Activation of HSF and selective increase in heat-shock proteins by acute dexamethasone treatment

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Sun, L., J. Chang, S. R. Kirchhoff, and A. A. Knowlton. Activation of HSF and selective increase in heat-shock proteins by acute dexamethasone treatment. Am J Physiol Heart Circ Physiol 278: H1091–H1097, 2000.—Heat-shock proteins (HSPs) are an important family of endogenous protective proteins, which increase in response to myocardial ischemia and other stresses. Overexpression of HSP72 is cardioprotective. We were interested in the regulation of heat-shock factor (HSF), the transcription factor for HSP genes. Previously we have observed that the inflammatory cytokine tumor necrosis factor-α increases HSP72 levels and postulated that dexamethasone might effect the heat shock response. In the adult rat cardiac myocyte we found that treatment with either low (10 μM)- or high (100 μM)-dose dexamethasone activated HSF by 2–6 h as determined by gel shift assay without evidence of cytotoxicity. Although HSF activation is a key step in expression of HSP72, this may not result in an increase in HSP72. We found that 10 μM dexamethasone increased HSP72 38%, and 100 μM dexamethasone increased HSP72 62% (P < 0.05). HSP27 and HSP60 were unchanged. The selective increase in HSP72 was associated with protection of the cardiac myocytes from hypoxia and reoxygenation. We conclude that dexamethasone is a novel inducer of the heat shock response.

METHODS

Isolation of adult rat cardiac myocytes. Adult rat cardiac myocytes were isolated from 3- to 4-mo-old male Sprague-Dawley rats weighing 250-300 g according to a method described by Ford and Rovetto (10) with modification. Briefly, hearts were removed from rats following anesthesia with a combination of ketamine, xylazine, and acepromazine, and

HEAT-SHOCK PROTEINS (HSPs) are an important family of endogenous protective proteins, which increase in response to a wide variety of stresses (5, 15, 36, 43). These proteins have specificity of function and are found in different locations within the cell. HSP70 has been the focus of cardiac HSP research. There are at least two forms of HSP70 in mammalian cells: HSC (heat-shock constitutive)-70, a protein expressed at high levels in normal cells and involved in many of the chaperon and protein-folding functions of HSP70 and HSP72, expressed at low levels in normal tissue and rapidly induced in response to stress. In the heart HSP72 is induced by ischemia (16). Heat pretreatment to induce the heat-shock response reduces infarct size. Overexpression of HSP72 in various settings, including an embryonic cardiac cell line and transgenic mice, will protect these cells and tissues against various forms of stress (12, 22, 24, 32, 33). Previously we have observed that blocking the endogenous increase in HSP72 by antisense to HSP72 genes increased susceptibility to hypoxia and reoxygenation in isolated adult feline cardiocytes (29). Overexpression of HSP60 in conjunction with HSP10 is protective (20). Likewise, increased expression of HSP27 is protective against cardiac injury (23). Thus the HSPs have cardioprotective properties.

HSP synthesis is controlled by a specific family of transcription factors, heat-shock factors (HSFs), of which four have been identified but only two of these have been shown to be important to date (25, 26, 35). The primary HSF involved in regulation of expression of HSPs is HSF-1. Both heat and hypoxia activate HSF-1, which is present in the cytoplasm in an inactive form as a monomer. With stress trimerization occurs as well as phosphorylation. HSF-1 migrates to nucleus where it binds to the heat-shock element (HSE), which is present in the promoter of the stress response gene, initiating HSP transcription and synthesis. HSF-2 has been shown only to activate HSP transcription in an erythroleukemia line (37, 38).

We were interested in upregulating HSP expression by a less noxious means than heat shock, which requires temperatures of 42°C or higher. Previously, we have observed that tumor necrosis factor-α cytokine associated with inflammatory pathways increases HSP72 levels in the absence of cellular injury (27, 28). We postulated that the anti-inflammatory glucocorticoid steroids would influence activation of HSF. In the present study, we report activation of HSF-1 by dexamethasone at medically relevant concentrations in isolated adult rat cardiac myocytes. Activation of HSF by dexamethasone represents a novel pathway of HSP regulation that is independent of any evidence of cell injury. The activation of HSF-1 is accompanied by an increase in HSP72 but not HSP27 or HSP60. Pretreatment with dexamethasone followed by hypoxia and reoxygenation protected cardiac myocytes from injury compared with controls. This is to our knowledge the first report of activation of the heat shock response by glucocorticoid hormones.
HSF, HSP, AND DEXAMETHASONE

Cardiac myocyte culture and dexamethasone treatment. Freshly isolated cardiac myocytes were cultured in M199 (GIBCO) in petri dishes precoated with 0.2% laminin (GIBCO) at 37°C in a humidified incubator with 5% CO2-95% air. When the cells became adherent to the dishes (after 2–4 h of culture), the medium was exchanged for fresh M199 medium containing either 10 or 100 µM dexamethasone (Sigma; St Louis, MO), or equal volume of diluent. At 2, 4, and 6 h of treatment, samples were collected for gel mobility shift assays. After 10 h of treatment, either samples were collected for Western analysis for HSP levels or the medium was changed and the cells were subjected to hypoxia. The time line for this and subsequent experimental manipulations are summarized in Fig. 1.

Gel shift. For the gel mobility shift assay, we used 5′-CTAGAAGGCTTCTAGAAGCTTCT-AG-3′ as our consensus HSE, and end-labeled with [γ-32P]ATP. Otherwise, our methods were as described by Benjamin et al. (3, 4). Because HSF is normally present in the cell in an inactive form, we were able to use whole cell lysates for our studies. Supershift studies were carried out using a mouse monoclonal anti-HSF-1 (Affinity Bioreagents) and anti-HSF-2 (the generous gift of R. Morimoto, Northwestern University). The sample-HSE mixes were incubated with antibody at 1:5 and 1:10 dilutions for 30 min. For cold compete experiments, the samples were incubated with a 50-fold molar excess of cold HSE for 15 min before the addition of labeled HSE. Images were collected using a PhosphorImager (Molecular Dynamics; Sunnyvale, CA).

Western blot analysis. Western blotting was performed as previously described (17). Briefly, the cells were washed twice with PBS and solubilized by scraping into ice-cold RIPA buffer (pH 7.4, 50 mM Tris, 150 mM NaCl, 2.5 mg/ml deoxycholic acid, 1 mM EGTA, 10 µl/ml Nonidet P-40) supplemented with protease inhibitors (2.5 µg/ml antipain, 2.5 µg/ml leupeptin, 1.75 µg/ml pepstatin A, 0.95 µg/ml aprozin, 2.5 mM phenylmethylsulfon fluoride) and sonicated. Protein concentrations were determined with a biocinchoninic acid assay (Pierce). Samples were stored at −80°C until analyzed. The antibodies to HSPs were purchased from StressGen (Victoria, Canada), including rabbit polyclonal antibody to HSP72 protein (1:5,000 dilution), mouse monoclonal antibody to HSP60 protein (1:70,000 dilution), and rabbit polyclonal antibody to HSP27 protein (1:5,000 dilution). The mouse monoclonal antibody to α-actin was purchased from Sigma (1:1,000 dilution). Anti-HSP72 and anti-HSP27 were incubated with anti-rabbit IgG-horseradish peroxidase (HRP) at 1:2,000 (Amersham; Arlington Heights, IL). Anti-HSP60 and anti-α-actin were developed with anti-mouse IgG-HRP at 1:1,000 (Amersham). Blots were washed and developed using a chemiluminescent system (ECL, Amersham). The films were scanned for densitometric analysis (SigmaGel, Jandel; San Rafael, CA).

Hypoxia Studies

After dexamethasone treatment the medium was changed to DMEM base (no glucose, glutamine, or phenol red to prevent switching to glycolysis) and the cells were subjected to hypoxia for 4 h as previously described (29). Briefly, cells were exposed to 90% nitrogen-10% CO2 in a specially designed chamber (Billups-Rothenberg; Del Mar, CA). The dissolved oxygen with this system is 30 to 35 torr (P02) with hypoxia, with a baseline of 140 torr during normoxia as previously reported (29).

Indexes of injury. Ratio of live to dead cells, lactate dehydrogenase (LDH) levels, and C, N-diaryl-N-4,5-dimethylthiazol-2-yltrazolium chloride (MTT) were measured as previously described (29). Briefly, LDH levels were measured on medium samples using a colorimetric assay (Sigma) measuring the

Fig. 1. Time line summarizes experimental manipulations used in this study. For gel shift studies, cells were pretreated with dexamethasone for 2 to 6 h. To determine changes in heat-shock factor (HSF) levels, cells were treated with dexamethasone for 10 h and then harvested. For viability experiments, cells were treated with dexamethasone for 10 h, medium was changed, and cells were subjected to 4 h of hypoxia followed by 4 h of reoxygenation. All measurements of viability were made at end of reoxygenation. All control samples were collected simultaneously with treatment samples. HSF, heat-shock factor; LDH, lactate dehydrogenase; MTT, C, N-diaryl-N-4,5-dimethylthiazol-2-yltrazolium chloride.
conversion of pyruvic acid to lactic acid by LDH. Mitochondri
drial function was determined using MTT. Tetrazolium salts
are reduced by the respiratory chain; in the reduced state
MTT turns blue, which can be quantified using a spectropho-
tometer. MTT is reduced in both the early and late portions
of the respiratory chain, so assessment of its reduction allows
evaluation of the entire respiratory chain. For our purposes
cells were grown in 96-well microtiter plates (Falcon, Becton
Dickinson; Franklin Lakes, NJ) coated with 0.2% laminin. A
second plate containing serial dilutions of normoxic myocytes
was used as a reference standard curve for mitochondrial
function. After hypoxia the cells were returned to M199, 20
µl/well of MTT stock (5 mg/ml in PBS) added, and the cells
returned to the incubator. SDS (10%, pH 7.2) was added after
4 h of incubation with MTT, the cells were incubated over-
night, and optical density was measured with a microtiter
plate reader at 600 nm (Molecular Devices). The optical
density for each well was compared against the standard
curve derived from the normoxic control serial dilution of
cells, and the number of cells obtained from the standard
curve was divided by the number originally plated to give
percent uptake of MTT.

The ratio of live to dead cells, a simple index of cell viability,
was determined by counting a minimum of 60 cells per plate
after incubating the cells for 30 min with 1.05 µmol/l calcein-
acetoxymethyl ester (AM) and 4.0 µmol/l ethidium ho-
modimer (Molecular Probes; Eugene, OR) in M199. The cells
were then viewed under ultraviolet light. Live cells take up
the calcein-AM and are stained green, whereas dead and
dying cells take up the ethidium homodimer and are stained
red. Cells were scored as live or dead by an investigator
blinded to treatment group.

Statistics and Data Analysis

All results are reported as means ± SE. Results represent
the mean of three or more experiments with multiple data
determinations in each experiment. Data were compared by
one-way ANOVA followed by a Student-Newman-Keuls test.
Data comparing normalized values with control values were
compared with an ANOVA on ranks (Kruskal-Wallis) followed
by a Dunn’s test; if data samples passed test of normality and
of equal variance, one-way ANOVA was performed. All statis-
tical analysis was performed with SigmaStat (Jandel). A
value of P < 0.05 was considered significant.

RESULTS

Activation of HSF by Dexamethasone

Activation of HSF was observed with both 10 and 100
µM dexamethasone as shown in Fig. 2. This activation
was seen as early as 2 h after treatment was started
(data not shown) and persisted through 4 and 6 h. Cold
competition with unlabeled probe showed the observed
gel shift changes to be specific (Fig. 2A). The addition of
antibodies to HSF-1 and HSF-2 showed a supershift
only with anti-HSF-1, indicating that it is HSF-1 that is
activated by dexamethasone treatment (Fig. 2B). As
shown below, neither dose of dexamethasone was
associated with any evidence of injury as assessed by three
different measurements: LDH release, ratios of live to
dead cells, and MTT uptake.

Effect of Dexamethasone on HSP Levels

HSP72 expression. Western blot analysis showed
that after 10 h of treatment with dexamethasone

HSP72 increased 38 (P < 0.05) and 62% (P < 0.05) with
10 and 100 µM dexamethasone treatment, respectively,
compared with the controls (Fig. 3). Levels of α-actin
were unchanged.
Expression of HSP60 and HSP27. HSP60 and HSP27 levels were examined by Western blotting on the same samples as for HSP72. There was no change in levels of either HSP60 or HSP27 after dexamethasone treatment (Fig. 4).

Hypoxia and Reoxygenation

To determine whether upregulation of HSP72 by dexamethasone pretreatment protected the cardiac myocytes, a series of hypoxia and reoxygenation experiments were performed. Pilot experiments were done to define the effect of hypoxia on the isolated adult rat cardiac myocytes. We selected 4 h of hypoxia followed by 4 h of reoxygenation to injure the cells. After pretreatment with 10 or 100 µM dexamethasone for 10 h, the medium was changed to DMEM base and the cells were subjected to 4 h of hypoxia followed by 4 h reoxygenation before assessment of cell injury. All samples for analysis of control, hypoxia, and reoxygenation were collected at the end of the 4-h reoxygenation period.

LDH levels. LDH levels were measured in the medium. During normoxia dexamethasone-treated cells had similar LDH levels in the medium as untreated cells. As shown in Fig. 5, the LDH medium levels with hypoxia in the 100 µM dexamethasone-treated cells were unchanged compared with all groups of normoxic cells. LDH medium levels were less in the 10 µM cells than in the control cells following hypoxia, but this difference was not significant. Both control hypoxia and reoxygenation and 10 µM dexamethasone hypoxia and reoxygenation groups had significantly increased LDH medium levels compared with normoxia. After hypoxia and reoxygenation the level of LDH was 1.86 ± 0.16 U/µg protein in controls, 1.65 ± 0.16 U/µg protein in 10 µM dexamethasone-treated cells, and 1.32 ± 0.11 U/µg protein in 100 µM dexamethasone-treated cells. LDH medium levels were 1.02 ± 0.07 U/µg protein in untreated normoxia control cells (P < 0.05 vs. hypoxic controls and 10 µM dexamethasone, P = not significant (ns) vs. 100 µM dexamethasone).

Live/dead assay. The live/dead assay showed no difference between dexamethasone-treated cells and controls in the absence of hypoxia. After hypoxia, there was higher viability in both 10 and 100 µM dexamethasone-treated cells compared with control following hypoxia. The viability in untreated hypoxia cells was

Fig. 3. HSP72 protein levels with 10 h dexamethasone (Dex) treatment. A: graph summarizes changes in levels. Densitometric measurements were normalized to mean controls (C) on same blot for each group of samples. Plot represents 4 sets of experiments. Expression level of HSP72 in 10 µM Dex-treated cells was 1.38 vs. normal (n = 15, *P < 0.05) and in 100 µM Dex-treated cells was 1.623 (n = 15, *P < 0.05). There was no significant difference between 10 and 100 µM Dex-treated cells (P = ns). B: representative Western blot showing increased HSP72 levels after 10 h of treatment with Dex. C: same blot as in B developed with anti-actin antibody. In contrast to HSP72, actin levels were unchanged with Dex treatment.

Fig. 4. A: representative Western blot for HSP60 showing that there were no significant differences in HSP60 levels among control and 10 or 100 µM Dex-treated cells. B: representative Western blot for HSP27 showing that there were no significant differences in HSP27 levels among control and 10 or 100 µM Dex-treated cells. Same samples were used as for HSP72 measurement, and HSP60 and HSP27 were analyzed on same blot.

Fig. 5. LDH release. Plot represents results from 5 different experiments. Results are expressed as LDH U/µg of protein. Open bars, treatment with diluent only (n = 24, 17). Hatched bars, 10 µM dexamethasone (n = 15, 17). Double-hatched bars, 100 µM dexamethasone (n = 15, 17). *P < 0.05 vs. normoxia.
The findings in the present study demonstrate the activation of HSF-1 and increased expression of HSP72 in isolated adult rat cardiac myocytes treated with dexamethasone. No change was observed in levels of HSP27 or HSP60. There was no evidence of cellular injury from treatment with dexamethasone in the absence of hypoxia and reoxygenation, indicating that activation of HSF-1 in this setting is not mediated by cellular injury and protein denaturation. Pretreatment with dexamethasone resulted in resistance to hypoxia and reoxygenation injury as measured by the ratio of live to dead cells and LDH release. In contrast, the decreased mitochondrial function posthypoxia as measured by MTT uptake was not prevented by pretreatment with dexamethasone. This may reflect the lack of increase in HSP60, a mitochondrial HSP vs. HSP72, which is found in the cytoplasm and in the nucleus with stress.

Pretreatment with 100 µM dexamethasone increased HSP72 levels by 60%. Although this increase is less than that of heat shock, this change was associated with protection. The higher dose of dexamethasone was associated with a greater increase in HSP72 and more protection than the lower dose. The increase in HSP72 with dexamethasone is similar to the increase we observed with mild hypoxia (8 h) and reoxygenation in isolated adult feline cardiac myocytes (29). Blocking this increase in HSP72 after hypoxia and reoxygenation was associated with increased injury (29). Thus the modest increase in HSP72 associated with dexamethasone treatment has physiological importance.

Differential HSP Induction

Although HSF-1 is the transcription factor for multiple HSPs, only HSP72 increased. HSP72 appears to be one of the more responsive of the HSPs and may be more readily upregulated than some of the other HSPs. Whereas most studies have focused on a single HSP, some investigators have examined levels of several HSPs in response to a given stress and have found differential changes in HSPs (2, 21, 31, 42). In our own laboratory we have observed upregulation of HSP60 and HSP27 but not HSP72 in the setting of end-stage cardiomyopathy (18). Why these differences in HSP expression occur will be better understood as we learn more about the transcriptional and posttranscriptional regulation of HSPs in mammalian tissue.

HSF-1 Activation

With dexamethasone, not only was HSF-1 activated, but activation was slower than the usual 10 or 15 min and was more sustained than the typical 1- or 2-h duration (3, 4, 13, 30). HSP72 is thought to be involved in turning off HSF-1 activation, and the very slow increase in HSP72 may be part of the reason for the prolonged activation of HSF-1 (1). However, the activation of HSF-1 by dexamethasone may be by a novel mechanism, and turn-off of activation may occur by a different mechanism.

Hormones and HSP Induction

Glucocorticoids have a plethora of effects, including inhibition of lipid peroxidation, inhibition of formation of arachidonic acid products and modulation of neutrophil and endothelial function (19). Chronic glucocorticoid administration is known to alter protein metabolism (7, 8). Short-term glucocorticoid treatment blocks the inflammatory response. In patient studies, acute

**DISCUSSION**

The findings in the present study demonstrate the activation of HSF-1 and increased expression of HSP72 in isolated adult rat cardiac myocytes treated with dexamethasone. No change was observed in levels of HSP27 or HSP60. There was no evidence of cellular injury from treatment with dexamethasone in the absence of hypoxia and reoxygenation, indicating that activation of HSF-1 in this setting is not mediated by cellular injury and protein denaturation. Pretreatment with dexamethasone resulted in resistance to hypoxia and reoxygenation injury as measured by the ratio of live to dead cells and LDH release. In contrast, the decreased mitochondrial function posthypoxia as measured by MTT uptake was not prevented by pretreatment with dexamethasone. This may reflect the lack of increase in HSP60, a mitochondrial HSP vs. HSP72, which is found in the cytoplasm and in the nucleus with stress.

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35.59 ± 4.47% (P < 0.05 vs. normoxia), 57.59 ± 7.61% (P < 0.05 vs. control hypoxia-treated cells) in 10 µM dexamethasone-treated hypoxia cells, and 67.79 ± 5.21% (P = ns vs. normoxia) in 100 µM dexamethasone-treated hypoxia cells (Fig. 6). Normoxic control cells had a viability of 84.18 ± 3.28%.

MTT. Hypoxia and reoxygenation significantly reduced MTT uptake; however, dexamethasone treatment had no effect on this measure of mitochondrial function in either normoxic or hypoxic groups (Fig. 7).

**Fig. 6. Live/Dead assay.** Plot represents results from 5 different experiments. Results are expressed as percentage of live to total cells counted (live + dead). Open bars, control (n = 10, 10). Hatched bars, 10 µM dexamethasone (n = 10, 10). Double-hatched bars, 100 µM dexamethasone (n = 10, 10). *P < 0.05 vs. normoxia. +P < 0.05 vs. control hypoxia-treated cells. **P < 0.001 vs. control hypoxia-treated cells.

**Fig. 7. MTT assay.** Plot represents results from 3 different experiments. Results are expressed as percent uptake of MTT compared with standard curve, as described in METHODS. Postexposure to hypoxia MTT uptake and metabolism, an index of mitochondrial function, was decreased in all groups compared with normoxia, whereas no significant difference in MTT uptake was found between control, 10 and 100 µM dexamethasone-treated cells after hypoxia. Open bars, control (n = 24, 24). Hatched bars, 10 µM dexamethasone (n = 24, 24). Double-hatched bars, 100 µM dexamethasone (n = 24, 24). *P < 0.05 vs. normoxia.
doses of dexamethasone and other glucocorticoids, in concentrations similar to the current study, reduced damage postcardioplegia, in spinal cord injury and in other acute injury states (9, 14, 39). Although these investigators did not examine HSP72, our results would suggest that HSP72 had been upregulated.

In the last few years, considerable interest has developed in the heat-shock response and myocardial protection. Multiple lines of evidence have suggested a link between induction of the heat-shock response and improved recovery of the myocardium from ischemic injury. The known methods to induce the HSPs, such as heating, have deleterious effects. As tumor necrosis factor-α, a cytokine associated with inflammatory pathways, increases HSP72 levels, we were interested in whether the anti-inflammatory glucocorticoids altered HSF activation. A number of reports have implicated hormones in regulation of HSP expression. Vasopressin activated HSF-1, increased HSP72 mRNA, and was associated with an increase in HSF72 in renal tubular cells (44). Surgical stress and restraint stress both increased HSP72 levels in the adrenal gland and the aorta but not in other organs (40, 42). Chronic treatment with dexamethasone decreased the restraint-stimulated increase in HSP72 (41). In healing wounds, chronic dexamethasone treatment blocked the increase in HSP25, HSP72, and HSC70 and inhibited fibroblast proliferation (11). The results of these previous investigations suggest that long-term treatment with glucocorticoids inhibits the stress response. The effect of short-term treatment, addressed by the current study, has not been described previously.

In the late 1970s a number of investigators reported deleterious effects with repeated glucocorticoid usage in acute myocardial infarction (6, 34). Single-dose glucocorticoid therapy was not associated with adverse effects. Thus these previous adverse findings with multiple-dose glucocorticoid usage in acute myocardial infarction do not contraindicate the use of a single dose of dexamethasone to increase HSP expression in the setting of myocardial injury.

We conclude that dexamethasone activates HSF-1 and upregulates HSP72. This activation of HSF-1 is not preceded by cellular injury. These results suggest that a novel pathway of activation is involved. Further studies are needed to elucidate the interaction between glucocorticoids and the stress response.

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