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

L. SUN, J. CHANG, S. R. KIRCHHOFF, AND A. A. KNOWLTON
Cardiology Research, Veterans Affairs Medical Center and Baylor College of Medicine, Houston, Texas 77030

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 protein (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.

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 HSF 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 HSF regulation that is independent of any evidence of cellular 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.

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 heat 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.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
immerged in ice-cold heparin-J oklik A buffer [modified MEM/J oklik (GIBCO; Grand Island, NY), 60 mM taurine, 20 mM creatine, 5 mM HEPES, 0.1% BSA, 1 IU heparin/ml, pH 7.4]. After a 3-min perfusion on a Langendorff apparatus with heparin-J oklik A to remove any blood, the perfusion solution was switched to J oklik A with 0.6 mg/ml collagenase (collagenase type 2, Worthington Biochemical; Lakewood, NJ). After perfusion with collagenase, the hearts were minced and digested for 5 min in a shaking water bath at 37°C. This step was repeated twice to achieve complete digestion. The cell suspension was filtered and washed twice with J oklik A buffer, and the myocytes were transferred to the top of a 6% BSA gradient to increase the percent yield of rod-shaped cells. To reintroduce calcium, 100 mM CaCl$_2$ was added to a final 1,000 µM calcium concentration in five steps with 5-min incubations. The cardiac myocytes were resuspended in medium 199 (M199) supplemented with 100 µU penicillin, 100 µg streptomycin, 20 µl human serum albumin, 5 µg insulin, and 5 µg transferrin per milliliter and transferred to a cell culture flask for 2 h in an incubator at 37°C. After this differential plating step to remove fibroblasts, the cells were plated on laminin-coated dishes. This procedure yielded on average 70% rod-shaped cardiac myocytes. In pilot studies, staining with anti-m20 antibody showed these cells were over 97% cardiac myocytes.

The animal protocol was approved by the Baylor College of Medicine Animal Research committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

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% CO$_2$−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'-CTAGAAGCTTCTAGAAGCTTCTAG-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 monoconal anti-HSF-1 (Affinity Bioreagents) and anti-HSF-2 (the generous gift of R. Morimoto, Northwestern University). The samples 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 µl/ml leupeptin, 1.75 µg/ml pepstatin A, 0.95 µg/ml aprotinin, 2.5 mM phenylmethylsulfonyl fluoride) and sonicated. Protein concentrations were determined with a bichoninic acid assay (Pierce). Samples were stored at −80°C until analyzed. The antibodies to HPSs were purchased from StressGen (Victoria, Canada), including rabbit polyclonal antibody to HSP72 protein (1:1,000 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 (Amerham). Blots were washed and developed using a chemiluminescent system (ECL, Amersham). The films were scanned for densitometric analysis (SilageGel, J andel; 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% CO$_2$ in a specially designed chamber (Billups-Rothenberg; Del Mar, CA). The dissolved oxygen with this system is 30 to 35 torr (PO$_2$) 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-diphenyl-N-4,5-dimethylthiazol-2-yltetrazolium chloride (MTT) were measured as previously described (29). Briefly, LDH levels were measured on medium samples using a colorimetric assay (Sigma) measuring the
conversion of pyruvic acid to lactic acid by LDH. Mitochondrial 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 spectrophotometer. 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 overnight, 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-acetoxyethyl ester (AM) and 4.0 µmol/l ethidium homodimer (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 statistical 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 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
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).

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.

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
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 HSP72 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 repeated glucocorticoid usage were not described previously.

REFERENCES


22. Martin, J. L., R. Mestril, R. Hilal-Dandan, L. L. Brunton, and W. H. Dillmann. Small heat shock proteins and protection...


