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Decreased expression of heat shock protein 70 mRNA and protein after heat treatment in cells of aged rats

(stress/aging)

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ABSTRACT **The effect of aging on the induction of heat shock protein 70 (HSP70)-encoding gene expression by elevated temperatures was studied in cultures of lung- or skin-derived fibroblasts from young (5 mo) and old (24 mo) male Wistar rats. Although the kinetics of the heat shock response were found to be similar in the two age groups, we observed lower levels of induction of HSP70 mRNA and HSP70 protein in confluent primary lung and skin fibroblast cultures derived from aged animals. Additional experiments with freshly excised lung tissue showed a similar age-related decline in the heat-induced expression of HSP70.**

Physiological aging is characterized by decreased responses to stress. In many cases baseline levels of physiological parameters do not change with aging, but when the systems are stressed, an age-related decrement is observed (1). Molecular approaches now permit the examination of the effect of aging on the response to stress at the molecular level.

The exposure of cells to a variety of metabolic or environmental stresses results in the preferential synthesis of a group of highly conserved proteins referred to as the heat shock proteins (2, 3). Although their precise function(s) are not known, these proteins are generally presumed to increase the ability of cells to recover from the toxic effects of heat and/or other physiological stresses. Of particular relevance to aging is the suggestion that the heat shock proteins might bind to denatured or abnormal proteins produced by stress and aid in their elimination (4). Importantly, several studies have demonstrated an accumulation of abnormal protein as a function of age (5, 6). Because some heat shock proteins are also present in the absence of stress, they have further been hypothesized to play a role in the normal assembly and disassembly of proteins (7).

The most frequently studied heat shock proteins are a group of proteins of approximately 70 kDa comprising the HSP70 gene family. The genes for several members of this family have been cloned in several mammalian species, as well as in lower organisms, and the transcriptional control elements responsible for their stress-induced expression are well characterized (8–10). However, relatively little is known about the role of these proteins in cellular physiology. In this study, we examined the effect of aging on the induction of HSP70 mRNA as well as the corresponding protein(s) in primary cultured fibroblasts obtained from young (5 mo) and aged (24 mo) male Wistar rats. This *in vitro* system was chosen for these initial experiments to avoid the influence of complex physiological mechanisms associated with thermoregulation in intact animals. We report a decline in the heat-induced expression of HSP70 mRNA and protein in primary fibroblasts with aging. Furthermore, in additional experiments with fresh lung tissue from old and young rats, we found a similar age-related decline in HSP70 expression in response to heat stress.

MATERIALS AND METHODS

Isolation and Culture of Primary Rat Fibroblasts. Fibroblast cultures were derived from male Wistar rats obtained from the Gerontology Research Center animal colony at the National Institute on Aging. The lifespan of these animals is 27–28 mo, and the average life expectancy is 24 mo. After sacrifice by decapitation, pieces of ventral skin (5×5 cm^2) or lung tissue were removed and washed with phosphate-buffered saline. Tissues were minced and washed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The total lung tissue from a single rat was transferred to a 10-cm^2 tissue culture dish containing 10 ml of complete growth medium and collagenase at 200 units/ml and incubated at 37°C for 20–24 hr. Cells were then centrifuged, washed, resuspended in fresh medium, and inoculated into four T175 tissue culture flasks. Cells generally reached confluency 5 to 6 days after inoculation and were maintained an additional 5 days before experiments.

Heat Shock Conditions. Sealed flasks were completely submerged in a circulating water bath at 42.5°C for 90 min. Flasks were returned to 37°C for 2 hr before RNA isolation. These conditions were chosen for the fibroblasts after initial time-course studies demonstrated that these conditions resulted in maximum levels of HSP70 mRNA in both old and young cells without affecting cell viability.

For experiments with fresh lung tissue, lungs were removed and cut into small fragments ≈0.5 cm^3. The tissue was placed in 2 ml of fresh DMEM medium and incubated in a water bath at 42°C for 90 min. After the heat treatment the tissues were immediately processed for RNA extraction. Control samples were left at room temperature for 90 min before RNA extraction.

RNA Isolation and Blot Hybridization Analysis. RNA was isolated by lysis and disruption of cells or tissue in guanidine isothiocyanate and subsequent centrifugation through $CsCl_2$ cushions according to Chirgwin *et al.* (11). For Northern (RNA) analysis, RNA samples were electrophoresed on 2.2 M formaldehyde gels. The RNA was transferred onto GeneScreen*Plus* membranes (DuPont) according to the manufacturer's recommended conditions. The cDNA probe used throughout these studies was isolated from a Chinese hamster ovary cell library as described (12). The probe was labeled with ^{32}P-labeled deoxycytidine triphosphate ([^{32}P]dCTP) by using the random-primer method of Feinberg and Vogelstein (13). Blots were hybridized overnight at 42°C and then

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Abbreviation: HSP70, heat shock protein 70.
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washed in 0.1× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) as described (14).

Dot Blot Analysis and RNA Quantitation. RNA was blotted onto GeneScreen*Plus* membranes and hybridized and washed as described by Church and Gilbert (15). To confirm that the levels of mRNA were equivalent from sample to sample, RNA was blotted (15–100 ng based on OD at 260 nm) and hybridized with an ^{35}S-labeled poly(thymidylic acid) probe (14). The amount of poly(thymidylic acid) hybridization, a measure of the poly(adenylic acid) content, was then compared among samples. Variation among samples based on densitometric analysis was minor and generally varied <10% from spectrophotometric quantitation. Any samples showing greater deviations were corrected for poly(adenylic acid) content.

HSP70 Protein Analysis. For HSP70 protein labeling, lung fibroblast cultures were placed at 42.5°C for 90 min as described above, but recovery was allowed to proceed for 4 hr at 37°C. In the last hour of recovery, a ^{14}C-labeled L-amino acid mixture at 50 μCi/ml (1 Ci = 37 GBq) was added to the flasks. Control flasks were similarly incubated with the ^{14}C-labeled amino acids. To isolate labeled proteins, cells were lysed in water with three cycles of freeze/thawing. Pellets were discarded after a 30-min spin in an Eppendorf microcentrifuge placed at 4°C, and ^{14}C-labeled amino acid incorporation into proteins in the remaining supernatants was determined by trichloracetic acid (TCA) precipitation. Samples were electrophoresed on 12% acrylamide gels in the presence of 0.1% $NaDodSO_4$ (16) and fluorographed with EN^{3}HANCE (New England Nuclear), according to the manufacturer's instructions.

RESULTS

HSP70 Expression During Cell Growth. Because HSP70 expression is growth-phase and/or cell-cycle dependent (10, 17, 18), it was important to first determine the effect of the growth phase of aged and young fibroblasts on the level of HSP70 induction. Primary cultures were established at low density from lung tissue of young (5 mo) and old (24 mo) animals, and cell proliferation and induction of HSP70 mRNA by heat treatment were examined over the following 12-day period. The growth curves for primary cell cultures established from the young and old animals were virtually identical with similar proliferation rates and cell densities obtained at confluency (Fig. 1). During logarithmic growth, high levels of HSP70 mRNA were induced by heat in both young and old lung fibroblasts (Fig. 1 *Inset*, days 3–5). When these cells reached confluency and proliferation slowed, heat-induced HSP70 mRNA levels fell in both young and old cells (Fig. 1, *Inset*, day 6). However, the decrease in induced HSP70 mRNA levels was comparatively much greater in cultures derived from aged animals than in those from young animals. After an additional week at confluency, HSP70 mRNA levels produced by heat shock in the young cell cultures returned to the peak levels seen during logarithmic cell growth (Fig. 1, *Inset*, PC). In contrast, cells derived from aged animals displayed no detectable HSP70 mRNA induction by heat shock at this postconfluent time point (Fig. 1, *Inset*, PC). In an independent experiment conducted with skin fibroblasts, similar results were obtained (data not shown). Therefore, all further studies were performed under postconfluent conditions where the greatest difference between fibroblasts derived from young and old rats was seen.

HSP70 mRNA Expression in Young and Aged Fibroblast Cultures. To further examine the age-related difference in expression of HSP70 after heat shock, pooled cultures were established from skin and lung tissues from three to five individual young or old Wistar rats. A total of seven different batches of lung and three different batches of skin fibroblast cultures from aged and young animals were examined for heat-induced expression of HSP70 mRNA. Five of the seven lung fibroblast cultures and all three of the skin fibroblast cultures demonstrated lower levels of heat-induced HSP70 mRNA in the cultures derived from aged animals. A representative blot displaying four such experiments is shown in Fig. 2. No differences were seen in the level of HSP70 mRNA between young and aged cells in the absence of heat shock. In the control populations, a single mRNA species was present. While this band is only faintly present in the pictured

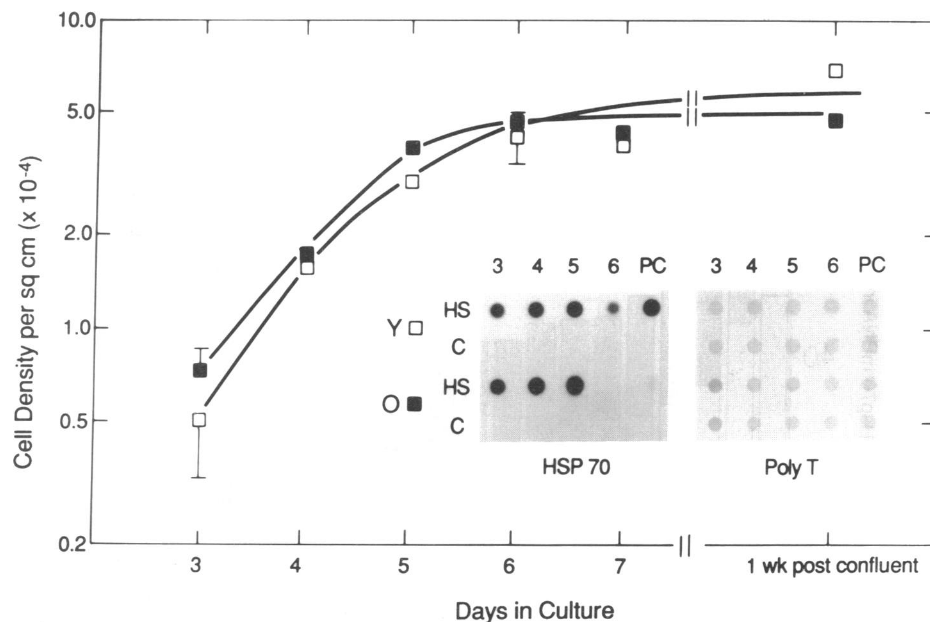

FIG. 1. Heat-induced HSP70 expression as a function of growth in fibroblast cultures from young (Y) and old (O) rats. Cells from triplicate flasks were counted with a hemacytometer beginning 2 days after isolation for a period of up to 1 week post-confluency (PC). Viability was assessed by trypan blue dye exclusion. The inset shows the corresponding mRNA levels in control (C) and heat-stressed (HS) cultures at each time period. One μg of RNA was blotted onto membranes and hybridized with ^{32}P-radiolabeled HSP70 cDNA or ^{35}S-radiolabeled poly(thymidylic acid) (T) (as a control for mRNA quantitation and loading).

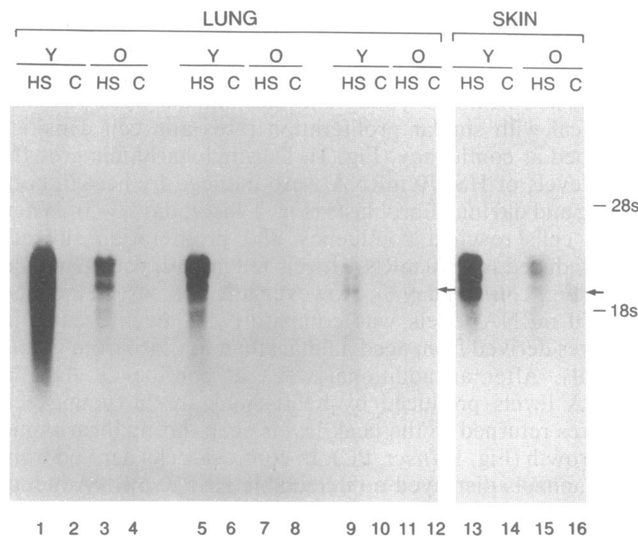

FIG. 2. Northern analysis of heat-induced HSP70 expression in fibroblast cultures. Total RNA (5 μg per lane) from cultures derived from three to five young (Y) and old (O) animals without heat treatment (C) and after heat shock (HS) was analyzed by using a ^{32}P-radiolabeled HSP70 cDNA probe. Shown are three experiments with lung fibroblasts and one experiment with skin fibroblasts. The arrow indicates the HSP70 cognate. 28s and 18s are the major ribosomal RNA markers.

autoradiograph, it is clearly detectable in all samples on longer exposures. In heat-stressed rat fibroblasts two additional mRNA species were apparent. These represent members of the HSP70-encoding gene family, the expression of which is strictly stress-inducible. Importantly, as shown here, the levels of induced HSP70 mRNA were found to vary considerably from one experiment to the next. Therefore, we surveyed the response in primary lung fibroblast cultures from a large number of individual animals. Fig. 3 shows the results of the densitometric analysis of HSP70 mRNA levels for heat-stressed fibroblast cultures derived from 22 old and 22 young animals. The results for individual animals are displayed in order of the highest to lowest level of HSP70 mRNA expression. As can be seen, individual animals demonstrated considerable variability in their heat shock responses. The cause for this variability is not clear but most likely reflects both experimental and endogenous components. In this respect it is important to note that these rats are not inbred. Despite the variability in response, the mean level of HSP70 expression was significantly lower in the heat-stressed fibroblasts from aged animals relative to that of fibroblasts from younger animals ($P < 0.01$). Several of these blots were stripped of hybridized HSP70 cDNA probe and rehybridized with a cDNA probe for β-actin. No appreciable differences in the levels of actin mRNA were observed between old and young animals, even in experiments where no HSP70 induction was observed (data not shown). Thus, the age-related decrease in HSP70 induction that we have observed cannot be attributed to differential gel loading, transfer, or degrees of mRNA degradation.

HSP70 Protein Levels After Heat Shock. The regulation of HSP70-encoding gene expression is known to occur at both transcriptional and translational levels (2, 9). Thus we examined whether the decrease in HSP70 mRNA levels in aged fibroblasts resulted in decreased HSP70 protein production. For these experiments both RNA and protein measurements were performed with replicate flasks of the same cultures. Four representative experiments are displayed in Fig. 4. In the absence of heat shock, HSP70 protein, like HSP70 mRNA, was barely detectable (lanes C and D). However, 4 hr after heat shock, HSP70 protein was clearly evident in extracts from both young and aged cell cultures (lanes A and B, arrow). Consistent with lower mRNA expression, lower levels of HSP70 protein were observed in heat-stressed cultures of aged animals compared with younger animals in three of the four experiments shown. In the fourth experiment, fibroblasts derived from an old animal displayed a higher level of HSP70 mRNA expression compared with its matched young counterpart, but little difference in the protein levels was evident (Fig. 4, experiment 4). Thus, in general, the HSP70 protein levels were found to be consistent with the mRNA levels, although the magnitude of the age-

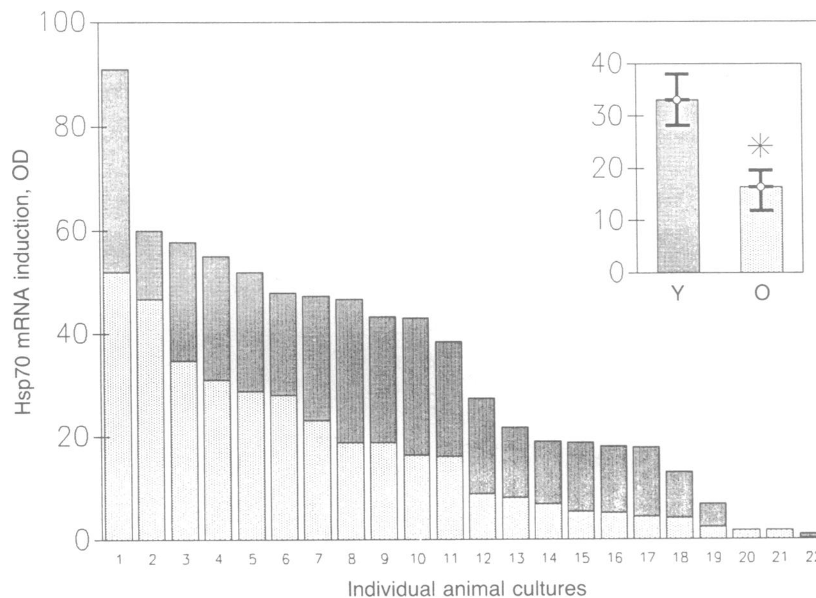

FIG. 3. Heat-induced HSP70 mRNA expression in cultures derived from individual rats. Relative levels of heat-induced HSP70 mRNA were measured by scanning optical densitometry of autoradiographs from Northern blots hybridized with radiolabeled HSP70 cDNA. The data for young (dark gray, Y) and old (light gray, O) animal cultures are organized and compared by order of highest to lowest level of HSP70 mRNA expression. (*Inset*) Mean level ± SE of HSP70 expression in the young versus old cultures. *$P < 0.01$, comparing expression in fibroblast cultures of young and old animals by using Student's t test for unpaired samples.

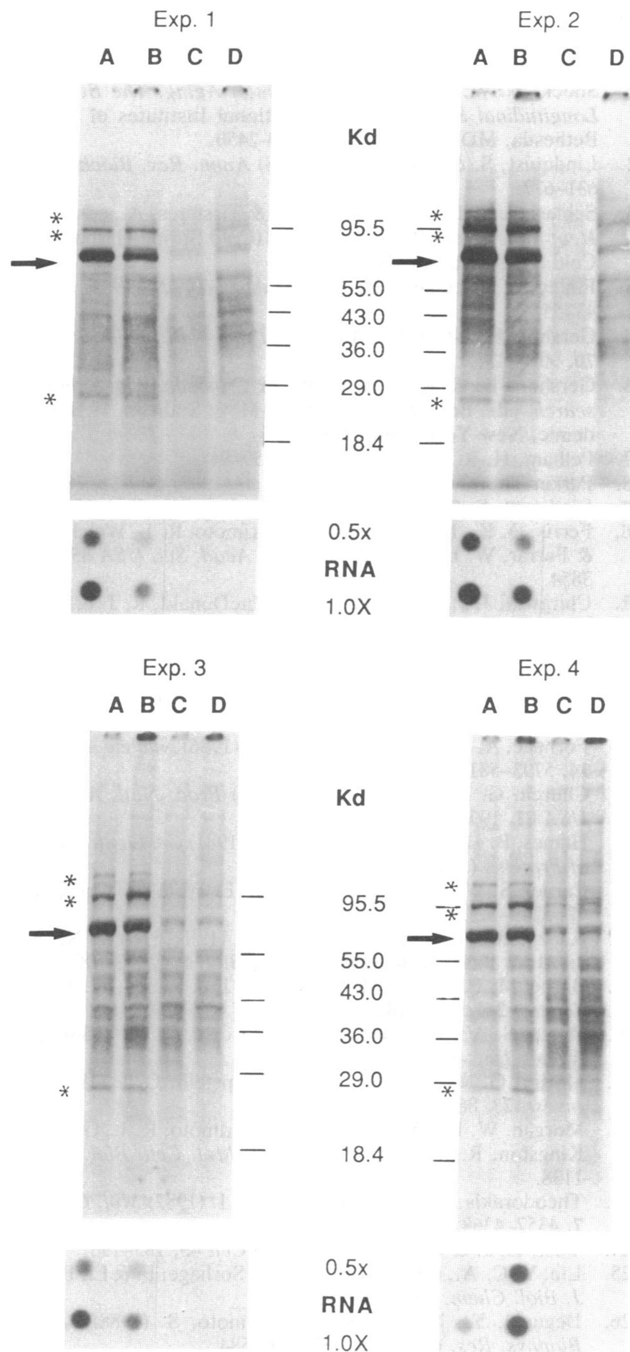

FIG. 4. Heat-induced HSP70 protein levels in fibroblast cultures from old and young animals (four representative experiments). Cells were labeled with ^{14}C-radiolabeled amino acids in the absence of (lanes C and D), or after (lanes A and B) heat shock. Cellular extracts were fractionated by SDS/polyacrylamide electrophoresis after loading equal amounts of cpms based on trichloroacetic acid-precipitated counts. The predominant HSP70 protein band migrating at ≈70 kDa (arrow) is compared between young (lane A) and old (lane B) animals. Asterisks mark other major heat-inducible proteins. The panel below each set of gels displays the results of dot-blot-hybridization analysis of RNA (1.0 and 0.5 μg) isolated from a duplicate set of heat-shocked cells. Kd, kDa.

related differences in protein induction at the time point examined were not as great as those for mRNA induction.

When the overall protein synthetic patterns between young and old cultures were compared, the levels of most other newly synthesized proteins, both before and after heat stress, were similar between the two age groups. However, several

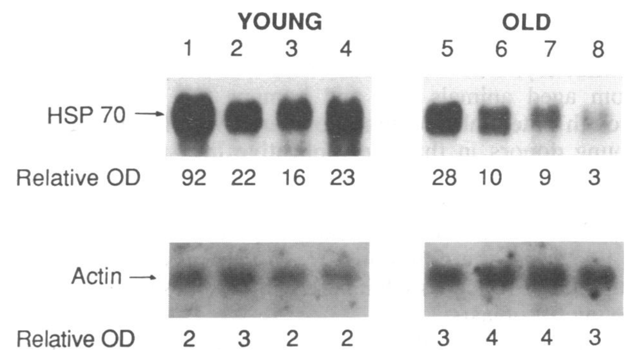

FIG. 5. HSP70 mRNA expression in fresh lung tissue subjected to heat shock. Levels of HSP70 and actin mRNA were examined by Northern analysis. Lanes 1, 2, 5, and 6 represent one experiment; lanes 3, 4, 7, and 8 are a second experiment. Numbers below each lane are the relative levels of hybridization intensity determined by scanning densitometry.

other proteins were specifically induced by the heat stress. Heat shock protein 27, a low-molecular-mass heat shock protein, was induced to similar levels in the young and old cells. In addition, two high-molecular-mass proteins (90 and 110 kDa) were also induced to similar levels in the young and old cell cultures. Thus, the age-related difference in the heat shock response appears to be specific for HSP70 under these conditions.

Age Differences in the Induction of HSP70 mRNA Levels Are Conserved in Freshly Isolated Lung Tissue. Because the cultured fibroblasts used in these studies were derived from lung tissue, we examined whether the difference in heat-induced HSP70 expression between young and old animals was present in freshly dissected lung tissue. The results obtained in two separate experiments, each comparing HSP70 mRNA expression in heat-treated lung tissue from two old and two young animals, are shown in Fig. 5. Consistent with our results with cultured fibroblasts, basal levels of HSP70 mRNA were low and essentially equivalent in cultures in the absence of heat shock (data not shown). The relative hybridization intensities for the heat-induced HSP70 expression are shown below the corresponding mRNA band on the autoradiograph (Fig. 5). The induced HSP70 mRNA levels after heat shock were higher in lung tissue from young animals relative to those of old animals. In these experiments, comparing lanes 1 and 2 with 5 and 6 (experiment 1), and lanes 3 and 4 with 7 and 8 (experiment 2), the mean induced HSP70 levels in aged lung tissue were 33% and 32%, respectively, of those found in young lung tissue. When actin mRNA levels were compared on the same blots, only slight differences were seen between the two age groups with higher levels seen in aged tissues.

DISCUSSION

While their name is derived from their response to heat stress, the heat shock proteins are induced by a variety of cellular stresses (2, 3). They appear to play a protective role in responding to these stresses and may also function to protect cells against subsequent challenges (4, 6, 19). Our studies demonstrating an age-related reduction in HSP70 expression after heat stress in fresh lung and skin and lung fibroblast cultures suggest that the protection afforded by HSP70 induction may be impaired with aging.

In addition to its proposed protective role during stress, there is increasing evidence for the importance of HSP70 in normal cellular growth and proliferation (10, 17, 18). This relationship to cellular growth may account for the finding that the decreased HSP70 mRNA expression seen in the

fibroblasts of aged rats after heat shock is highly dependent on growth phase and can be best elicited when cells are at confluency. It is well documented that fibroblasts obtained from aged animals (20) and humans (21) exhibit altered growth potential in culture compared with fibroblasts from young donors in that the replicative life span of cultured fibroblasts is inversely related to the age of the donor. These findings are, in fact, the basis for the widespread use of such fibroblasts as a model for senescence at the cellular level. It is likely that the differential HSP70 expression observed in our studies reflects such inherent differences in the aged and young fibroblasts.

The age-related alteration in HSP70 expression in primary cultures of rat fibroblasts appears to be specific for this heat shock protein rather than being representative of a generalized alteration in protein synthesis and/or altered responsiveness to heat stress. We observed no clear differences in the induced expression of any of the other major heat shock proteins at the protein level (Fig. 4). In addition, we have examined mRNA levels for heat shock protein 27 in a limited number of cultures and have not observed any age-related differences in its expression. However, it is important to point out that our culturing and treatment conditions were optimized for detecting differences in HSP70 expression and may not be optimal for other heat shock proteins.

The regulation of HSP70 gene expression is complex. While there is a significant body of evidence demonstrating control at the transcriptional level (8, 9, 22), the importance of posttranscriptional and translational mechanisms in controlling HSP70 protein levels has also been demonstrated (23, 24). At the present time we have no information regarding the mechanism of the age-related decline in HSP70 expression we have seen. However, Liu *et al.* (25) recently reported a decline in the heat-induced expression of HSP70 in human diploid fibroblasts as a function of cell passage (*in vitro* aging) and provided clear evidence for a transcriptional mechanism. They have proposed that there is an age-associated dysfunction in the signaling mechanism of the heat shock response. In a preliminary report Deguchi *et al.* (26) also reported a 30% retardation of heat-induced HSP70-encoding gene transcription in human peripheral blood mononuclear cells of old donors relative to those of young individuals. Future studies will focus on exploring the cause of the age-related alteration in heat shock protein expression in our primary fibroblasts and lung tissues.

At the physiological level, there have been several reports of age-dependent altered thermoregulation in humans as well as in experimental animals (27, 28). On a clinical level, hyperthermia is an increasingly recognized disorder in older individuals exposed to high temperatures, although not affecting all aged individuals uniformally (29, 30). The significance of our *in vitro* findings to these physiological alterations remains to be determined, as few studies have examined the regulation of HSP70 expression *in vivo* and no such studies are available with respect to aging. However, HSP70 is clearly induced *in vivo* in response to a variety of stresses (31–33), and recent studies have provided evidence for the role of heat shock proteins in enhancing the stress tolerance of tissues *in vivo* (19). Thus, our *in vitro* observation of an age-related decrement in HSP70 expression suggests important new avenues to be explored *in vivo*.

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