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Research Article

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Dexamethasone Protection of Rat Intestinal Epithelial Cells against Oxidant Injury Is Mediated by Induction of Heat Shock Protein 72

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Abstract

Although the therapeutic actions of glucocorticoids are largely attributed to their anti-inflammatory and immunosuppressive effects, they have been implicated in enhancing tissue and cellular protection. In this study, we demonstrate that dexamethasone significantly enhances viability of IEC-18 rat small intestinal cells against oxidant-induced stress in a dose-dependent fashion. This protective action is mediated by induction of hsp72, the major inducible heat shock protein in intestinal epithelial cells. Dexamethasone stimulates a time- and dose-dependent response in hsp72 protein expression that parallels its effects on cell viability. Furthermore, the induction of hsp72 is tissue dependent, as nonintestinal epithelioid HeLa cells show differential induction of hsp72 expression in response to the same dexamethasone treatment. Antisense hsp72 cDNA transfection of IEC-18 cells abolishes the dexamethasone-induced hsp72 response, without significantly affecting constitutive expression of its homologue, hsc73. Dexamethasone treatment also significantly induces hsp72 protein expression in rat intestinal mucosal cells in vivo. These data demonstrate that glucocorticoids protect intestinal epithelial cells against oxidant-induced stress by inducing hsp72. (*J. Clin. Invest.* 1998. 102: 1860–1865.) Key words: glucocorticoids • stress proteins • inflammatory bowel diseases • cytoprotection • intestinal epithelial cells

Introduction

Glucocorticoids are commonly used as therapeutic agents for many acute and chronic inflammatory illnesses such as ulcerative colitis, Crohn's disease, rheumatoid arthritis, psoriasis, multiple sclerosis, and autoimmune hepatitis. The therapeutic actions of these compounds have largely been attributed to their anti-inflammatory and immunosuppressive effects. For instance, glucocorticoids are believed to inhibit arachidonic acid metabolism and activation of complement and vasoactive substances as well as to prevent neutrophilic chemotaxis by affecting intercellular adhesion molecules (1). Recent studies have suggested that glucocorticoids decrease expression of TNF-α, interleukin-1α, nitric oxide, and IL-2 through regulation of transcription factors (2–6).

However, previous reports have suggested that glucocorticoids have additional effects in enhancing tissue and cell survival under stressful and injurious conditions. For instance, administration of pharmacological doses of glucocorticoids prevents stress-induced gastric ulcers in rats (7). Glucocorticoids have also been reported to be beneficial in treating certain patients with sepsis and adult respiratory distress syndrome, and their ability to stabilize endothelial and lysosomal membranes is thought to be part of this protective action (8–11). More directly, dexamethasone (DEX)[1] has been shown to protect mouse fibroblast cells against cytotoxic concentration of TNF-α in vitro (12). However, the cellular bases for these observed effects are unknown.

Because inducible heat shock proteins, particularly hsp72, appear to be important in conferring protection to intestinal epithelial cells against oxidant and thermal injury, we speculated that the protective actions of glucocorticoids might be mediated through the induction of these proteins. Of note, studies of nonepithelial tissues including retroocular fibroblasts (13), Chinese hamster ovary cells (14), and mouse fibroblasts (12, 15) have suggested and inhibitory or negligible effect of glucocorticoids on heat shock protein expression. Nevertheless, we embarked on re-exploration of this issue in intestinal epithelial cells, in light of marked tissue and cellular differences in the hsp72 response we have observed (16, 17).

Methods

Cell culture. Rat small intestinal epithelial IEC-18 cells were studied since they are nontransformed, normal diploid cells, which exhibited consistent hsp72 induction to thermal stress (16–18). HeLa cells, which are commonly used in the study of heat shock proteins, were used as nonintestinal control cells. Both types of cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD). IEC-18 cells were cultured in high-glucose DMEM with 5% vol/vol fetal bovine serum, 0.1 U/mL insulin, 50 μg/mL streptomycin, and 50 U/mL penicillin; HeLa cells were grown in high-glucose DMEM with 10% vol/vol heat-inactivated fetal bovine serum and penicillin/streptomycin in same concentrations as above. IEC-18 cells were used at or near confluence between passages 16 and 32, and HeLa cells at passages 3–20 after receipt from ATCC.

Time- and dose-dependent effects of DEX on hsp72 expression. Cells were treated with various pharmacologically relevant concentrations of DEX (19): 10^{-9} M to 10^{-4} M or 10^{-10} M to 10^{-4} M in complete media. Cells and proteins were harvested by a standard technique from 60-mm petri dishes by scraping in phosphate-buffered saline and pelletted after lysis in homogenization buffer (10 mM Tris pH 7.4, 5 mM $MgCl_2$, 1 mM PMSF, 10 μg/mL leupeptin and aprotinin, and 50 U/mL DNase and RNase). Samples were analyzed on SDS-PAGE and Western blots performed using 1 × Towbin buffer

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1. *Abbreviations used in this paper:* DEX, dexamethasone; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt.

(25 mM Tris pH 8.8, 192 mM glycine with 15% vol/vol methanol) in a standard fashion (18). Blots were blocked with 5% Blotto (5% wt/vol nonfat dry milk in phosphate-buffered saline with 0.2% vol/vol Nonidet P40) and then incubated with a specific mouse monoclonal anti–hsp72 antibody, C92 (Stressgen, Victoria, BC, Canada). Blots were washed, incubated with horseradish peroxidase-conjugated secondary anti–mouse antibody, and developed using an enhanced chemilluminescence system. Relative quantification of hsp was done by densitometric measurements of fluorographs by using NIH Image 1.54 (National Institutes of Health, Bethesda, Maryland). A time course expression of hsp72 was also performed with DEX treatment of cells up to 96 h (0, 24, 48, 72, and 96 h) at concentration of 10^{-7} M. As controls, samples obtained from thermally stressed cells as previously described (18) as well as non-DEX–treated, nonthermally stressed cell extracts were simultaneously loaded in gels.

Cell viability assays. Cells were grown to an approximate cellular concentration of 10^4/well in 96-well tissue culture plates (Costar, Cambridge, MA) and treated for 6 and 96 h at 37°C with various concentrations of DEX, as described above. They were then washed and incubated in phenol red-free DMEM without supplements with 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) for 90 min. Cellular viability was determined by the generation of a formazan dye from the XTT substrate (monitored at 490 nm on a scanning multiwell spectrophotometer [ELISA reader]), a reaction that is dependent on metabolism by mitochondrial dehydrogenases of viable cells. After 90 min, the cell-permeant and stable oxidant, monochloramine, was added at a concentration of 0.3 mM. Increases in absorbance were then measured over the next 90 min. A paired control group that did not receive any DEX was simultaneously exposed to the oxidant. Changes of absorbance after 90-min exposure to monochloramine were measured among the cells with varying DEX concentration exposure. Also, the assay was run on IEC-18 cells without DEX or monochloramine exposures as a negative control.

Using IEC-18 cells, we performed separate radiolabeled-chromium-release assays as a comparison of the viability assays as previously described (16). Cells were exposed for 60 min by using variable concentrations of monochloramine (0, 0.03, 0.1, 0.3, 1, and 3 mM), parallel XTT assays were performed, and absorbance measurements were taken after 60 min of monochloramine exposure.

Inhibition of the hsp72 response of IEC-18 cells by antisense transfection. The full-length rat-inducible hsp72 containing 55 bases of the 5′ untranslated region (G12, a generous gift of Dr. Ruben Mestril, University of California at San Diego) was cloned in the antisense direction into mammalian expression vector pCEP4 (pCEP4 drives the expression of its insert under the control of a strong, constitutive cytomegalovirus promoter and contains hygromycin resistance gene). IEC-18 cells were transfected by electroporation at the setting of 250 μF and 250 V. Cells were selected by resistance to the antibiotic hygromycin (200 μg/mL) and tested for functional insertion of the antisense construct by measuring hsp72 levels under glutamine treatment as previously described (16). Clones that demonstrated the most complete inhibition of glutamine-induced hsp72 production were propagated for further study.

Measurement of constitutive heat shock protein hsc73. Western blot technique was applied as described above using anti-hsc73, a specific rat monoclonal antibody to constitutive hsc73, 1B5 (Stressgen, Victoria, Canada).

Induction of hsp72 intestinal epithelial cells by DEX treatment, in vivo experiment. The animal usage protocol for this experiment was approved by the Institutional Animal Research Committee. Three normal Sprague-Dawley rats (250—275 g) were injected daily for 4 d with 0.2 mg/kg/d of DEX, a dose approximating clinically relevant therapeutic dosage, intraperitoneally (19). An additional three rats were used as a control group receiving daily intraperitoneal sterile saline injections. After the completion of the treatment regimen, intestinal mucosal cells were harvested from jejunum, ileum, and proximal colon and from spleen, which served as a control tissue. Tissues were kept in cold phosphate-buffered saline immediately after harvest followed by homogenization and incubation in the homogenization buffer at concentrations as noted in the Western blot protocol above. Protein extracts from the harvested cells were analyzed by Western blot using 10 μg protein and mouse monoclonal anti-hsp72 and anti-hsc73 as described above.

Statistics. Results were presented as means±SEM. Three separate experiments were incorporated for the data analysis as noted. Student's *t* test was used for comparison of the XTT assay measurements in DEX-dose effect against the control (i.e., monochloramine-exposed, non-DEX–treated group). Student's paired *t* test was used for comparing XTT measurements of antisense and vector-only transfectants at each concentration of DEX. The criterion for statistical significance was a $P < 0.05$.

Results

XTT assay as a measure of cell viability. As shown in Fig. 1, the percentage of radiolabeled chromium release, a well-established viability assay, correlates well with the nonradioactive XTT assay, which was used for all subsequent studies. The linear correlation with a coefficient of 0.996 was observed between percentage of ^{51}Cr release and percentage of difference in absorbance readings of the XTT from the nonoxidant exposed group, obtained from cells exposed to variable concentrations of monochloramine (0–3 mM) for 60 min.

Effects of DEX on intestinal epithelial cell viability after exposure to oxidant, monochloramine. We have previously shown that monochloramine causes injury to IEC-18 cells in a time- and concentration-dependent fashion (18). As shown in Fig. 2, monochloramine (0.3 mM) significantly decreases the viability of IEC-18 cells compared with untreated (non-DEX, nonoxidant-exposed) cells. Treatment with DEX for 96 h at various concentrations (10^{-8} M to 10^{-4} M) significantly reduces oxidant-induced cell injury in a concentration-sensitive fashion. Interestingly, at higher concentrations of DEX, a sig-

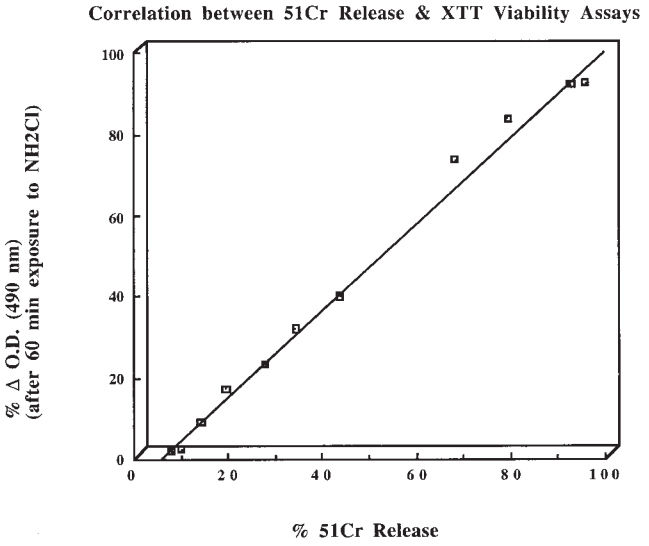

Figure 1. Correlation of two viability assays in IEC-18, ^{51}Cr release, and XTT assays. Cells were treated with variable concentrations of monochloramine (0, 0.03, 0.1, 0.3, 1, and 3 mM in duplicates except 0 mM, $n = 11$) for 60 min. Plot of the percentage of released ^{51}Cr and difference of XTT measurements from nonoxidant-exposed group is shown on x- and y-axes, respectively. $r = 0.996$.

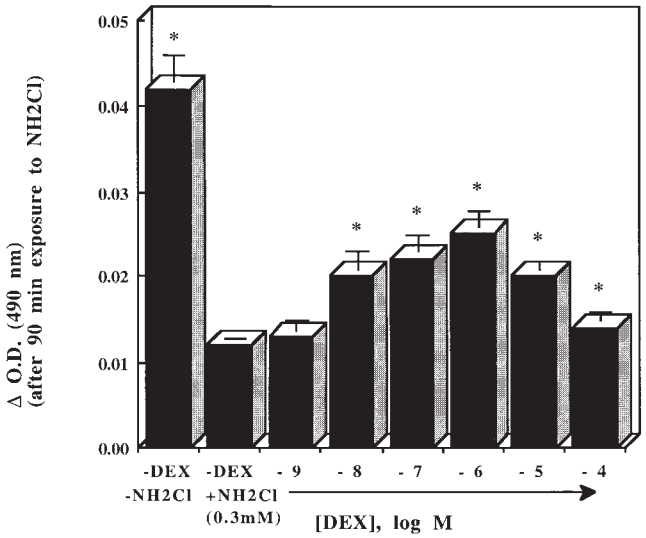

Figure 2. Effect of DEX on monochloramine-induced injury of IEC-18 cells. Cells were treated with varying concentrations of DEX for 96 h in 96-well plates in complete medium. Medium was changed to phenol red and serum-free medium without DEX with the addition of reagents for the XTT assay. Cumulative baseline conversion of XTT to formazan dye was followed for 90 min to assure no difference in cell number and viability. Monochloramine was then added (0.3 mM) and changes in 490 nm absorbance monitored for the next 90 min on ELISA reader. Data shown represents changes after monochloramine introduction. Data shown are means±SEM for three experiments. The asterisks indicate significant difference from (−) DEX, (+) oxidant-exposed group.

nificant diminishment of the protective effect is seen represented by decreased changes of the absorbance. DEX treatment for up to 96 h has no effects on IEC-18 conversion of XTT to the formazan dye, indicating no adverse effects on cell survival (data not shown).

Time course and concentration dependence of DEX induction of hsp72. To determine if the induction of heat shock proteins mediated the increased cellular protection conferred by DEX, the time- and concentration-dependence of DEX effects on IEC-18 hsp72 expression were examined, as hsp72 is the major inducible heat shock protein in these cells (16–18). The cells were treated with 10^{-7} M DEX in complete DMEM, and protein samples were harvested and analyzed for hsp72 expression at 0, 24, 48, 72, and 96-h exposure times. A time-dependent increase in hsp72 expression was observed after DEX treatment, which was first evident at 24 h, and significantly higher expression was seen at 96 h as depicted in Fig. 3. Using the densitometric analysis and setting the value at 96-h exposure as the maximal effect (100%) for each experiment, we determined that the relative values at each time point were as follows: at 24 h, 18±8%; at 48 h, 48±11%; and at 72 h, 83±12% (n = 3). In contrast, the expression of hsc73 was present at baseline without DEX exposure, and no apparent induction of the protein expression was noted upon exposure.

This relatively delayed hsp72 protein expression appears to be temporally associated with its cellular protective effects, as shown in Fig. 4. After 6-h exposure to DEX, where minimal induction of hsp72 protein is observed, no significant enhancement of cellular protection was seen. In contrast, after 96-h exposure to DEX (Fig. 2), cellular viability to oxidant stress was

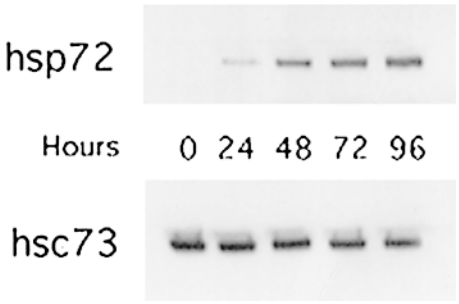

Figure 3. Time-course effect of DEX on IEC-18 cell hsp72 production. Cells were treated with 10^{-7} M DEX in complete DMEM for varying times, and hsp72 and hsc73 expressions were analyzed by Western blot as described in Methods. Image shown is representative of those obtained in three different experiments.

significantly increased, suggesting involvement of hsp72 in conferring this cellular protective effect.

The concentration dependence of DEX effect was investigated using IEC-18 cells after 96-h exposure, a time when hsp72 expression appeared at its peak. As shown in Fig. 5, increased expression of hsp72 induced by DEX could be observed at concentrations as low as 10^{-9} M (1 nM). The maximal effect was observed at $10^{-7} \sim 10^{-6}$ M (1 μM), although decreased expression occurred at greater concentrations of 10^{-5} and 10^{-4} M. These changes closely paralleled the dose effect of this agent on cell viability after oxidant exposure.

DEX specifically affected hsp72 and had no dose effect on the expression of the constitutive homologue to hsp72, hsc73 (Fig. 5). Analyses were performed on the same samples used for hsp72 measurements. Thus, DEX induction of hsp72 expression appeared specific.

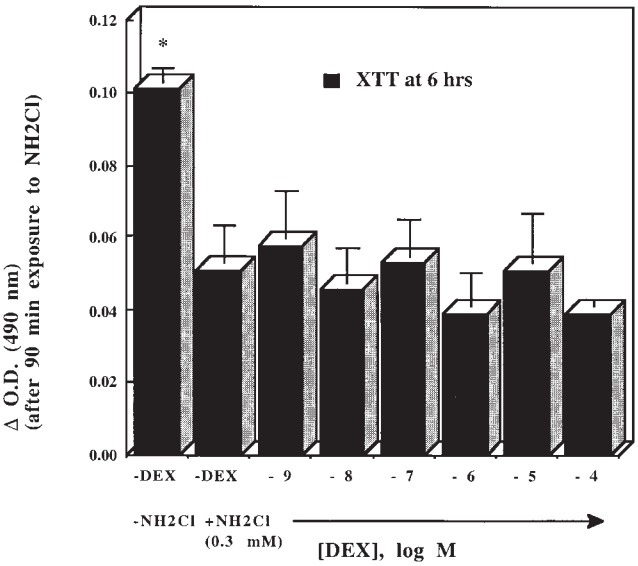

Figure 4. Cellular viability after acute (6 h) exposure to DEX. XTT assays were performed as described for Fig. 2. Graph represents the optical density measurements after 6-h treatment with DEX followed by exposure to monochloramine (compare with Fig. 2). Data shown are means±SEM for three separate experiments. Asterisk indicates significant difference from (−) DEX, (+) oxidant-exposed group.

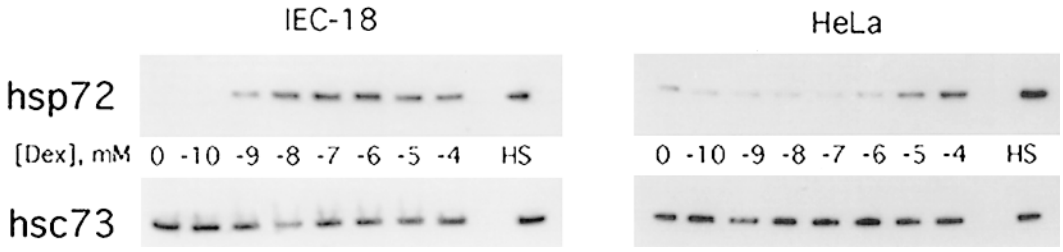

Figure 5. Concentration and tissue-sensitive effect of DEX on hsp72 vs. hsc73 expression. Cells (IEC-18 or HeLa) were treated with varying concentrations of DEX in complete DMEM for 96 h, and hsp72 and hsc73 expressions were analyzed by Western blot as described in Methods. Image shown is representative of those obtained in three difference experiments. HS lane represents protein extracts from heat-shocked cells of respective cell line.

Differences in cellular response to glucocorticoid treatment. To determine if a similar hsp72 induction by DEX could be observed in other types of cells, nonintestinal HeLa cells were studied. These cells were treated with DEX for 96 h and analyzed for hsp72 and hsc73 expression (Fig. 5). In the HeLa cells, the induction of hsp72 expression was substantially less compared with IEC-18 cells at the same pharmacological concentrations of DEX as well as to the thermally treated cells. (Lanes were loaded with equal amount of protein to those for IEC-18 measurements.) Significant increases in hsp72 expression were only observed after exposure of HeLa cells to higher concentrations of DEX (10^{-5} to 10^{-4} M). So there is a greater sensitivity of the hsp72 response in IEC-18 cells to the DEX treatment compared with that in HeLa cells. As in IEC-18, DEX treatment had no substantial effects on hsc73 expression in HeLa cells (Fig. 5).

Antisense inhibition of DEX-stimulated hsp72 response in IEC-18 cells. To determine if there is a cause-effect relationship between DEX induction of hsp72 and increased cellular protection, a full-length rat hsp72 antisense cDNA was stably transfected into IEC-18 cells. As shown in Fig. 6, antisense expression effectively inhibits hsp72 protein expression at all tested concentrations of DEX. However, hsp72 is still inducible after thermal stress, which apparently can overwhelm the anti-inhibitory effects of antisense transfection. In contrast, hsp72 antisense transfection had no effects on hsc73 expression, indicating the specificity of the inhibitory response on hsp72.

The functional effect of inhibiting DEX-induced hsp72 expression was determined by the XTT assays. The basal viability of hsp72 antisense and vector-transfected cells was not different, as determined by conversion of XTT to formazan when cells were not injured. As seen in Fig. 7, the injury was greater in DEX-treated hsp72 antisense cells than in DEX-treated vector-transfected cells. These findings suggest that the DEX-induced hsp72 response plays a major role in mediating the enhanced cellular protective effects of this agent in intestinal epithelial cells.

Dexamethasone induces intestinal mucosal hsp72 expression in vivo. To determine if the in vitro effects of DEX on intestinal epithelial hsp72 expression could be reproduced in vivo, rats were treated with DEX (0.2 mg/kg/d) for 4 d, a dose that approximates therapeutic ranges used clinically in humans. As shown in Fig. 8, DEX treatment of rats causes a significant increase in hsp72 protein expression throughout the small and large intestinal mucosa. The ratio of densitometric measurement of DEX-induced to control of hsp72 expression were as follows (expressed as means±SEM, $n = 3$): 2.98±0.55 (spleen), 4.11±0.96 (colon), 7.34±0.71 (jejunum), and 11.80±1.79 (ileum). Thus, in vivo evidence of induction of hsp72 by DEX in intestinal mucosal cells is demonstrated.

Discussion

This study demonstrates that DEX specifically increases the expression of hsp72, both in vivo and in vitro and appears to

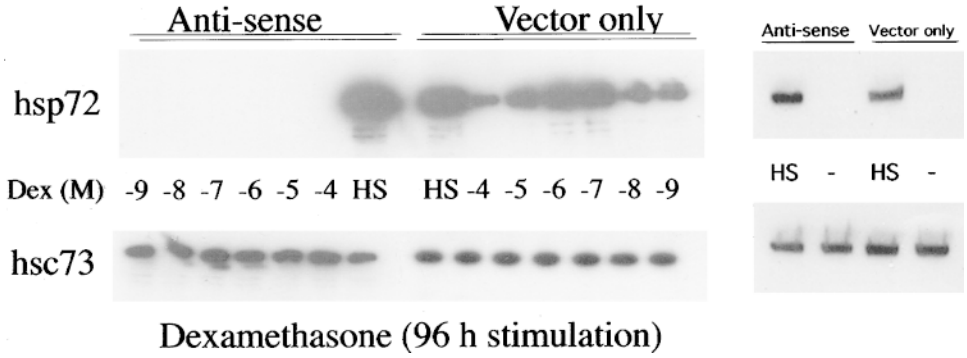

Figure 6. Effect of antisense hsp72 on ability of DEX to induce hsp72 vs. hsc73. Cells (IEC-18) were transfected with either antisense (pG12AS) or vector alone (pCEP4) and selected as described in Methods. Cells were treated with varying concentrations of DEX for 96 h, and then hsp72 and hsc73 expressions were analyzed by Western blot. Image shown is representative of those obtained on three separate occasions. HS lane represents protein extracts from heat-shocked cells. Shown on the right are hsp72 and hsc73 protein expressions of antisense and vector-only cells under thermal stress (HS) and basal conditions (−).

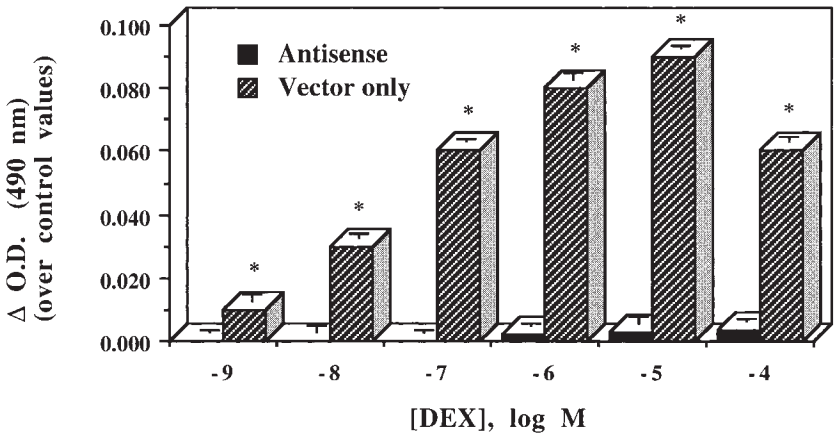

Figure 7. Effect of antisense hsp72 on protective effect of DEX. Antisense or vector-transfected cells were treated for 96 h with varying concentrations of DEX in 96-well plates in complete DMEM. Media was changed to phenol red-free, serum-free DMEM with the addition of reagents for XTT assay. Conversion of XTT to formazan was followed by cumulative increases at 490 nm and at 90 min; monochloramine (0.3 mM) was added and increases in absorbance measured for 90 minutes to measure viability. Data shown are differences of means±SEM from control cells for three experiments. Asterisks indicate significant difference ($P < 0.05$) between antisense and vector-only cells at each concentration of DEX.

confer cellular protection to intestinal epithelial cells against oxidant stress. Stable introduction of antisense hsp72 to the IEC-18 and the consequent loss of the cytoprotective pattern conferred by the glucocorticoid suggest that this is an important mechanism of its protective actions. Although glucocorticoids have been shown to enhance mouse fibroblast viability against cytotoxic concentration of TNF-α, the mechanism of this protective action is unclear (12, 15). Our findings define a unique action and effect of glucocorticoids that may have relevance to the therapeutic actions of these compounds in certain tissues.

Glucocorticoids are known to have numerous cellular effects and sites of action. Inhibition of arachidonic acid metabolite formation, for instance, is thought to be secondary to glucocorticoids' direct dephosphorylation of active phospholipase A2 and to their induction of lipocortin/annexin family of proteins. This induction, in turn, regulates the prostaglandin synthase expression at the level of translation (20, 21). Recent studies demonstrate that glucocorticoids also have a role in transcriptional regulation, in part, by inducing the inhibitory protein, I-κB α, which binds cytoplasmic NF-κB and prevents its translocation into the nucleus (22, 23). Since NF-κB appears to have a major role in the transcriptional activation of several cytokines involved in inflammation, this potentially represents an important anti-inflammatory mechanism of glucocorticoids' action. In the case of nitric oxide synthesis, recent evidence suggests that the ligand-bound glucocorticoid receptor complex prevents the binding of NF-κB, through protein–protein interaction, to the promoter regions of nitric oxide synthase gene. This further minimizes the synthesis of subsequent inflammatory mediators (4). In addition, glucocorticoids appear to repress RelA-mediated activation of the adhesion molecule, ICAM-1, which plays an essential role in recruiting and migration of leukocytes to sites of inflammation (1).

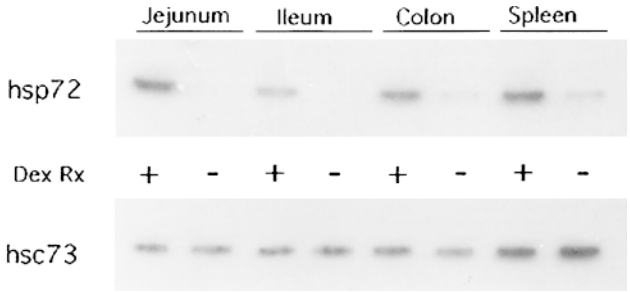

Figure 8. Induction of hsp72 protein expression in rat intestinal mucosal cells upon exposure to DEX. Western analysis of protein extracted from spleen and various intestinal mucosal cells from rats in which one group was DEX treated for 4 d with daily intraperitoneal injections (0.2 mg/kg/d) and the other a control group receiving intraperitoneal sterile saline injections. Image shown is representative of blots obtained from three separate experiments.

Our current study demonstrates a previously unreported mechanism of action of glucocorticoids, i.e., increased cellular protection that is mediated by the induction of hsp72. The mechanism of glucocorticoids' cellular protection may involve stabilization of key intracellular proteins by hsp72. In preliminary investigations, for instance, we have found that hsp72 associates with and stabilizes cytoskeletal proteins under conditions of thermal- and oxidant-induced stress (24). Heat shock proteins have also been known to protect active intermediate forms of intracellular proteins by associating with and preventing them from forming irreversible aggregates. These actions may contribute to the enhancement of cell protection induced by glucocorticoids during oxidant-induced stress.

Two other observations from our studies bear some discussion. The loss of cell viability and diminished hsp72 response at higher concentrations of DEX are consistent findings. A similar dose response to DEX treatment was observed in glucocorticoid regulation of rat hepatocyte glutathione S-transferase genes (25), which appeared to be mediated at the transcriptional level. Possibly, a similar mechanism is involved in DEX-induced hsp72 expression. We also speculate that there may be still unknown mechanisms at play involving the action of glucocorticoids in inducing apoptosis (26–28) at higher concentrations. We also found the differential glucocorticoid-stimulated hsp72 response of tissues to be interesting, as this has not been a well-reported or studied phenomenon. However, we speculate that there may be tissue- and cell-specific factors that account for the differential response of hsp72 to various stimuli. In support of this, we observed that 5-aminosalicylic acid and sodium salicylate augment and accelerate the hsp72 response to thermal stress in intestinal epithelial IEC-18 cells by accelerating the binding of the heat shock factor to the heat shock element (17). In contrast, these agents have a different effect and action on the thermal response of HeLa cells, where they lower the activation threshold for thermal induction but do not augment the response (16). Thus, these data and the findings of this study suggest that there may be critical intrinsic differ-

ences between cell types that determine the nature and magnitude of the inducible heat shock protein response. In the gastrointestinal tract, particularly in the colon, which is constantly under a state of physiological inflammation from various antigenic load, the increased responsiveness of the intestinal epithelium to stress may be teleologically important to ensure preservation of critical ion transport and barrier function. The increased responsiveness of the intestinal epithelium to agents such as glucocorticoids and glutamine (16) compared with nonintestinal cells may also have therapeutic significance. These agents have been proven useful in the treatment of patients with inflammatory bowel disease, albeit their mechanisms of action are incompletely understood. It is possible that part of their therapeutic actions are mediated by the induction of cytoprotective heat shock proteins such as hsp72.

Acknowledgments

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