EFFECTS OF PROTEIN MALNOURISHMENT AND CORTICOSTERONE ON THYMOCYTE APOPTOSIS

By Erin Crowgey

Previous research in this laboratory demonstrated that protein malnourishment causes increased levels of serum corticosterone and thymic atrophy in mice. The purpose of this study was to determine the effects of up-regulated serum corticosterone on thymocyte apoptosis, and the effects of a stress response on corticosterone-induced apoptosis. This study confirmed that protein malnourishment induces thymic atrophy. When mouse thymocytes were treated in vitro with corticosterone and assayed for apoptosis by quantifying phosphatidylserine externalization, mitochondrial permeabilization, and DNA fragmentation, corticosterone was shown to induce thymocyte apoptosis. When thymocytes from protein deficient mice were assayed for apoptosis, phosphatidylserine externalization and mitochondrial permeability were significantly altered, but DNA fragmentation was not. To determine how a stress response could alter thymocyte apoptosis, protein sufficient thymocytes were heat shocked and then treated with corticosterone in vitro. Heat shock decreased corticosterone-induced apoptosis and increased heat shock protein (hsp) 70 and hsp90 levels in these normal mouse thymocytes. However, when hsp70 and hsp90 were quantified in thymocytes from protein deficient mice, hsp70 or hsp90 were not significantly increased. In conclusion this study describes the significance of corticosterone-induced thymocyte apoptosis during protein malnourishment.
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INTRODUCTION

Protein malnourishment is a severe problem in both developed and developing countries because it influences the body’s ability to fight infectious agents (40). This laboratory has developed a model system, using weanling mice, to study the effects of protein malnourishment on the immune system, focusing on both adaptive and innate immune responses. Previous studies have demonstrated that mice maintained on a protein deficient diet have increased levels of serum corticosterone, increased thymic atrophy, and up-regulation of stress proteins in thymocytes (1, 7, 8). The current study focuses on how corticosterone and stress proteins influence thymocyte apoptosis.

To understand the effects of protein malnourishment on the thymus, it is first essential to understand the normal development of thymocytes. Lymphoid progenitor cells that migrate into the thymus undergo a series of phenotypic and genotypic differentiations that allow them to develop into mature T cells, the regulators of the immune system (4). When lymphoid progenitor cells enter the thymus they are referred to as double-negative cells, because these thymocytes do not express either CD4 or CD8 on their surface. CD4 and CD8 interact with major histocompatibility complexes (MHC) class II and MHC class I, respectively, and act as co-receptors with the T cell receptor (TCR), which is composed of two chains, $\alpha$ and $\beta$. As they begin development in the cortex of the thymus, double-negative cells undergo rearrangement of their TCR $\beta$ chain genes, and express the newly synthesized $\beta$ chain on their surface together with pre-T$\alpha$ and CD3 (6). Next, these double-negative cells undergo positive selection, and those cells expressing a functional TCR $\beta$ chain will be induced to proliferate, otherwise known as clonal expansion. These cells will then up-regulate the expression of both CD4 and CD8 (6), followed by rearrangement of their TCR $\alpha$ genes. The double-positive cells able to express functional TCR $\alpha$ and $\beta$ chains will migrate across the corticomedullary junction into the medulla of the thymus (38). As these cells migrate they undergo negative selection, which eliminates cells expressing a TCR that interacts with self-antigen/MHC with too high an affinity (6). If they survive negative selection, they differentiate into single-positive cells expressing either CD4 or CD8. These naïve mature T cells then leave the thymus to circulate throughout the body and populate the peripheral lymphoid tissues.
Corticosterone, a glucocorticosteroid hormone secreted by the adrenal cortex and locally synthesized in the thymus of the mouse, is thought to play an essential role during both positive and negative selection by regulating thymocyte apoptosis (29). Corticosterone diffuses across the plasma membrane and binds to the glucocorticoid receptor (GR) complex. Each cytoplasmic GR complex contains one molecule of GR associated with molecular chaperones, such as hsp90, and immunophilins, such as Cyp40 (cyclophilin). The molecular chaperones facilitate folding of the hormone-binding domain of the GR into its high affinity glucocorticoid binding conformation (34). The immunophilins are thought to play a role in signaling transduction pathways that influence cell cycle progression (40). Once the glucocorticoid binds to the GR complex, the complex undergoes a conformational change, releasing the ligand-bound GR from the other components of the receptor complex and exposing the nuclear localization sequence (NLS), which allows it to translocate into the nucleus (40).

The GR complex belongs to the large family of nuclear receptors that act as transcription factors to control gene expression in a ligand-dependent manner by binding to DNA or to other transcription factors. These interactions are dependent on coactivators and corepressors that regulate the structure of chromatin and recruitment of the basal transcriptional machinery (24). The GR has a hinge region that contains the NLS, which allows the GR complex to interact with molecules of the nuclear pore, and permits its translocation into the nucleus. The GR also has a conserved C-terminal ligand-binding region, which associates with another ligand-bound GR. The GR dimer binds to DNA sequences termed glucocorticoid response elements (GREs), and acts as a co-activator in gene transcription. The GR protein also has an N-terminal variable domain region that allows it to interact with other transcription factors, and thus acts as a transrepressor (4) because it limits their ability to bind DNA. It is thought that both the transactivation and the transrepression properties of the GR contribute to activation of the apoptotic pathway (17). Once these interactions take place inside the nucleus, they trigger numerous events that occur outside the nucleus.

One of the earliest measurable events in apoptosis is the translocation of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma
membrane (Fig. 1A). PS is synthesized in the endoplasmic reticulum (ER) by a base exchange reaction in which serine is exchanged for the ethanolamine moiety of phosphatidylethanolamine or for the choline moiety of phosphatidylcholine (45). These reactions do not require metabolic energy; however, they are dependent on calcium. The mechanism of this translocation process during apoptosis is not completely understood, although current research has indicated the importance of cytoplasmic calcium flux as a triggering event, the loss of function of aminophospholipid translocase in the membrane, and the activation of a scramblase in the membrane (11). Recently, Annemiek et al. demonstrated that loss of phospholipid asymmetry, which occurs during externalization of PS, causes sphingomyelin to translocate from the outer leaflet to the inner leaflet of the plasma membrane (3). Once sphingomyelin has translocated to the inner leaflet, it serves as a substrate for intracellular sphingomyelinases. The hydrolysis of sphingomyelin by sphingomyelinases releases ceramide, an important secondary signaling molecule. Ceramide is involved in numerous signaling pathways, such as activation of protein phosphatase 1, induction of mitochondrial permeabilization, and caspase activation (46).

Another key feature of apoptosis induced by corticosterone is mitochondrial permeabilization (Fig. 1B). During apoptosis, the Bcl-2 family of proteins act as anti-apoptotic or pro-apoptotic molecules that mostly interact with each other and associate with the membranes of mitochondria and the ER (2). The voltage-dependent anion channel (VDAC), an important complex involved in maintaining the potential of the mitochondrial membrane, interacts with both anti-apoptotic and pro-apoptotic molecules. Pro-apoptotic molecules, such as Bak, Bid, and Bim, are thought to cause a disruption in membrane potential as a result of their interaction with the VDAC. This allows certain molecules, such as apoptosis inducing factor (AIF) and cytochrome c (Fig. 1), to be released from the mitochondria into the cytoplasm, where they interact with other molecules that help in the destruction of the cell (2). This is countered by anti-apoptotic molecules, such as Bcl-2 and Bcl-xL, that sequester pro-apoptotic molecules, preventing their interactions with the mitochondrial membrane. Activation of both pro- and anti-apoptotic molecules is thought to involve phosphorylation of active sites and dephosphorylation of inhibitory sites on these proteins (2). Interestingly, current research
Figure 1. Schematic representation of three major events induced by corticosterone in thymocytes. 

A. Translocation of PS (red rectangle) is an early apoptotic event induced by corticosterone in thymocytes. 

B. Mitochondrial permeabilization, which allows cytochrome c (blue circle) to be released into the cytoplasm, is an intermediate apoptotic event induced by corticosterone in thymocytes. 

C. DNA fragmentation, induced by caspases activation (orange hexagon), is a late apoptotic event induced by corticosterone in thymocytes.
has also shown the generation of free oxygen radicals during apoptosis induced by
corticosterone (39). These highly reactive oxygen free radical species cause significant
damage to the cell by causing further mitochondrial permeabilization and oxidation of
other cellular components.

When mitochondrial permeabilization occurs, cytochrome c and an endonuclease,
AIF, are released into the cytoplasm of the thymocytes. Once in the cytoplasm,
cytochrome c binds to apoptosis peptidase activating factor-1 (APAF-1) to form a
complex termed the apoptosome. This complex activates procaspase 9, which can then
cleave other procaspases, inducing a cascade effect. The proteolytic activities of caspases
cause the degradation of proteins, which contributes to the destruction of the cell.
Eventually, caspases activate the endonucleases that, together with AIF, are responsible
for DNA fragmentation in the nucleus, yet another key feature of apoptosis (Fig. 1C).
Corticosterone-induced apoptosis is a complicated pathway that is intertwined to cause
the destruction of not only the cell as a whole, but also the individual organelles within
the cell.

This apoptotic pathway can be regulated by numerous proteins that act as anti-
apoptotic molecules. Current research has shown that hsps, molecular chaperones that
facilitate the correct folding of proteins and protein-containing structures, are capable of
down-regulating apoptosis (1, 35, 36). The anti-apoptotic actions of hsps are complex
and thought to involve interactions with pro-apoptotic molecules in the cytoplasm, such
as the apoptosome and caspases (35, 36). Hsps are normally expressed constitutively, but
they can be up-regulated. This up-regulation is controlled by the heat shock transcription
factors (HSFs). Under normal cellular conditions, HSFs exist as monomers or dimers;
however, certain stress stimuli induce conformational changes in HSF1 and HSF3 (32).
These conformational changes allow the transcription factors to form trimers, which bind
to DNA with a high affinity, and cause up-regulation of the expression of heat shock
proteins.

This study first confirmed that protein malnourishment induces thymic atrophy.
The next goal of the present research was to assess how corticosterone influences
thymocyte apoptosis in vitro as measured by PS externalization, mitochondrial
permeabilization, and DNA fragmentation. It was found that corticosterone, at the
concentration seen in serum of protein malnourished mice, induced thymocyte apoptosis in vitro. Next, thymocyte apoptosis was quantified in protein deficient mice. These results indicated that protein malnourishment induces thymocyte apoptosis after 7 days on the diet. Previous research from this laboratory had indicated that protein malnourishment also induced a stress response in mice that was characterized by elevated levels of hsp70 and hsp90, and further indicated that these proteins could influence corticosterone-induced apoptosis (1). In the current study, it was found that heat shock, which up-regulates hsp70 and hsp90, can down-regulate corticosterone-induced thymocyte apoptosis in vitro as measured by decreased PS externalization, mitochondrial permeabilization, and DNA fragmentation. To determine a role for heat shock proteins in thymocyte apoptosis during protein malnourishment, both hsp70 and hsp90 were quantified in mouse thymocytes. It was found in this study that protein malnourishment did not increase hsp70 or hsp90 in mouse thymocytes.
MATERIALS AND METHODS

Mice, Diets, and Thymocyte Preparation

Protein malnourishment studies were conducted in a manner similar to those described by Barone et al. (7, 8). Weanling (21 days old +/- 3 days) female CD2F1 mice (Harlan Sprague, Dawley, Inc., Indianapolis, IN) were maintained on either a protein sufficient diet (TD.86495 with 20% egg white solid), or a protein deficient diet (TD.88088 with 1.25% egg white solid) (Harlan-Teklad, Madison, WI). The in vitro corticosterone and heat shock experiments used thymocytes from adult female CD2F1 mice (Harlan Sprague Dawley). These mice were maintained on Purina Mills Laboratory Rodent Diet 5001 (23.4% protein; Richland, IN). In all cases, the mice had unlimited access to food and deionized water.

Mice were killed by carbon dioxide inhalation. The thymus was then aseptically removed from each mouse and placed into 10ml of tissue culture medium (TCM) comprised of RPMI-1640 (Cellgro, Herndon, VA) containing 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO). The thymus was disaggregated by gentle compression between the frosted ends of glass slides. The resulting thymocytes were washed twice by centrifugation at 300xg for 10min, followed by suspension in 10 ml of fresh TCM. The number of viable cells per ml was determined by trypan blue exclusion. All thymocyte suspensions were adjusted to 10^6 cells/ml, and 1ml aliquots were placed in sterile 12x75 mm culture tubes with snap caps (USA Scientific, Ocala, FL).

Corticosterone Induction of Apoptosis

Corticosterone was added to thymocyte cultures at a final concentration of 600ng/ml, which is the average concentration of corticosterone measured in the serum of protein malnourished mice (1, 6, 7). The corticosterone (Sigma-Aldrich) was dissolved in 95% ethanol, filter-sterilized, and then diluted with TCM to a working concentration of 6000ng/ml. To control for the effects of ethanol on cells, all corticosterone controls contained the same amount of ethanol (0.006%). Unless indicated otherwise, all incubation of cells was carried out 37°C in a 95% air, 5% CO₂ atmosphere. After incubation for the appropriate times, the cells were assayed for apoptosis using Annexin V, MitoTracker Orange, and TUNEL assays as described below.
**PS Externalization**

Annexin V-FITC Apoptosis Detection Kits 1 (BD Biosciences, San Jose, CA) were used to quantify PS externalization. The protocol provided with the kits was followed. In brief, cells were pelleted by centrifugation at 300xg for 10 min at 4°C followed by suspension in 1ml of phosphate buffer solution (PBS; 137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2). The cells were then pelleted as before, followed by suspension in 1ml of Annexin V Binding Buffer (10mM HEPES, 140mM NaCl, 2.5 mM CaCl₂, pH 7.4). Next, 300µl of the total cell suspension was used for staining by adding 3µl of Annexin V-FITC and 3µl of propidium iodide staining solution. The cells were incubated for 15min at 25°C in the dark, then they were transferred into 12x75 mm polystyrene tubes and analyzed on a FACScan flow cytometer using Cellquest 5.1.1 software (Becton Dickinson, San Jose, CA) as depicted in Figure 2A. Cells in quadrant 1 have bound propidium iodide only, and would therefore be considered necrotic debris. Cells in quadrant 2 have bound both propidium iodide and Annexin-V-FITC, and would therefore be considered necrotic. Cells in quadrant 3 have not bound either propidium iodide or Annexin-V-FITC, and would therefore be considered viable. Cells in quadrant 4 have bound only Annexin-V-FITC, and would therefore be considered apoptotic.

**Mitochondrial Permeabilization**

MitoTracker Orange (CMTMRos, Molecular Probes, Eugene, Oregon) was used to quantify mitochondrial permeabilization. Briefly, a 1.25x10⁻⁵ M solution of MitoTracker Orange was prepared in TCM and added to the thymocyte suspensions at a final concentration of 1.25x10⁻⁷ M. After they had been incubated for 30min at 37°C as previously described, the cells were pelleted by centrifugation at 300xg for 10min at 37°C followed by suspension in 1ml of pre-warmed TCM. The cells were then incubated for 30min at 37°C as previously described. Finally, the cells were transferred to 12x75 mm polystyrene tubes and analyzed on a FACScan flow cytometer using Cellquest 5.1.1 software as depicted in Figure 2B. Thymocytes with mitochondria able to retain the MitoTracker Orange dye were considered viable, whereas thymocytes with mitochondria unable to retain the MitoTracker Orange dye were considered apoptotic.
Figure 2. Interpretation of flow cytometry data for Annexin V, MitoTracker Orange, and TUNEL assays. Panel A represents a four quadrant analysis for the Annexin-V-FITC assay. The X-axis (Fl-1) represents the fluorescent intensity for Annexin V-FITC and the Y-axis represents the fluorescent intensity for propidium iodide. The topological lines represent the densities of cells. Cells in quadrant 1 have bound propidium iodide only, and would therefore be considered necrotic debris. Cells in quadrant 2 have bound both propidium iodide and Annexin-V-FITC, and would therefore be considered necrotic. Cells in quadrant 3 have not bound either propidium iodide or Annexin-V-FITC, and would be considered viable. Cells in quadrant 4 have bound only Annexin-V-FITC, and would therefore be considered apoptotic. Panel B represents a histogram for the MitoTracker Orange assay. The X-axis represents the fluorescent intensity for the MitoTracker Orange dye, and the Y-axis represents number of cells. Cells in peak 1 have mitochondria unable to retain MitoTracker Orange in their membranes, and would therefore be considered to be positive for apoptosis. Cells in peak 2 have mitochondria able to retain MitoTracker Orange in their membranes, and would therefore be considered as negative for apoptosis. Panel C represents a histogram of the TUNEL assay. The X-axis represents the fluorescent intensity for the Streptadivin bound dNTPs conjugated with biotin. The Y-axis represents number of cells. Cells in peak 1 do not have dNTPs conjugated with biotin incorporated into their DNA, and would therefore be considered negative for apoptosis. Cells in peak 2 have dNTPs conjugated with biotin incorporated into their DNA, and would therefore be considered positive for apoptosis.
DNA Fragmentation

FlowTACS Apoptosis Detection Kits (R&D Systems, Inc., Minneapolis, MN), otherwise referred to as TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling), were used to quantify DNA fragmentation. Briefly, thymocytes were pelleted by centrifugation at 300xg for 10min at room temperature and suspended in a 3.7% formaldehyde solution (Sigma-Aldrich). After a 10min incubation at room temperature, the cells were pelleted by centrifugation at 300xg for 10min at 25°C and suspended in 1ml of PBS. The cells were incubated for 2min at room temperature and then pelleted by centrifugation as described above. The cells were then suspended in 100µl of Cytonin and incubated for 30min at room temperature to permeabilize them. Next, the cells were pelleted as described above and suspended in 1ml of TdT Labeling Buffer Solution. The cells were then pelleted as described above and suspended in 25µl of Labeling Reaction Mix, which contained dNTPs conjugate with biotin and TdT, the enzyme that incorporates the dNTPs onto the 3’hydroxyl end of fragmented DNA. Positive controls were generated by adding 25µl of a TACS-Nuclease Working Solution to cells, followed by 15min incubation at 37°C. Next these cells were pelleted as described above and suspended in 1ml of Labeling Buffer. The cells were again pelleted as described above and suspended in 25µl of Labeling Reaction Mix. The cells were incubated in the Labeling Reaction Mix for 1h at 37°C, after which the labeling reaction was stopped by adding 1ml of Stop Buffer. Next, the cells were pelleted, as described above, followed by suspension in 25µl of diluted Streptavidin-FITC Working Solution. The cells were then incubated for 10min in the dark at room temperature to allow binding of the Streptavidin-FITC Working Solution to the biotin on the dNTPs that had been incorporated into fragmented DNA by the enzymatic activities of TdT. After incubation, the cells were pelleted as described above, followed by suspension in 500µl of PBS. The cells were then transferred to 12x75 mm polystyrene tubes and analyzed on a FACScan flow cytometer using Cellquest 5.1.1 software as depicted in Figure 2C.

Heat Shock

Heat shock experiments were initiated by placing thymocyte suspensions in a water bath at 42°C for 20min, followed by the addition of corticosterone at a final concentration of 600ng/ml. The cells were then incubated at 37°C as previously
described until assayed for apoptosis using Annexin V at 8h, MitoTracker Orange at 18h, and TUNEL at 24h. Furthermore, hsp70 and hsp90 levels were quantified at 0, 3, 6, 12, 18 and 24 h as described below.

**Intracellular Staining of Hsp70 and Hsp90**

Thymocyte hsp70 and hsp90 concentrations were quantified using an intracellular flow cytometric assay. Thymocyte suspensions were pelleted by centrifugation at 300xg for 10min at 25°C and suspended in 100µl of Fixation Medium Reagent A (Serotec, Oxford, United Kingdom). After a 15min incubation at room temperature, 2ml of PBS were added to each tube. The cells were washed twice by centrifugation at 300xg for 10min at 25°C followed by suspension in 1ml of PBS, and then suspended in 100µl of Permeabilization Medium Reagent B (Serotec, Oxford, United Kingdom). Next, 0.5µl of anti-mouse hsp70 antibody (2µg/µl; Stressgen, British Columbia, Canada) or 0.5µl anti-rat hsp90 antibody (2µg/µl; Stressgen) was added to the cells, followed by 1µl of goat anti-mouse IgM antibody conjugated with FITC (2µg/µl; Sigma-Aldrich) for hsp70, or 1µl of goat anti-rat IgG antibody conjugated with PE (2µg/µl; Sigma-Aldrich) for hsp90. The cells were incubated with these antibodies for 15min in the dark at 25°C, then washed twice by centrifugation at 300xg for 10min at 25°C followed by suspension in 2ml of fresh PBS. After the final centrifugation, the cells were suspended in 500µl of PBS, transferred to 12x75 mm polystyrene tubes, and analyzed on a FACScan flow cytometer using Cellquest 5.1.1 software.

Hsp70 expression was determined by quantifying the mean fluorescent intensity (MFI) of cells that increased in their fluorescence, from the total population of cells, due to increased expression of hsp70. Furthermore, for the heat shock experiments, hsp70 induction was determined by dividing the MFI of heat shocked cells that expressed hsp70, by the MFI of non-heat shocked cells that expressed hsp70. Hsp90 expression was determined by quantifying the MFI for the total population of cells, because hsp90 was constitutively expressed. Additionally, hsp90 induction was determined by dividing the MFI of heat shocked cells, by the MFI of non-heat shocked cells.
Analysis of Data and Statistics

Michael R. Hughes, Statistical Consulting Service, Department of Mathematics and Statistics, Miami University, performed the statistical analyses. Statistical significance of differences among experimental values was determined using a 2–factorial factor analysis of variance. Values of $p \leq 0.05$ were considered significant.
RESULTS

Protein Malnourishment Causes Thymic Atrophy

Previous research in this laboratory demonstrated that protein malnourishment causes thymic atrophy (1, 7, 8). To confirm these results, mice were maintained on either a protein sufficient diet or a protein deficient diet, and thymic cell yields were determined on days 0, 7, and 14 of the dietary period. Mice maintained on the protein sufficient diet had a 1.3-fold increase in thymocyte numbers on days 7 and 14, compared to day 0 (Fig. 3). In contrast, mice maintained on the protein deficient diet had a 2.7-fold decrease in thymocyte numbers on days 7 and 14, compared to day 0 (Fig. 3). Overall, mice maintained on the protein deficient diet for 14 days had a 10-fold lower number of thymocytes compared to mice maintained on the protein sufficient diet. These data support earlier findings indicating that protein malnourishment causes thymic atrophy (1, 7, 8).

Corticosterone Induces Thymocyte Apoptosis In Vitro

Previous research in this laboratory demonstrated that mice maintained on protein deficient diets have increased thymic atrophy correlated with high levels of serum corticosterone (1, 7, 8). Corticosterone is thought to aid in the recycling of amino acids to help the body maintain protein synthesis, as well as stimulating glycogen production (4). However, high levels of corticosterone have also been found to induce thymocyte apoptosis (1). To determine how corticosterone is influencing thymic atrophy in protein malnourished mice, an in vitro model system was used. Thymocytes from protein sufficient mice were treated in vitro with corticosterone at the average concentration seen in the serum of protein deficient mice, 600ng/ml, and then assayed for apoptosis using three techniques. Phosphatidylserine externalization, an early apoptotic event, was measured after 0, 3, 6, and 12 h of incubation with corticosterone. It was found that corticosterone induced phosphatidylserine externalization in mouse thymocytes starting at 3h exposure (Fig. 4A). Furthermore, after a 12h exposure there was a 6-fold increase in the amount of phosphatidylserine externalization on mouse thymocytes treated with corticosterone compared to mouse thymocytes not treated with corticosterone. These findings indicate that corticosterone, at the concentration seen in the serum of protein...
Figure 3. **Protein malnourishment causes thymic atrophy.** Weanling mice were maintained on either a protein sufficient or a protein deficient diet. On days 0, 7, and 14 days of the dietary period, thymic cell yields were determined. Data are expressed as mean +/- standard deviation. Values represent data from 3 mice per group on each day of the dietary period.
Thymocyte Yields

Number of Cells per Thymus ($\times 10^7$)

Time on Diet (days)

protein sufficient
protein deficient
Figure 4. Corticosterone induces apoptosis in mouse thymocytes in vitro. Mouse thymocytes were treated in vitro with 600ng/ml of corticosterone, as indicated. A. After 0, 3, 6, and 12 h exposure to corticosterone, thymocytes were assayed for PS externalization using an Annexin-V kit. Thymocytes that bound Annexin-V-FITC only were considered positive for apoptosis, whereas thymocytes that bound propidium iodide only, or bound both propidium iodide and Annexin-V, were considered necrotic. B. After 0, 6, 12, 18, and 24 h exposure to corticosterone, thymocytes were assayed for mitochondrial permeabilization using MitoTracker Orange. Values represent thymocytes with mitochondria unable to retain MitoTracker Orange. C. After 24 h exposure to corticosterone, thymocytes were assayed for DNA fragmentation using a TUNEL assay. All data were obtained by flow cytometry, and are expressed as mean +/- standard deviation (n=3).
A. PS Externalization

![Graph showing PS Externalization](image)

B. Mitochondrial Permeabilization

![Graph showing Mitochondrial Permeabilization](image)
C. DNA Fragmentation

Percent of Cells with Fragmented DNA

Untreated | Corticosterone

0 | 0
10 | 0
20 | 0
30 | 0
40 | 0
50 | 0
60 | 0
70 | 0
80 | 0
90 | 0
malnourished mice, induces this early event in the apoptotic pathway in thymocytes in vitro. Thymocytes were assayed for mitochondrial permeabilization, an intermediate apoptotic event, after 0, 6, 12, 16, and 24 h exposure to corticosterone. Corticosterone induced mitochondrial permeabilization after 6 h of exposure (Fig. 4B). Furthermore, corticosterone caused a 2-fold increase of mitochondrial permeabilization at 24 h exposure. These findings indicate that corticosterone, at the concentration seen in the serum of protein malnourished mice, induces this intermediate event in the apoptotic pathway in thymocytes. Thymocytes were assayed for DNA fragmentation after 24 h of exposure to corticosterone. Corticosterone caused almost a 4-fold increase in DNA fragmentation in mouse thymocytes (Fig. 4C). This finding indicates that corticosterone, at the concentration seen in protein malnourished mice, is able to induce this late event in the apoptotic pathway. Taken together, the results of these three in vitro assays demonstrate that corticosterone at the concentration seen in the serum of protein malnourished mice is able to induce thymocyte apoptosis.

**Apoptotic Events Are Differentially Regulated by Protein Malnourishment**

To further investigate the relationship between elevated serum corticosterone concentration and thymic atrophy, thymocyte apoptosis was quantified in mice maintained on a protein deficient diet for up to 14 days and compared to thymocyte apoptosis in mice maintained on a protein sufficient diet. After 8 h in culture, thymocytes were assayed for PS externalization. On day 7 of the dietary period, thymocytes from mice maintained on the protein deficient diet had more PS externalization than thymocytes from mice maintained on a protein sufficient diet. However, on day 14 of the dietary period this trend was reversed; thymocytes from mice maintained on the protein sufficient diet had more PS externalization than thymocytes from mice maintained on the protein deficient diet (Fig. 5A). After 18 h in culture, the thymocytes were assayed for mitochondrial permeabilization. On both day 7 and day 14 of the dietary period, thymocytes from mice maintained on the protein deficient diet had more thymocytes with permeabilized mitochondria than thymocytes from mice maintained on the protein sufficient diet (Fig. 5B). After 24 h in culture, the thymocytes were assayed for DNA fragmentation. On both day 7 and day 14, DNA fragmentation in
Figure 5. Apoptotic events are differentially regulated by protein malnourishment. Weanling mice were maintained on either a protein sufficient or a protein deficient diet for 0, 7, or 14 days, then thymocytes were placed in culture. A. After 8 h in culture, thymocytes were assayed for PS externalization using an Annexin-V kit. Thymocytes that bound Annexin-V-FITC only were considered positive for apoptosis, whereas thymocytes that bound propidium iodide only, or bound both propidium iodide and Annexin-V, were considered necrotic. B. After 18 h in culture, thymocytes were assayed for mitochondrial permeabilization using MitoTracker Orange. Values represent thymocytes with mitochondria unable to retain MitoTracker Orange. C. After 24 h in culture, thymocytes were assayed for DNA fragmentation using the TUNEL assay. All data were obtained by flow cytometry, and are expressed as mean +/- standard deviation (n=3). Statistical analysis was done by a 2-factor factorial analysis of variance, and * represents a statistical difference with $p \leq 0.0001$. 
A. PS Externalization

B. Mitochondrial Permeabilization
DNA Fragmentation

C.

Percent of Cells with Fragmented DNA

Time on Diet (days)

protein sufficient
protein deficient
thymocytes was not significantly different between the two groups of mice. Taken together, these results indicate that both an early and an intermediate apoptotic event induced by corticosterone were occurring at elevated rates in thymocytes from protein malnourished mice; however, a late apoptotic event was not detected.

**Heat Shock Up-Regulates Hsp70 and Hsp90, but Down-Regulates Corticosterone-Induced Apoptosis**

Based on previous research (1, 7, 8), it was speculated that protein malnourishment induces a mechanism that modulates corticosterone-induced apoptosis. These observations were based on a study that implanted bioerodable pellets that released corticosterone into mice. Mice with these inserts, but maintained on a protein sufficient diet, had significantly more thymic atrophy than mice maintained on either a protein sufficient or protein deficient diet (8). Furthermore, thymocytes from mice maintained on a protein deficient diet were found to have higher expression levels of hsp70 and hsp90 than mice maintained on a protein sufficient diet (1). Heat shock proteins are chaperones that facilitate the proper folding of proteins and have been shown to down-regulate apoptotic pathways. Therefore, it was hypothesized that hsps could act as anti-apoptotic molecules during corticosterone-induced apoptosis. An in vitro system was used to study this hypothesis. Thymocytes were assayed for hsp70 or hsp90 using intracellular fluorescent antibody staining at 0, 3, 6, 12, and 24 h after heat shock. It was found that heat shock caused a gradual increase of hsp70 expression in mouse thymocytes, reaching a 2-fold increase by 12 h. Furthermore, hsp70 remained at this elevated level at the 24 h time point (Fig. 6A). Expression of hsp90 was also increased by heat shock, reaching almost a 2-fold higher expression level by 6 h. Hsp90 expression gradually decreased from the 12 h time point to basal levels by the 24 h time point (Fig. 6B). These results indicated that heat shock was useful as an vitro model system to study increased levels of hsps in thymocytes.

To determine if hsps could provide protection against corticosterone-induced apoptosis, thymocytes were heat shocked, then treated with corticosterone (600ng/ml). After 8 h in culture, these thymocytes were assayed for PS externalization; after 16 h in culture, they were assayed for mitochondrial permeabilization; and after 24 h in culture, they were assayed for DNA fragmentation. When thymocytes were heat shocked and
Figure 6. Heat shock up-regulates hsp70 and hsp90 expression. Mouse thymocytes were heat shocked at 42°C for 20 min and hsp70 and hsp90 expression levels were quantified 0, 3, 6, 12, and 24 h after heat shock. A. Hsp70 expression was quantified by intracellular staining using anti-hsp70 antibody and FITC-conjugated anti-mouse IgG antibody. Hsp70 induction was determined by dividing the MFI of heat shocked cells that expressed hsp70, by the MFI of non-heat shocked cells that expressed hsp70.

B. Hsp90 expression was quantified by intracellular staining using anti-hsp90 IgM antibody and PE-conjugated anti-rat IgM antibody. Hsp90 induction was determined by dividing the MFI from heat shocked cells, by the MFI of non-heat shocked cells (n=3).
A. Hsp70 Expression

Fold Induction of Hsp70 Levels vs. Time in Culture (h)

B. Hsp90 Expression

Fold Induction of Hsp90 Levels vs. Time in Culture (h)
then treated with corticosterone, PS externalization decreased by 10% compared with thymocytes not heat shocked but treated with corticosterone (Fig. 7A). Heat shock also caused decreased corticosterone-induced mitochondrial permeabilization by about 5% (Fig. 7B). Additionally, heat shock decreased DNA fragmentation induced by corticosterone by 52% (Fig. 7C). Taken together, the results of these three assays support the idea that increased levels of hsp70 and hsp90 correlate with a reduction in apoptotic events in thymocytes.

**Protein Malnourishment Does Not Significantly Increase Hsp70 or Hsp90**

The above results, combined with results from previous research (1), suggested a potential role for heat shock proteins in modulating thymocyte apoptosis induced by corticosterone during protein malnourishment. Therefore, weanling mice were maintained on either a protein sufficient diet or a protein deficient diet for 0, 7, or 14 days, and then thymocytes were removed for quantification of hsp70 and hsp90 levels on each of these days. Due to increase variability of hsp70 and hsp90 expression at the time of thymus removal, thymocytes were cultured for 3 hr prior to measuring hsp70 and hsp90. Intracellular fluorescent antibody staining 3h after thymocytes were placed into culture indicated that the expression level of hsp70 in thymocytes from mice maintained on the protein deficient diet was not significantly different than the hsp70 expression in thymocytes from mice maintained on the protein sufficient diet (Fig. 8A). Furthermore, there was no significant difference in hsp90 expression in thymocytes from mice maintained on the protein deficient diet and that in thymocytes from mice maintained on the protein sufficient diet (Fig. 8B). Taken together, these results suggest that neither hsp70 nor hsp90 expression in thymocytes is influenced by protein malnourishment in this mouse model.
Figure 7. Heat shock down-regulates corticosterone-induced apoptosis. Mouse thymocytes were heat shocked at 42°C for 20 min and then treated with 600ng/ml of corticosterone (Cort), then cultured until assayed for apoptosis. A. After 12 h in culture, thymocytes were assayed for PS externalization using an Annexin-V kit. Thymocytes that bound Annexin-V-FITC only were considered positive for apoptosis, whereas thymocytes that bound propidium iodide only, or bound both propidium iodide and Annexin-V, were considered necrotic. B. After 18 h in culture, thymocytes were assayed for mitochondrial permeabilization using MitoTracker Orange. Values represent thymocytes with mitochondria unable to retain MitoTracker Orange. C. After 24 h in culture, thymocytes were assayed for DNA fragmentation using a TUNEL assay. All data were obtained by flow cytometry, and are expressed as mean +/- standard deviation. * represents a statistical difference based on ANOVA $p \leq 0.0001$. 
A.

**PS Externalization**

**Percent of Cells Binding Annexin-V-FITC**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PS Externalization</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Cort No Heat</td>
<td>*</td>
</tr>
<tr>
<td>Cort No Heat</td>
<td>60</td>
</tr>
<tr>
<td>Cort Heat</td>
<td>50</td>
</tr>
<tr>
<td>No Cort Heat</td>
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B.

**Mitochondrial Permeabilization**

**Percent of Cells without MitoTracker Orange Fluorescence**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitochondrial Permeabilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Cort No Heat</td>
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<tr>
<td>Cort No Heat</td>
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<tr>
<td>Cort Heat</td>
<td>90</td>
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<tr>
<td>No Cort Heat</td>
<td>*</td>
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* denotes statistical significance.
C.

DNA Fragmentation

Percent of Cells with Fragmented DNA

No Cort  Cort  Cort  No Cort
No Heat  No Heat  Heat  Heat

* *
Figure 8. Protein malnourishment does not significantly increase hsp70 or hsp90 expression. Weanling mice were maintained on either a protein sufficient diet or a protein deficient diet for 0, 7, or 14 days then thymocytes were placed in culture. A. After 3 h in culture, hsp70 expression in thymocytes from these mice was quantified by intracellular protein staining using anti-mouse hsp70 antibody and FITC-conjugated anti-mouse IgG antibody. Hsp70 expression was determined by the MFI of cells that were expressing hsp70. B. After 3 h in culture, hsp90 expression in thymocytes from these mice was quantified by intracellular protein staining using anti-rat hsp90 antibody and PE-conjugated anti-rat IgM antibody. Hsp90 expression was determined by the MFI of the total population of cells. All data were quantified by flow cytometry.
A. Hsp70 Expression

Hsp70 Expression

- protein sufficient
- protein deficient

Time on Diet (days)

B. Hsp90 Expression

Hsp90 Expression

- protein sufficient
- protein deficient

Time on Diet (days)
DISCUSSION

Protein malnourishment is a severe problem, due to its detrimental effects on the immune system, for humans around the world. The purpose of this study was to determine how protein malnourishment influences thymic atrophy. Previous research from this laboratory has demonstrated that protein malnourishment causes increased levels of serum corticosterone (1,7-8). Corticosterone is a multi-faceted regulatory molecule of the immune system that influences metabolism, inflammation, apoptosis, and cell proliferation (4). First, this study confirms previous research that protein malnourishment causes thymic atrophy. One possible explanation for thymic atrophy in protein malnourished mice is that elevation of serum corticosterone leads to increased levels of apoptosis in thymocytes. To test the hypothesis that corticosterone can induce apoptosis, which could lead to thymic atrophy, mouse thymocytes were treated with corticosterone at the average concentration seen in the serum of protein malnourished mice, and assayed for apoptosis. In this in vitro system, corticosterone induced three key characteristics of apoptosis, PS externalization, mitochondrial permeabilization, and DNA fragmentation (Fig. 4). In conclusion, these results demonstrated that corticosterone at the concentration found in the serum of protein malnourished mice induces thymocyte apoptosis in vitro.

From these results it was hypothesized that mice maintained on a protein deficient diet would have higher levels of thymocyte apoptosis than mice maintained on a protein sufficient diet. To test this hypothesis, mice were maintained on either a protein sufficient or protein deficient diet, and thymocyte apoptosis was quantified using the same assays used in the in vitro experiments. It was found that protein malnourishment significantly increases the number of thymocytes expressing PS on the outer leaflet of the plasma membrane in protein deficient mice on day 7 of the dietary period (Fig. 5A). In contrast, it was found that on day 14 of the dietary period mice maintained on a protein deficient diet had significantly fewer thymocytes with PS externalization than mice maintained on a protein sufficient diet (Fig. 5A). To be accurately analyzed, these data need to be correlated with thymic cell yields calculated for each day of the diet. The thymic cell yield results (Fig. 3) indicate that the majority of thymic atrophy occurs during the first 7 days of the dietary period. From this and the PS externalization results,
it was concluded that the highest rate of thymocyte apoptosis occurs during the first 7 days of the dietary period. In contrast, on day 14 of the dietary period thymocytes from protein deficient mice do not appear to be undergoing detectably higher rates of apoptosis than thymocytes from protein sufficient mice.

Mitochondrial permeabilization was found to be significantly higher in thymocytes from mice maintained on a protein deficient diet on both day 7 and day 14 of the dietary period (Fig. 5B). The mitochondrial data provided further support for increased thymocyte apoptosis during protein malnourishment on day 7 of the dietary period, but the day 14 results are not consistent with those of other apoptosis assays. Mitochondrial permeabilization is an essential characteristic of apoptosis. However, mitochondrial permeability within a cell, in the absence of other corticosterone-induced characteristics of apoptosis, suggests that a different mechanism is influencing the mitochondrial integrity. Therefore, it was hypothesized that on day 14 of the dietary period increased mitochondrial permeability measured in thymocytes from protein deficient mice may not be indicative of corticosterone-induced apoptosis. Rather, it is hypothesized that this phenomenon was due to a stress response related to amino acid deprivation, possibly influencing the integrity of the ER (37). Cells have adapted an ER mechanism termed the unfolded protein response or UPR, which allows the cell to deal with a variety of stress stimuli, such as amino acid deprivation, that influence protein synthesis. UPR signals to the cytoplasm and the nucleus of the cell to either adapt to the stress or to undergo apoptosis (37). The survival signals cause the up-regulation of genes that aid in protein folding, protein export, protein degradation, metabolism, and protein synthesis. These changes within the cell provide a mechanism for survival. However, over time, if a cell is in a prolonged state of UPR activation, apoptotic effectors, such as caspases, can be induced (37). Therefore, it was hypothesized the mitochondrial permeabilization results on day 14 of the diet were due to survival cross talk between ER and mitochondria, which was induced by the UPR trying to induce proper protein synthesis. However, additional studies would be required to further analyze the potential role of ER stress influencing mitochondrial permeabilization in this system. Future experiments could quantify the activation of ER caspases, such as caspases 8 and 12, to determine the potential role of ER stress during protein malnourishment.
During protein malnourishment, DNA fragmentation, a late event in apoptosis, was not significantly different in thymocytes from mice maintained on a protein deficient diet and thymocytes from mice maintained on a protein sufficient diet (Fig. 5C). These results, at first glance, seemed to contradict the results of the other apoptotic assays on day 7 of the dietary period, until the structure of the thymus is considered. As thymocytes undergo apoptosis and externalize PS, they are engulfed by macrophages that line the corticomedullary junction. Therefore, it is hypothesized that DNA fragmentation, a late event during apoptosis, could not be detected with the TUNEL technique used in this experiment because thymocytes undergoing apoptosis would be inside macrophages during later stages of apoptosis. Once inside the macrophages, the apoptotic thymocytes will be degraded, preventing the labeling of their fragmented DNA using this type of TUNEL technique. It is hypothesized that an in situ TUNEL assay, which stains the cytoplasmic contents of macrophages within a tissue section of the thymus, would be necessary to accurately quantify DNA fragmentation in thymocytes of mice maintained on a protein deficient diet. Research on apoptosis during positive and negative selection supports this hypothesis (38). In this study, macrophages, which line the corticomedullary junction, had increased cellular debris in their cytoplasm. This cellular debris stained positive for fragmented DNA. However, an in vitro TUNEL technique, identical to the one used in the present study, was not able to detect DNA fragmentation in thymocytes undergoing positive and negative selection. These results suggested that during later stages of apoptosis thymocytes are inside macrophages being degraded, which prevents the in vitro DNA fragmentation TUNEL assay from properly identifying later stages of apoptosis.

One of the purposes of this study was to investigate the regulation of thymic atrophy during protein malnourishment. The above results indicated that corticosterone, at the average concentration seen in the serum of protein malnourished mice, can induce thymocyte apoptosis in vitro, and that thymocytes of protein deficient mice undergo higher rates of apoptosis on day 7 of the dietary period, which provided correlations between thymic atrophy and apoptosis. Furthermore, previous results from this laboratory suggested that protein malnourishment induced a response that down-regulated thymic atrophy in mouse thymocytes (7, 8). Mice were implanted with
bioerodable pellets that maintained a concentration of serum corticosterone similar to that of protein malnourished mice. These mice had had more thymic atrophy than mice maintained on a protein deficient diet, indicating that corticosterone induces thymic atrophy, and that corticosterone-induced thymic atrophy is down-regulated in protein malnourished mice. Subsequent research in this laboratory demonstrated that hsp70 and hsp90 were up-regulated in thymocytes from protein deficient mice (1). Furthermore, current research of others supports the idea that hsps can act as anti-apoptotic molecules (9). To establish a correlation between increased levels of hsps and a decrease in corticosterone-induced apoptosis, an in vitro model system was used. Briefly, thymocytes from mice maintained on a protein sufficient diet were placed in culture, heat shocked, and then treated with corticosterone. Heat shock was found to significantly decrease corticosterone-induced PS externalization, mitochondrial permeabilization, and DNA fragmentation in these mouse thymocytes. DNA fragmentation was decreased the most, followed by PS externalization, and then by mitochondrial permeabilization (Fig. 7). These results suggest that heat shock provides the most protection during later stages of apoptosis. Interestingly, hsp70 and hsp90 were increased by 2-fold by 3 h after heat shock (Fig. 6), further suggesting that the expression of these proteins correlates with a decrease in later apoptotic events induced by corticosterone.

These results supported a possible role for heat shock proteins down-regulating corticosterone-induced apoptosis. Therefore, hsp70 and hsp90 were quantified in thymocytes from mice maintained on protein deficient and protein sufficient diets. However, there was no significant difference between hsp70 and hsp90 expression in thymocytes from mice maintained on the protein deficient diet compared to that of thymocytes from mice maintained on the protein sufficient diet (Fig. 8). These results contradict those of previous studies done in this laboratory (1), which showed an increase in both hsp70 and hsp90 levels in thymocytes from mice maintained on the protein deficient diet. The antibodies used to quantify hsp70 and hsp90 in this study were different from those used in the previous study. Antibodies recognize specific epitopes on their corresponding antigens. Since the antibodies used in these two contradicting experiments were purchased from different companies, the epitopes they recognize may have differed. These possible differences in epitope binding could alter the ability of the
antibodies to recognize hsp70 and hsp90. In addition, because hsp70 and hsp90 are usually found complexed with other proteins, the epitopes needed for antibody binding may be altered or hidden, and antibodies that recognize different epitopes might be differentially affected. Perhaps these differences explain why the results differ. To determine if these differences account for the contradictory results, an experiment that uses both techniques comparing the results simultaneously would be necessary.

In conclusion, protein malnourishment dramatically influences the immune system by causing the up-regulation of serum corticosterone (1, 7, 8), which inhibits the proper development of the thymus. This study provides insight into this mechanism by demonstrating that corticosterone at the level seen in protein malnourished mice, is able to induce thymocyte apoptosis in vitro, and that mice maintained on a protein deficient diet have higher levels of thymocyte apoptosis than mice maintained on a protein sufficient diet. Furthermore, this study investigated the potential role of a stress response regulating corticosterone-induced apoptosis. A correlation was established between increased levels of hsp70 and hsp90 and a decrease in corticosterone-induced apoptosis using an in vitro model system. However, neither hsp70 nor hsp90 were significantly increased in mice maintained on a protein deficient diet. This study provides future direction for studying the effects that protein malnourishment has on the immune system.
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