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Stress-induced heat shock protein 70 expression in adrenal cortex: An adrenocorticotropic hormone-sensitive, age-dependent response

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ABSTRACT **The induction of heat shock proteins (HSP) by cellular stress and the activation of the hypothalamic–pituitary–adrenal axis by physiologic stress are biological responses that aid in the maintenance of cellular and organismal homeostasis, respectively. In this report, restraint stress, known to activate the hypothalamic–pituitary–adrenal axis, is shown to induce expression of HSP70 mRNA selectively in the adrenal cortex of the rat. Restraint-induced HSP70 expression in the adrenals is rapid and is preceded by the activation of a protein factor capable of binding to the heat shock transcriptional control element. The ability of restraint to induce HSP70 expression in the adrenal is virtually eliminated in hypophysectomized rats but can be restored by the exogenous administration of adrenocorticotropic hormone. The magnitude of this induction declines as a function of increasing age, which may contribute to a reduced stress tolerance by aged animals. These results support a role for HSP70 in the physiologic stress response mediated by the hypothalamic–pituitary–adrenal axis.**

The ability of an organism to adapt to stress is a requisite for its survival in an everchanging environment (1). In higher organisms stress results in the activation of the hypothalamic–pituitary–adrenal (HPA) axis. This response is characterized by the secretion of corticotropin-releasing hormone from hypothalamic nuclei into the hypophyseal portal system, which, in turn, stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the peripheral circulation. ACTH binds to specific adrenocortical receptors, resulting in the rapid release of glucocorticoids into the bloodstream. Glucocorticoids act on a variety of tissues to give rise to their numerous homeostatic effects (2).

At the cellular level, heat and other metabolic stressors induce the synthesis of a set of highly conserved proteins termed heat shock proteins (HSPs) (3). This enhanced gene expression occurs as a result of stress-induced activation of one or more heat shock transcription factors (HSFs) (4–6). These proteins bind to a specific DNA sequence, the heat shock element (HSE) (7–10), in the promotor regions of the HSP genes to increase their rate of transcription. HSPs interact with other cellular proteins under normal and stress conditions and are thought to aid in the maintenance of cellular homeostasis (3).

Our current knowledge of HSP function is derived largely from studies in cultured cells. Few investigations have used *in vivo* stress models that might have direct physiologic relevance. In a previous study we demonstrated that, in response to heat stress, HSP70 is preferentially induced in specific brain regions involved in the regulation of HPA function and hypothesized that HSPs are an integral component of the mammalian physiologic stress response (11). In support of this hypothesis, this report demonstrates that a mild physiologic stress, restraint, induces the expression of the major HSP, HSP70, in rat adrenal cortex. We provide additional evidence that the response is mediated through the activation of the HPA axis and is dependent on the presence of ACTH. Additionally, the magnitude of restraint-induced HSP70 expression is attenuated with age.

MATERIALS AND METHODS

Animals and Treatment Conditions. Male Wistar rats ranging from 2 to 24 mo of age were obtained from the Gerontology Research Center colony and maintained on a light/dark (12 hr/12 hr) cycle at 24°C and given food and water ad libitum except during experiments. The average life-span of the rats in the Gerontology Research Center colony is ≈23 mo. Over 95% of these rats die before reaching 26 mo of age. Stress treatment consisted of restricting the mobility of rats by placing them in ventilated Plexiglass restraints. These stress conditions were approved by the Animal Care and Use Committee of the National Institute on Aging and are in accordance with the National Institutes of Health guidelines on the care and use of laboratory animals. Restrained animals were placed in a forced air incubator (24°C) for the indicated lengths of time before sacrifice. Animals sacrificed immediately after removal from the housing facility served as "unstressed" controls.

RNA Isolation and Analysis. Total RNA was isolated from tissue samples by using the RNAzol B method (TM Cinna Scientific, Friendswood, TX). RNA was quantified spectrophotometrically and confirmed by ethidium bromide staining of 18S and 28S ribosomal RNA. For Northern (RNA) analysis, RNA was denatured in formaldehyde and fractionated on 1.0% agarose gels and then transferred to GeneScreen*Plus* membranes (DuPont). Hybridization and wash procedures were conducted by using published methods (12).

Gel-Retardation Assay. Total cellular protein was isolated from lung, kidney, and adrenal glands by homogenization on ice in 20 mM Hepes, pH 7.5/1.5 mM MgCl/0.2 mM EDTA/0.2 mM dithiothreitol/0.4 M NaCl/20% (vol/vol) glycerol/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM leupeptin. Cellular debris was pelleted by centrifugation at 140,000 × *g* (4°C, 30 min). Supernates were removed, and protein content was determined (Bio-Rad). Gel-retardation assays were done by using the Gelshift kit purchased from Stratagene (catalog no. 201001) and a ^{32}P-labeled oligonucleotide corresponding to the consensus HSE sequence (5′-GCCTCGAATGTTCGCGAAGTTTCG-3′) (13).

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Abbreviations: HSP, heat shock protein; HSE, heat shock transcriptional control element; HSF, heat shock transcription factor; ACTH, adrenocorticotropic hormone; HPA, hypothalamic–pituitary–adrenal.

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***In Situ* Hybridization.** After restraint, adrenal glands were removed and frozen in embedding matrix (−20°C). Whole adrenals were sectioned (14 μm) through the midportion of the gland, thaw mounted on glass slides, dried on a slide warmer (50°C), and stored desiccated and frozen (−80°C) until used. Hybridizations were conducted according to published procedures (14). After hybridization washes, slides were dried and apposed to x-ray film (Cronex, DuPont) for visualization.

Probes and Labeling Reactions. An HSP70 cDNA probe isolated from a Chinese hamster ovary cell line (15) and shown to recognize at least three HSP70 mRNA transcripts in the rat (16) was used as probe for Northern analysis. cDNA probes corresponding to the human HSP89β and HSP27 genes were obtained from Stress-Gen Biotechnology (Sidney, BC, Canada). Isolated inserts were labeled with [^{32}P]dCTP (Amersham) by using the random-primer method of Feinberg and Vogelstein (17). For *in situ* hybridization, a synthetic oligonucleotide (5′-CGATCTCCTTCATCTTGGTCAGCACCATGG-3′) was used that has been shown to recognize heat-induced HSP70 transcripts but not HSC73 in the rat (11). Terminal deoxynucleotidyltransferase (Bethesda Research Laboratories) was used to label 3′ ends of the oligonucleotide with ^{35}S-labeled dATP (New England Nuclear).

Data Analysis. Optical densities were determined from autoradiograms by using the IN-SITU GRAIN analysis software on an Amersham RAS 3000 microdensitometer. The combined OD value corresponding to both the 2.3-kilobase (kb) and 3.0-kb transcripts of each sample was obtained from autoradiograms. Statistical analysis to test for differences between treatments was done by using analysis of variance with $P < 0.01$. The Student's t test was used to assess differences between individual groups.

RESULTS

Restraint Induces HSP70 Expression in the Adrenal Gland. HSP70 mRNA expression was examined by Northern analysis in 10 tissues of control and restrained rats (Fig. 1). In both control and stressed animals, a 2.3-kb HSP70 transcript, corresponding to the constitutively expressed HSC73 gene in rat (5, 20), was present in all tissues, although the magnitude of this expression varied (Fig. 1). Only the adrenal gland displayed a restraint-dependent increase in expression of this transcript. In addition, restraint also resulted in the appearance of a second, larger (3.0 kb) HSP70 transcript in adrenal tissue.

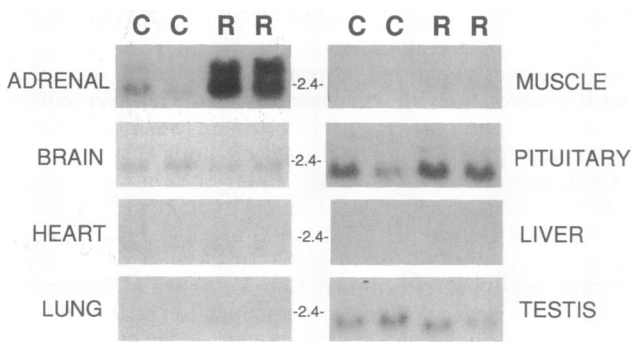

FIG. 1. Restraint induces the expression of HSP70 mRNA in rat adrenal tissue. Total RNA was isolated from adrenal gland, brain, heart (apical ventricle), lung, skeletal muscle (gracilis), pituitary, liver (right medial lobe), testis, kidney, spleen, and thymus of 6-mo-old adult rats, either exposed to 90 min of restraint (R) or sacrificed immediately after removal from the housing facility (C). Tissues from two different control and restrained animals were analyzed separately as shown. Northern blots (10 μg of RNA per lane) were hybridized with a hamster HSP70 cDNA probe (18) that has been shown to recognize at least three HSP70 mRNA transcripts in rat (19). Kidney, spleen, and thymus were not shown due to very low levels of constitutive expression and a lack of induction by restraint.

The restraint-induced elevation in HSP70 expression occurred rapidly (Fig. 2). Maximum HSP70 mRNA levels (9-fold greater than those of unstressed controls) were achieved after as little as 30 min of restraint but declined with extended exposure, perhaps reflecting an adaptation by the animal to the stressful conditions. Importantly, this effect appeared specific to the HSP70 family of HSPs, as neither HSP89β (a member of the HSP90 gene family) nor HSP27 expression was significantly affected by restraint. It is also important to note that with restraint, the body temperature of the rats did not deviate by >1°C at any time point (data not shown), suggesting that the induction of HSP70 in adrenals occurs through mechanisms not involving heat.

Activation of HSF by Restraint Stress. To determine whether restraint induces HSP70 mRNA through the activation of HSF, tissue lysates from adrenals of restrained and control animals were assayed for the presence of HSE-binding activity by using a gel-retardation assay (Fig. 3). The HSE consensus sequence used in this assay was derived from the *Drosophila* HSP70 gene (13) and has been shown to bind to heat shock-activated HSF of a variety of mammalian species including human and rodent. No HSE-binding activity was apparent in lung or kidney (Fig. 3), tissues that also failed to show induction of HSP70 mRNA. In contrast, HSE-binding activity was evident in adrenal tissue, even in the absence of restraint (Fig. 3, fifth lane). The level of binding activity was markedly increased in the adrenal gland after 20 min of restraint (Fig. 3, sixth lane). The restraint-induced increase in binding activity was specific for the HSE, as increased concentrations of unlabeled HSE effectively competed for binding to the factor (Fig. 3, HSE lanes), whereas unrelated DNA sequences known to bind other transcriptional activators did not (Fig. 3, Sp1, AP-1, and NF-κB lanes). As with HSP70 mRNA induction, this HSE-binding activity occurred in the absence of a measurable rise in body temperature, again indicating that restraint activates HSP70 transcription through mechanisms other than heat.

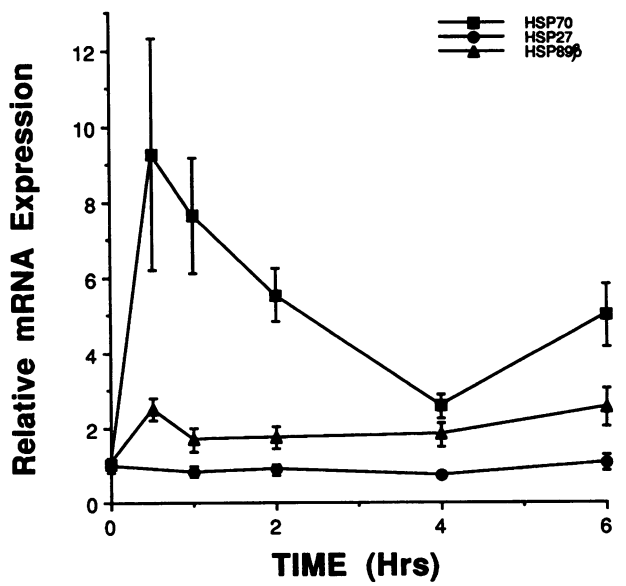

FIG. 2. Time course of restraint-induced HSP70 expression. Total RNA (10 μg) from adrenal glands of 6-mo-old rats sacrificed immediately upon removal from the housing facility (0 hr) or restrained for 30 min, 1 hr, 2 hr, 4 hr, or 6 hr (n = four per group) was assayed for HSP70, HSP27, and HSP89β expression by Northern analysis. Values shown for each time point are the mean increase in expression (relative to unrestrained animals) ± SEMs.

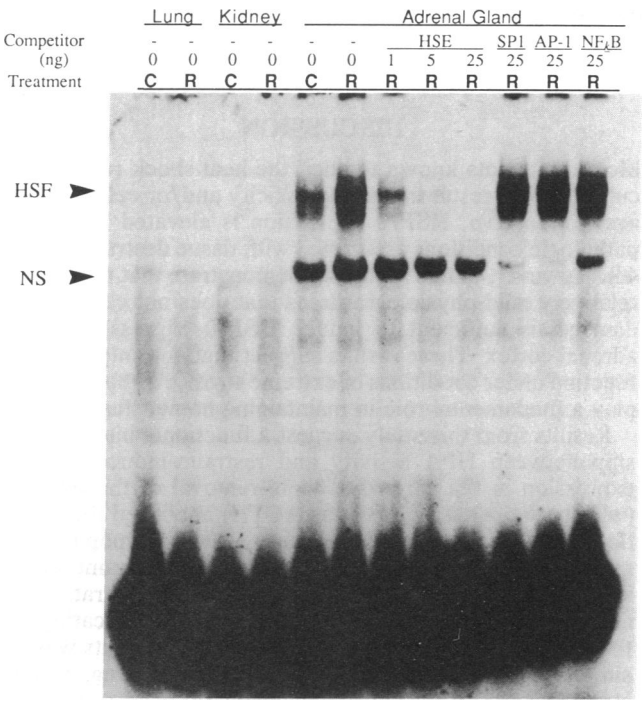

FIG. 3. Increased levels of HSE-binding proteins in adrenal tissue of restrained animals. Tissue lysates from lung, kidney, and adrenal glands of control (C) and restrained (R) rats were incubated with 1 ng of ^{32}P-labeled oligonucleotide corresponding to the HSE (first six lanes). To assess specific binding, similar incubations with adrenal lysates from restrained rats were conducted in the presence of 1, 5, and 25 ng of unlabeled HSE or in the presence of 25 ng of unlabeled Sp1, AP-1, and NF-κB oligonucleotides. HSF, specific HSE-binding activity; NS, nonspecific binding activity.

Endocrine Regulation of Restraint-Induced HSP70 Expression. Restraint is known to stimulate HPA axis activity characterized by the secretion of pituitary ACTH and culminating in the release of glucocorticoids from the adrenal cortex into the peripheral circulation (21–23). Acute stress also results in increased sympathetic nervous system activity and the release of catecholamines from the adrenal medulla (24). Therefore, it was important to determine the localization of restraint-induced HSP70 expression within the adrenal gland. *In situ* hybridization was performed on tissue sections from adrenals of control and restrained rats using a 30-base-pair (bp) oligonucleotide probe that recognizes only the induced 3.0-kb HSP70 transcript (11). Fig. 4*B*, Sham, shows that after restraint, HSP70 expression was clearly evident in the cortex but absent in the medulla.

Removal of the pituitary gland eliminates endogenous ACTH. Therefore, were ACTH important in mediating restraint-induced expression in the adrenal, hypophysectomized animals might be expected to show a diminished response. By using both Northern analysis (Fig. 4*A*) and *in situ* hybridization (Fig. 4*B*), hypophysectomy was shown to virtually eliminate HSP70 mRNA expression in the adrenal glands. The adrenals of hypophysectomized rats showed severe atrophy, which is characteristic of the loss of trophic stimulation by ACTH. Although restraint failed to induce HSP70 expression in the residual cortical tissue of these animals, we have shown in other experiments that adrenals of hypophysectomized animals do show induction of HSP70 mRNA in response to heat stress (data not shown).

The finding that hypophysectomy eliminated restraint-induced HSP70 expression in the adrenal indicated that either the loss of ACTH or the disuse atrophy associated with this loss was responsible for the reduced expression. To distinguish between these possibilities, hypophysectomized animals were given 10 daily injections of ACTH at a dose of 2.5 μg/day (0.25 United States Pharmacopoeia unit). These treatments were given to prevent the adrenal atrophy that normally occurs in hypophysectomized animals by stimulating daily production and release of glucocorticoids. On the 11th day, rats were injected with either saline or their daily dose of ACTH. Half of the rats from both groups were then either restrained or left unrestrained in their cages for 1 hr after the injections. After treatments, animals were immediately sacrificed, and HSP70 mRNA levels were assessed.

Saline-injected hypophysectomized rats showed only basal levels of HSP70 expression, even in the restrained group (Fig. 5). In contrast, animals receiving ACTH injections displayed high levels of HSP70 mRNA expression. Restraint did not increase this effect. Thus, these findings demonstrate that ACTH is both necessary and sufficient to induce adrenal cortical HSP70 expression.

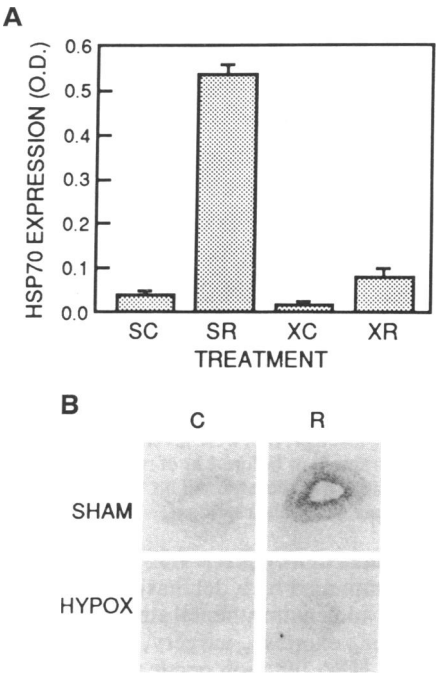

FIG. 4. HSP70 expression in adrenal glands of normal and hypophysectomized rats. Hypophysectomized (Hypox) and sham-operated control (Sham) rats were either exposed to restraint for 90 min (R) or sacrificed immediately after removal from the housing facility (C) 2 weeks after surgery. (*A*) Effect of hypophysectomy on restraint-induced expression by Northern analysis ($n = 4$ per group). HSP70 expression was determined by autoradiographic analysis of the Northern blots. Each bar represents the mean OD (± SEM) corresponding to the HSP70 transcripts (2.3 and 3.0 kb) for each treatment condition. SC, sham, control; SR, sham, restrained; XC, hypophysectomized, control; XR, hypophysectomized, restrained. Expression in restrained, hypophysectomized rats (XR) was significantly less than in restrained, sham-operated animals (SR) ($P < 0.01$). (*B*) Similar effect of hypophysectomy assessed by *in situ* hybridization with a synthetic oligonucleotide that binds only to the 3.0-kb RNA transcript in adrenals of restrained animals. Fourteen-micron sections taken from the middle of adrenal glands of animals from the four treatment groups were all mounted on glass slides and hybridized together under a single coverslip. After hybridization, the slides were apposed directly to x-ray film to generate the image of the actual adrenal sections; the photographic image is magnified ≈10 times. The darker areas in the restrained sham animal (upper right image) correspond to the adrenal cortex. This degree of hybridization did not occur in the sham control group (upper left image) or in the hypophysectomized animals under either control or restraint conditions. There was no evidence of HSP70 expression in the inner medullary region in any of the animals.

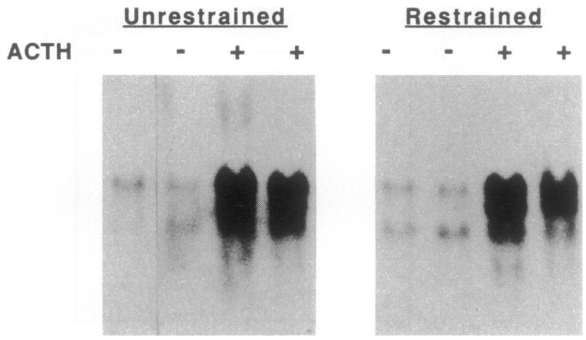

FIG. 5. ACTH is necessary for restraint-induced HSP70 expression in the adrenal gland. Hypophysectomized rats were maintained on daily injections (2.5 μg; 0.25 United States Pharmacopoeia unit) of ACTH. On the last day, rats were given ACTH or saline 1 hr before sacrifice (unrestrained) or before 1 hr of restraint (restrained). HSP70 mRNA expression was determined in the adrenal glands of two individual animals for each treatment.

Decline of Restraint-Induced HSP70 Expression with Age. Aging is accompanied by a decreased tolerance to a variety of physiologic and environmental stressors including temperature extremes, exercise, surgery, and infection (25, 26). Alterations in HPA function occur with age (18, 19, 27–32), and recently, we (33, 34) and others (35, 36) have provided evidence for an age-related decline in HSP expression in response to stress. Therefore, we determined whether the adrenal HSP70 expression in response to restraint was influenced by age. As shown in Fig. 6, adrenal HSP70 expression in restrained rats decreased as a function of age. Levels of both the 2.3- and 3.0-kb HSP70 transcripts were highest in rats 2–4 mo of age. A significant decrease ($P < 0.01$) in overall restraint-induced HSP70 expression was observed when comparing 2- to 4-mo-old rats with rats 6 mo of age. Restraint-induced levels remained constant through 14 mo and then began to fall again at 18 mo. A further significant decline ($P < 0.01$) was observed between 6 and 24 mo of age.

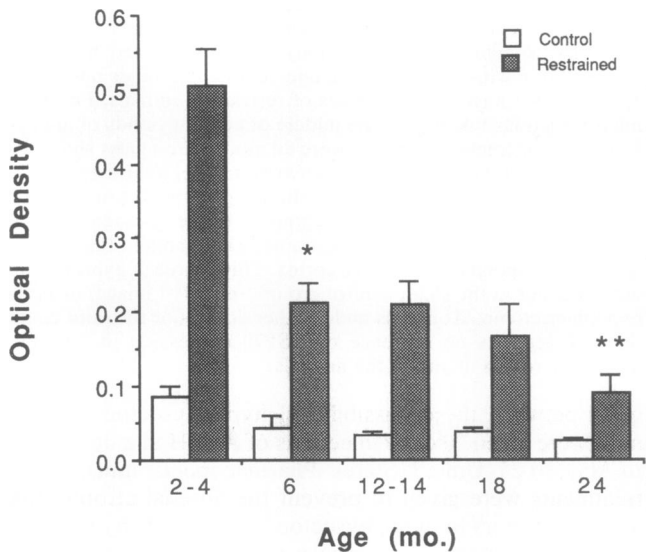

FIG. 6. Restraint-induced HSP70 mRNA expression declines with age. RNA was isolated from adrenals of restrained and unstressed control rats from five different age groups and assayed for HSP70 expression by Northern analysis. The average OD corresponding to both constitutive (2.3 kb) and induced (3.0 kb) HSP70 transcripts was obtained from individual samples. Bars represent the mean OD ± SEM. Number of animals in each group ranged from 8 to 12 in restrained groups and from 5 to 8 in control groups. Analysis of variance statistical analysis indicated a significant effect of age on restraint-induced HSP70 expression at $P < 0.0001$. *Post hoc* Student's *t* test indicated a significant difference ($P < 0.01$) in HSP70 expression between restrained animals in the 2- to 4-mo and 6-mo age groups (*) and between the 6-mo and 24-mo age groups (**).

DISCUSSION

Most treatments known to elicit the heat shock response in cultured cells result in marked toxicity and/or cellular damage (3). *In vivo*, HSP70 expression is elevated in various pathologic conditions associated with tissue destruction (37–40). However, in this report we demonstrate that restraint, a relatively mild physiologic stress that does not cause tissue damage, is sufficient to induce HSP70 expression in the adrenal cortex. These results suggest that not only do HSPs function under conditions of extreme stress but that they also play a fundamental role in maintaining homeostasis.

Results from this study suggest a functional interrelationship between HPA activity and restraint-induced HSP70 expression in the adrenals. While removal of the pituitary (which eliminated endogenous ACTH) abolished the induction, administration of exogenous ACTH to hypophysectomized animals induced HSP70 expression. Restraint did not induce HSP70 expression in hypophysectomized rats in the absence of the acute ACTH injection (Fig. 5), indicating that the loss of this response in hypophysectomized rats was not simply due to adrenal atrophy. Thus, these findings demonstrate that ACTH is a physiologic regulator of adrenal HSP70 expression *in vivo*. Presumably, other stressors capable of activating HPA activity would produce similar HSP70 expression in the adrenals.

In one of the first reports addressing HSP70 expression in mammalian systems with hyperthermia, it was demonstrated that adrenal glands contained high levels of HSP70 protein in the absence of heat stress (41). In that report, the treatment of non-heat-stressed controls was not described in detail. We have observed that the handling and exposure to a changed environment experienced by the rats when they are removed from their home cage is sufficient to induce HSP70 expression in the adrenals. Thus, the relatively high levels of HSP70 protein reported in non-heat-stressed rats in the above mentioned report may actually reflect induction of HSP70 through physiologic stress response mechanisms similar to what we have observed with restraint.

The induction of HSP70 mRNA is associated with increased HSE-binding activity in adrenal lysates. HSP89β and HSP27 mRNAs were not significantly induced (Fig. 2), yet these genes also contain an HSE in their 5′ regulatory regions. HSP27 mRNA levels showed no change with restraint, and HSP89β expression was increased, at most, 2 fold. This observation is somewhat surprising because the HSP70 and HSP90 gene families are generally found to be coregulated, although this is not always true for HSP27 (42–44). We have, in fact, demonstrated a discordance in the expression of HSP70 and HSP27 mRNA in tissues of heat-stressed rats (16). The lack of any significant increase in HSP89β expression in restrained rats may reflect a differential threshold for induction of the various HSPs. In other experiments conducted in this laboratory, we found that both HSP89β and HSP27 mRNAs are induced in the adrenal with hyperthermia, indicating that these genes can be induced in adrenals by using other stress conditions.

We have demonstrated that restraint-induced HSP70 expression declines throughout the life-span of the rat. If HSP induction is important in maintaining homeostasis, then a deficit in its expression could contribute to an age-related decrease in stress tolerance. Alterations in HPA function are known to occur with age (18, 19, 28–32). Although it has been reported that there is no age-related deficit in eliciting an adrenocortical response to acute stress, it has been suggested that reduced HPA activity occurs in aged animals after

repeated stress exposure (45). Thus, the decline in HSP70 expression with age could reflect a change in HPA activity rather than an intrinsic alteration in HSP70 gene regulation. The elucidation of the mechanism whereby the ACTH modulates HSP70 expression may provide important insights into why this response declines with age. Restoration and maintenance of this homeostatic response in aged animals may improve their tolerance to stressful conditions.

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