, there is no information on the thermosensitivity of cells or tissues of animals as they age in vivo. We would predict that cells and tissues of old organisms that show a reduced capacity to express hsp70 in response to hyperthermia are more thermosensitive than cells and tissues of young organisms. Several studies have shown a strong correlation between age and mortality from hyperthermia in human subjects (50); the number of deaths associated with high environmental temperatures has been reported to increase progressively with age; e.g., deaths increase 10-fold between 80 and 90 years of age (40). Our studies also suggest that cells and tissues of old rats fed the CR diet are more thermoresistant than cells and tissues of old rats fed ad libitum because the induction of hsp70 expression by heat shock is significantly higher for old rats fed a CR diet than for old rats fed ad libitum. Thus, old rats fed a CR diet would be predicted to be less vulnerable to hyperthermia than old rats fed ad libitum. This prediction is supported by the data that we obtained from an accident that occurred in our animal facility because of a power failure. Twenty-month-old rats fed ad libitum and a CR diet were exposed simultaneously to elevated temperatures for several hours. As shown in Table 3, only 16% of the rats fed ad libitum survived this heat shock. In contrast, 75% of the rats fed the CR diet survived this same period of hyperthermia. Thus, old rats fed the CR diet showed a much greater ability to survive hyperthermic conditions than did old rats fed ad libitum. It is inviting to suggest that the increased survival of the 20-month-old rats fed the CR diet might be due, at least partially, to the increased ability of cells from the rats fed the CR diet to express hsp70. However, changes in a variety of compensatory physiological processes, e.g., vasodilatation and alterations in heart rate and respiration, also could play a role in the ability of rats fed the CR diet to survive this hyperthermic stress.

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