

Glucocorticoid Modulation of Bcl-2 Family Members A1 and Bak during Delayed Spontaneous Apoptosis of Bovine Blood Neutrophils

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Neutrophils are critical for innate immune defense against microbial invasion but can also cause inflammatory tissue damage if their life span is not tightly regulated. Antiinflammatory glucocorticoids delay spontaneous apoptosis in human, rodent, and bovine neutrophils, but mechanisms involved are unknown. We hypothesized here that glucocorticoids delay neutrophil apoptosis by altering expression of key Bcl-2 apoptosis regulatory proteins, A1 and Bak, via activation of the cell's glucocorticoid receptors. To test this hypothesis, isolated bovine blood neutrophils were exposed to dexamethasone with and without glucocorticoid receptor antagonism (RU486) and aged *ex vivo* over 0–24 h for assessment of various spontaneous apoptosis pathway indicators and A1 and Bak abundance. Results show that dexamethasone preserved neutrophil mitochondrial membrane integrity, delayed caspase-9 activation, and reduced the rate of spontaneous apoptosis. Also, dexamethasone increased A1

and decreased Bak mRNA abundance. RU486 pretreatment of the cells abrogated each of these dexamethasone effects. Dexamethasone-induced increases in A1 mRNA were reflected in A1 protein increases, which also were observed in circulating neutrophils of dexamethasone-treated animals. Bak protein decreases were observed in neutrophils of the dexamethasone-treated animals but not in isolated neutrophils, suggesting that stimuli additional to (and perhaps regulated by) glucocorticoid are required to affect Bak protein expression changes in neutrophils. Collectively, our results are unique in demonstrating a mechanism behind glucocorticoid regulation of spontaneous apoptosis and implicate steroid receptor activation and subsequent regulation of A1 and Bak as contributors to mitochondrial membrane stability, reduced caspase-9 activity, and delayed apoptosis in bovine neutrophils exposed to glucocorticoids. (*Endocrinology* 147: 3826–3834, 2006)

NEUTROPHILS ARE GRANULOCYTIC leukocytes integral to the first line of immune defense against invading bacteria and other pathogens. Equally important to their antimicrobial activities is the tight regulation of their death, which occurs rapidly under normal physiological scenarios by a process of spontaneous (mitochondrial induced) apoptosis and prevents unnecessary inflammatory tissue damage. Accordingly, neutrophils normally have a short half-life of approximately 6–10 h in blood (1), which is extended up to 24 or 48 h when the leukocytes enter infected tissues to perform their bactericidal functions (2).

Regulation of neutrophil apoptosis in blood and tissues occurs through both intrinsic and extrinsic signaling pathways. Whereas extrinsic signals are initiated by death receptor ligation at the cell's surface, intrinsic signaling is controlled at the level of mitochondria with the aid of several Bcl-2 family proteins (3). Bak and Bax are two proapoptotic family members expressed in neutrophils (4, 5). In contrast to most other cell types that express Bcl-2 as the classical antiapoptotic protein, mature neutrophils use family mem-

bers A1/Bfl-1 (referred to as A1 in the remainder of the text) and Mcl-1 as their main survival inducing Bcl-2 family members (4, 5). Mcl-1 has been shown to be up-regulated in neutrophils during cytokine-induced apoptosis delay (5), but effects of Mcl-1 loss on neutrophil apoptosis have yet to be elucidated. The importance of A1, Bak, and Bax in neutrophil spontaneous apoptosis regulation has begun to be revealed through the use of knockout mice (reviewed in Ref. 3). For example, abolishment of A1 function results in accelerated neutrophil apoptosis (6) with correspondingly diminished acute inflammatory responses (7). In contrast, Bak/Bax double-knockout mice exhibit increased blood neutrophil numbers and multiple developmental defects and die perinatally (8). A1 is so potently antiapoptotic that its overexpression can rescue nuclear factor- κ B-deficient cell lines from apoptotic cell death (9). Thus, regulated expression of Bcl-2 family proteins appears to be an essential control point of spontaneous apoptosis in neutrophils and other cells.

In most cell systems, it is the ratio of antiapoptotic to proapoptotic Bcl-2 family members that is critical in determining apoptotic status (10). When proapoptotic members such as Bak are in excess, this signifies a state of apoptosis. On the other hand, high levels of antiapoptotic family members, including A1, heterodimerize with the proapoptotic family members such as Bak and Bax (11, 12) to limit their death-initiating actions and delay apoptosis. This life/death balance through the mitochondrial pathway is in constant flux in neutrophils. It can be altered at multiple signaling

First Published Online May 4, 2006

Abbreviations: FITC, Fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; LPS, lipopolysaccharide; pNA, p-nitroanilide; Q-RT-PCR, quantitative real-time RT-PCR; ROS, reactive oxygen species; sFasL, soluble Fas ligand.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

check points, is amplified during death receptor ligation via a BH3-only Bcl-2 family member called Bid (13, 14), and is highly dependent on the cytokine and steroid milieu of the neutrophils' surrounding blood or tissue environment.

Proinflammatory cytokines such as granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-8, TNF α , and interferon (IFN)- γ delay neutrophil apoptosis by changing the relative abundance and (or) subcellular location of expressed Bcl-2 family members (reviewed in Refs. 3, 15). For example, neutrophil A1 mRNA increases after exposure to G-CSF, GM-CSF, TNF α , and IFN γ (5, 16). Exposure to bacterial products, such as lipopolysaccharide (LPS), also increases A1 mRNA and protein in neutrophils located in both blood (5, 16) and lung (17). In contrast, proapoptotic Bax protein levels were decreased after GM-CSF (18) and TNF α (5) stimulation of neutrophils. G-CSF treatment did not alter neutrophil Bax abundance but dramatically reduced Bax translocation from the cytosol to mitochondrial membranes (19). Whereas Bak was unchanged by GM-CSF (18) or TNF α (5), conditioned culture medium from IL-8-treated neutrophils decreased Bak abundance in freshly isolated neutrophils (20). Summarizing across these studies, more of the tested inflammatory mediators targeted A1 than Bak or Bax. This may be due to the fact that the antiapoptotic Bcl-2 family members are extremely labile (A1 and Mcl-1 mRNA half-life \sim 3 h, Mcl-1 protein half-life \sim 6 h) and thus readily altered in abundance relative to proapoptotic family members (Bak and Bax protein half-life $>$ 22 h) (5). In addition, A1 is potently inducible through nuclear factor- κ B signaling (9, 17), such as occurs in neutrophils exposed to proinflammatory cytokines and LPS (21). Thus, it may be more efficient for short-lived neutrophils to alter the relative ratios of key pro- to antiapoptotic Bcl-2 family members and consequently apoptotic status through acute changes in A1 expression *vs.* more delayed changes in Bak or Bax expression. Once neutrophils have served their purpose of pathogen clearance in tissues and the inflammatory response wanes, the cells' A1 to Bak and A1 to Bax ratios revert back to levels that favor apoptosis, and the mitochondrial death pathway is resumed.

Paradoxically glucocorticoid hormones are potently antiapoptotic for neutrophils (22–26) despite their well-known antiinflammatory activities that decrease the cells' endothelial adhesion, trafficking, and bacteria killing functions (27–30). Neutrophils exposed to glucocorticoids possess depressed immune functions that are more typical of apoptotic cells (31) when in fact the cells' program of spontaneous apoptosis is temporarily delayed. Reasons for this apparent contradiction in cell status are unknown. However, it is possible that delayed apoptosis enables the body to cope with glucocorticoid-induced neutrophilia (27, 28), potentially lessening the burden on phagocytic networks for a sudden need to clear huge numbers of senescent neutrophils and limiting their resultant necrotic cell death and systemic inflammatory damage (32). Thus, a clear understanding of how glucocorticoids regulate neutrophil apoptosis status will be important to preventing and treating inflammation.

Although it has been demonstrated that the glucocorticoid-induced delay in neutrophil spontaneous apoptosis requires new gene expression and protein synthesis (33), spe-

cific molecular information about how glucocorticoids achieve this delay in cell death is limited. In an *in vivo* model of glucocorticoid challenge, namely bovine parturition, neutrophil A1 and Bak mRNA profiles were changed in such a way as to favor a temporary delay in spontaneous apoptosis (34). The peak increase in A1 and decrease in Bak mRNA occurred during peak cortisol concentrations in blood serum (35). Thus, it is possible that glucocorticoids delay neutrophil spontaneous apoptosis via effects on A1 and Bak gene expression and subsequent regulation of downstream events initiated at the cells' mitochondrial membranes. This was the hypothesis of the current study.

Materials and Methods

Animals and blood neutrophil isolations

Bovine neutrophils for the main *in vitro* experiments of this study (see below) were obtained as needed from five young male Holstein cattle donors that were castrated at 1 month of age (steers). The steers were 3–6 months of age during the blood collection period. In a supplementary experiment, blood neutrophils from three additional steers were collected before and 9 h after im injections of dexamethasone (0.10 mg/kg body weight at 0 and 6 h) (28) to evaluate protein abundance changes of A1 and Bak in response to *in vivo* glucocorticoid administration. All animals were fed and housed according to standard operating procedures at the Dairy Teaching and Research Facility, and their use for the described experiments was approved by the All University Committee on Animal Care and Use (approval no. 07/04-104-00), both organizations of Michigan State University.

For all experiments, blood was drawn by jugular venipuncture into ACD anticoagulant, and neutrophils were isolated according to our published Percoll density gradient centrifugation protocol (28). Total neutrophils were enumerated by electronic counting using a Z1 Coulter particle counter system (Beckman Coulter Particle Characterization, Miami, FL). Purity of the neutrophil preparations was always greater than 95% as assessed flow cytometrically (FACSCaliber flow cytometer and CellQuest software; Becton Dickinson, San Jose, CA) using our published G₁ immunostaining protocol (29). Viability of freshly isolated neutrophils, assessed flow cytometrically by propidium iodide uptake, was always greater than 98%.

Culture of isolated bovine blood neutrophils

Isolated neutrophils were cultured in basic medium consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 1.0% fetal bovine serum (low endotoxin fetal bovine serum; Hyclone, Logan, UT), and 25 U/ml penicillin plus 25 μ g/ml streptomycin (Invitrogen). Cultures were incubated at 39 C (normal deep body temperature for cattle) in humidified 5% CO₂ for up to 24 h with or without added glucocorticoid. The glucocorticoid used was dexamethasone (Azium; Schering Plough, Animal Health, Kenilworth, NJ) because it binds with high specificity and affinity to glucocorticoid receptors (36). We have shown in previous studies that bovine neutrophils express glucocorticoid receptor mRNA (28, 29) and protein (26). Hormone action was blocked in relevant experiments by pretreatment (30 min) of neutrophils with mifepristone (RU486; Sigma Chemical Co., St. Louis, MO), a known glucocorticoid receptor antagonist (37). Concentrations of dexamethasone and RU486 used are described in the various experiments below.

Apoptosis dose response to glucocorticoid receptor agonism and antagonism

Neutrophil apoptotic status was assessed by dual annexin V-fluorescein isothiocyanate (FITC)/propidium iodide staining with fluorescence-activated flow cytometric analysis (34). To determine glucocorticoid dose responsiveness of the delay in apoptosis, duplicate cultures of 5×10^5 neutrophils were treated with increasing molar concentrations of dexamethasone (0, 10^{-9} , 10^{-8} , 10^{-7} , and 10^{-6} M) in 96-well culture plates. At each assay time point (0, 6, 12, and 24 h), culture plates were placed on ice for 5 min to ensure maximum neutrophil retrieval from the

wells. Neutrophils were transferred to 5-ml polystyrene round-bottom tubes (Becton Dickinson) and assayed for apoptosis using the protocol contained in a commercial kit (annexin V-FITC apoptosis detection kit; BD Biosciences PharMingen, San Diego, CA). Resulting flow cytometric data were recorded as percent nonapoptotic (*i.e.* viable) neutrophils, which were the percentage of cells that were negative for both annexin V-FITC and propidium iodide staining in two-color density dot plots (34). Next, apoptotic status was assessed in neutrophils pretreated for 30 min with RU486 at 0-, 10-, 100-, or 1000-fold the molar concentrations of the most biologically effective dose of dexamethasone in cells aged for 0, 6, 12, and 24 h as before.

Assessment of neutrophil apoptotic status by mitochondrial membrane staining

Mitochondria membrane integrity was assessed in freshly isolated neutrophils and after treatment of the cells with 0 or 10^{-7} M of dexamethasone for 12 h using MitoTracker Green FM staining (referred to in remainder of the text as MitoTracker; Invitrogen-Molecular Probes, Carlsbad, CA). Neutrophils (2.5×10^5 cells per treatment scenario) were treated with 100 nM MitoTracker for 30 min at 5% CO₂ and 39 C. The cells were then centrifuged at $500 \times g$, suspended in basic medium, and cytocentrifuged onto glass microscope slides (Shandon Cytocentrifuge; Thermo Shandon Cytospin 4, Pittsburgh, PA) before microscopic evaluation and photography using a Leica DM IL microscope fitted with a Leica DFC480 digital camera system (Leica Microsystems Inc., Bannockburn, IL).

Assessment of neutrophil apoptotic status by caspase-9 activity

Neutrophil caspase-9 activity was assessed after dexamethasone treatment (0 or 10^{-7} M) \pm RU486 (10^{-6} M) for 0, 0.75, 1.5, 3, 6, and 9 h using a commercially available assay kit (APOPCYTO caspase-9 colorimetric assay kit; MBL International Corp., Woburn, MA). Control treatments included neutrophils incubated for 9 h in 50 μ M caspase-9 inhibitor (negative control; z-LEHD-fmk; Calbiochem, La Jolla, CA) or 100 ng/ml soluble Fas ligand (sFasL; positive control; recombinant human sFasL; Axxora Life Sciences, San Diego, CA) for the final 3 h of the 9-h culture time. At each time point, 1×10^7 neutrophils were transferred from 12-well culture plates to microfuge tubes and pelleted by centrifugation ($500 \times g$) for 5 min at 4 C. Supernatants were discarded and the cell pellets stored at -80 C until the time of assay.

Assessment of caspase-9 activity was performed in duplicate according to the manufacturer's protocol. Briefly, cell pellets were thawed on ice for 15 min, suspended in 115 μ l ice-cold cell lysis buffer, and incubated on ice for 10 min. Samples were centrifuged at $10,000 \times g$ for 5 min at 4 C to pellet debris, and supernatants (cell lysates) were transferred to new tubes. The following were then added to the wells of a flat-bottom, 96-well plate: 50 μ l/well of the various cell lysates, 50 μ l/well of $2 \times$ reaction buffer containing 0.01 M dithiothreitol, and 5 μ l/well of 0.01 M LEHD-*p*-nitroanilide (*p*NA) substrate. Plates were incubated at 37 C for 19.5 h after which the absorbance of *p*NA freed by caspase-9 activity was measured at 405 nm (Benchmark plate reader and Microplate Manager III analysis software; Bio-Rad Laboratories, Hercules, CA). Specific activity (micromole-free *p*NA divided by the product of milligrams per milliliter protein and assay time of incubation) was calculated using a standard curve for absorbance of *p*NA (supplied with the kit), and total cell lysate protein added to each well was determined by Lowry assay. Caspase-9 activity was normalized against total protein added per well.

RNA isolation and quantitative real-time RT-PCR analysis of A1 and Bak mRNA

Analysis of A1 and Bak mRNA abundance was assessed in neutrophils cultured with and without dexamethasone (10^{-7} M) \pm RU486 (10^{-6} M) for 0, 1, 2, and 4 h. Neutrophils were lysed in TRIzol Reagent (Invitrogen) at a concentration of 1×10^7 cells/ml TRIzol for 10 min at room temperature and stored in the same reagent at -80 C. RNA was isolated according to the manufacturer's instruction and treated with RQ1 RNase-free DNase (Promega, Madison, WI) as described by the manufacturer. Total RNA concentration and purity were determined with a

ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA was synthesized from 2 μ g total RNA using Superscript II RnaseH⁻ reverse transcriptase (Invitrogen).

Quantitative real time RT-PCR was performed using the SYBR Green PCR master mix system for real-time fluorescence detection in a PE7700 thermal cycler (PerkinElmer Applied Biosystems, Foster City, CA) and gene-specific primers for A1 and Bak (34). A1 and Bak PCR amplicons used for the development of standard curves in these assays were created with the following primers: A1 (284 bp) forward primer, 5'-CCAGGCAGAAGATGACAG-3' and reverse primer, 5'-GGTTACAATCCTGCCCCAGTT-3'; and Bak (306 bp) forward primer, 5'-AGGAGCAGGTAGCCCCAGGAC-3' and reverse primer, 5'-CCAGTTGATCCGCTCTCAAAC-3'.

The PCR products were amplified in a reaction mixture containing $1 \times$ PCR buffer, 3 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 0.2 μ M forward primer, 0.2 μ M reverse primer, 35 ng cDNA template (from bovine total leukocytes), and 1 U/reaction Taq DNA polymerase (Invitrogen), brought to a final volume of 25 μ l with sterile Milli-Q water. Reaction conditions used to generate A1 and Bak amplicons were: denature at 95 C for 5 min followed by 35 cycles of 95 C for 30 sec (denature), 52 C for 30 sec (anneal), and 72 C for 30 sec (extend), and a final extension at 72 C for 10 min. The resulting single-band amplicons were gel purified, ligated into the pGEM-T Easy vector (Promega), and the recombinant plasmids transformed into JM109 competent *Escherichia coli* cells (Promega). Positive clones containing the A1 and Bak cDNA inserts were selected by blue/white colony screening and confirmed by DNA sequence analysis. Plasmids from white colonies were isolated using the miniprep plasmid DNA isolation kit (Promega). A1 and Bak were then amplified from their respective plasmids with the primers described above and gel purified before dilution for their use as templates in the quantitative real-time RT-PCR (Q-RT-PCR) standard curves. Seven concentrations of the A1-284 bp amplicon (0.004–4000 fg/ μ l), five concentrations of the Bak-306 bp amplicon (0.4–4000 fg/ μ l), or 20 ng neutrophil cDNA were added as templates to Q-RT-PCR mixtures that contained Q-RT-PCR primers (A1 or Bak from Ref. 34) and SYBR Green PCR master mix. Amplification efficiency, observed as parallel slopes of the amplification curves, was similar between the two templates (amplicon *vs.* neutrophil cDNA) within each gene of interest. A1 and Bak mRNA abundance in treated neutrophil samples was calculated using the equation from the linear standard curves for A1-284 bp and Bak-306 bp, which were plotted as the number of PCR cycles to threshold *vs.* starting amplicon concentration (in femtograms). All reactions, including a negative control (no cDNA template), were run in triplicate.

Western blot analysis of A1 and Bak

To assess protein abundance changes of A1 and Bak, neutrophil cytosolic fractions were prepared with a protocol published elsewhere (modified from Ref. 28). Isolated neutrophils were treated *in vitro* for 3, 6, and 9 h in the presence or absence of dexamethasone (10^{-7} M) \pm RU486 (10^{-6} M) and also were examined before and 9 h after administration of dexamethasone *in vivo*. To obtain cytosol preparations, pelleted neutrophils were suspended in cell disruption solution [0.34 M sucrose, 0.01 M Tris-HCl (pH 6.8), 0.005 M EGTA, and Complete, miniprotease inhibitor cocktail tablet (1 tablet per 10 ml disruption solution; Roche Applied Science, Indianapolis, IN)] and sonicated before centrifugation at $2000 \times g$ for 15 min at 4 C. Resulting cell lysates were then centrifuged at $100,000 \times g$ for 30 min at 4 C. Supernatants were boiled after addition of $5 \times$ sample buffer [0.25 M Tris-HCl (pH 6.8), 50% glycerol, 10% sodium dodecyl sulfate] to result in cytosolic preparations containing 0.05 M Tris-HCl (pH 6.8), 10% glycerol, and 2% sodium dodecyl sulfate. Cytosol preparations (40 μ g total protein per lane) were subjected to SDS-PAGE using 15% gels for A1 detection and 12.5% gels for Bak detection. Proteins were transferred to nitrocellulose membranes overnight at 4 C and blocked for 1 h at room temperature with SuperBlock blocking buffer (in Tris-buffered saline; Pierce Biotechnology, Rockford, IL). After washing once with buffer of Tris-buffered saline and Tween 20, membranes were incubated with either anti-Bak antibody (rabbit polyclonal IgG; catalog no. H-211: sc7873; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-A1 antibody (rabbit polyclonal IgG; catalog no. FL-175: sc8351; Santa Cruz Biotechnology) for 1 h at room temperature, washed again, and incubated with detection antibody (goat antirabbit IgG horseradish

peroxidase conjugated; Pierce Biotechnology) at room temperature for 1 h. Blots were developed using the SuperSignal West Pico chemiluminescent substrate system (Pierce Biotechnology), photographed, stripped, and reprobed with anti- β -actin antibody (mouse monoclonal IgG₁; catalog no. AC-15: ab6276; Abcam Ltd., Cambridge, MA) as a lane-loading control (28). Resulting bands from chemiluminescent detection were analyzed with a scanning densitometer (GS-710 calibrated imaging densitometer and Multi-Analyst software; Bio-Rad) and A1 and Bak protein abundance recorded as density ratios to β -actin.

Statistical analysis

Data are summarized in the results as least squares means \pm SEM. When necessary for statistical analysis, data were log transformed for better normal distribution approximation. Statistical analysis was performed using the MIXED procedure of SAS (38), with a model that included the fixed effect of experimental group (no treatment, dexamethasone, RU486, and RU486 plus dexamethasone) and the random effects of steer ($n = 3$ –5) and steer \times group interaction. Regression analysis was used in the dexamethasone and RU486 dose-response experiments to determine the most biologically effective concentrations of each for use in all subsequent experiments. When the steer \times group interaction was significant, such as with the mRNA abundance data, the effect of one factor (*e.g.* RU486) was examined within each level of the other factor (*e.g.* dexamethasone 0 M vs. 10^{-7} M) using the SLICE function within the MIXED procedure of SAS. Significant differences between treatments were declared when $P \leq 0.05$.

Results

Dexamethasone caused a dose-dependent delay in spontaneous neutrophil apoptosis that was inhibited by all doses of RU486 tested

Before examining direct effects of dexamethasone on mitochondrial membrane stability, caspase-9 activity, and A1 and Bak abundance, we determined the most effective dose of the steroid at which spontaneous neutrophil apoptosis was delayed. Neutrophil cultures containing 10^{-9} , 10^{-8} , 10^{-7} , or 10^{-6} M dexamethasone all had significantly more nonapoptotic (*i.e.* viable) cells at 24 h relative to cultures with no dexamethasone (0 M, Fig. 1A). The dose response was curvilinear (quadratic) in shape, and the dose of dexamethasone that most effectively delayed neutrophil apoptosis at 24 h was estimated by regression analysis to lie between 10^{-8} and 10^{-7} M. The 10^{-7} M dose was selected for use in all subsequent experiments because it also best approximated the concentration of serum glucocorticoid achieved *in vivo* during bovine parturition, the animal model in which we first observed neutrophil gene expression changes in A1 and Bak (34, 35). Pretreatment of neutrophils with 10^{-6} , 10^{-5} , or 10^{-4} M of RU486 removed the apoptosis delaying effect of 10^{-7} M dexamethasone. Figure 1B demonstrates that as little as 10-fold excess of RU486 abrogated the dexamethasone effect without itself inducing apoptosis and, as a result, 10^{-6} M RU486 was used in all subsequent experiments. Although RU486 antagonizes both glucocorticoid and progesterone receptors, it is unlikely that the antagonistic effect on glucocorticoid-induced apoptosis delay occurred via the progesterone receptor because this receptor has not been detected in bovine neutrophils (see Ref. 39) (Burton, J. L., S. A. Madsen-Bouterse, and P. S. D. Weber, manuscript in preparation). Thus, the dexamethasone-induced delay in bovine neutrophil spontaneous apoptosis we observed was mediated via glucocorticoid receptor activation and inhibited by RU486 antagonism of this receptor.

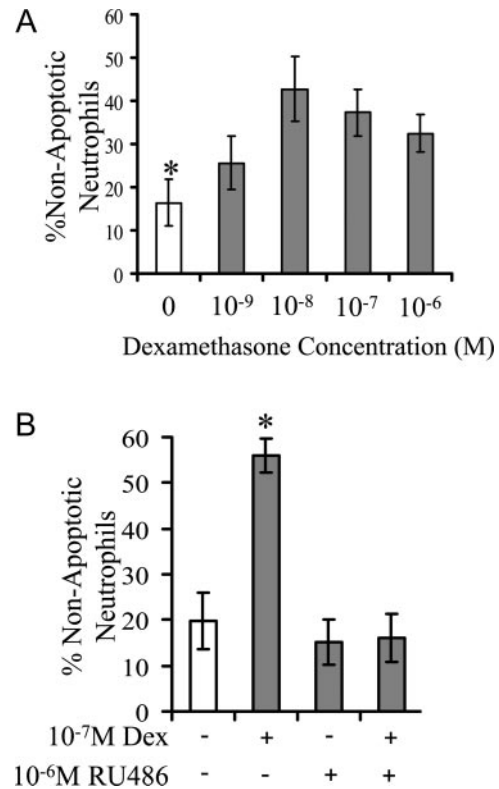


FIG. 1. Glucocorticoid delayed spontaneous apoptosis in bovine neutrophils is dose responsive and inhibited by RU486. A, A significantly higher percent of nonapoptotic neutrophils (determined flow cytometrically as negative for annexin V-FITC/propidium iodide staining) was detected after 24 h of *ex vivo* aging in the presence of dexamethasone. A quadratic relationship [$\hat{y} = -254.12 - 80.28(x) - 5.4518(x^2)$ where $x = \log(\text{concentration})$] was observed for the dexamethasone dose response. Using this regression equation, the dose of dexamethasone that was biologically most effective in preserving neutrophil viability was estimated to be between 10^{-8} and 10^{-7} M. B, A 30-min pretreatment with 10^{-6} M of RU486 before the addition of 10^{-7} M dexamethasone (Dex) for 24 h removed the neutrophil apoptosis delaying effect of the glucocorticoid (steer $n = 5$). *, $P < 0.05$ relative to all other treatments.

Dexamethasone treatment maintained neutrophil mitochondria membrane integrity during ex vivo aging

The integrity of neutrophil mitochondrial membranes was examined in this study with the aid of MitoTracker (19). This fluorescent dye preferentially accumulates in the lipid environment of mitochondrial membranes regardless of overall membrane potential (40). Freshly isolated neutrophils demonstrated a crisp, bright green staining of their tubular mitochondria (Fig. 2A, top panel) and did not stain with annexin V-FITC or propidium iodide (Fig. 2B, top panel). After 12 h of *ex vivo* aging without added dexamethasone, most neutrophils showed a diffuse cytosolic staining pattern with MitoTracker, indicative of mitochondrial membrane demise, and this correlated with advanced apoptosis (annexin V-FITC/propidium iodide staining) in the cells (Fig. 2, A and B, middle panels). In contrast, neutrophils aged for 12 h in the presence of 10^{-7} M dexamethasone showed crisp, bright green MitoTracker staining, similar to that of freshly isolated cells, indicating that the organelles' membranes remained intact and correlating well with their significantly reduced

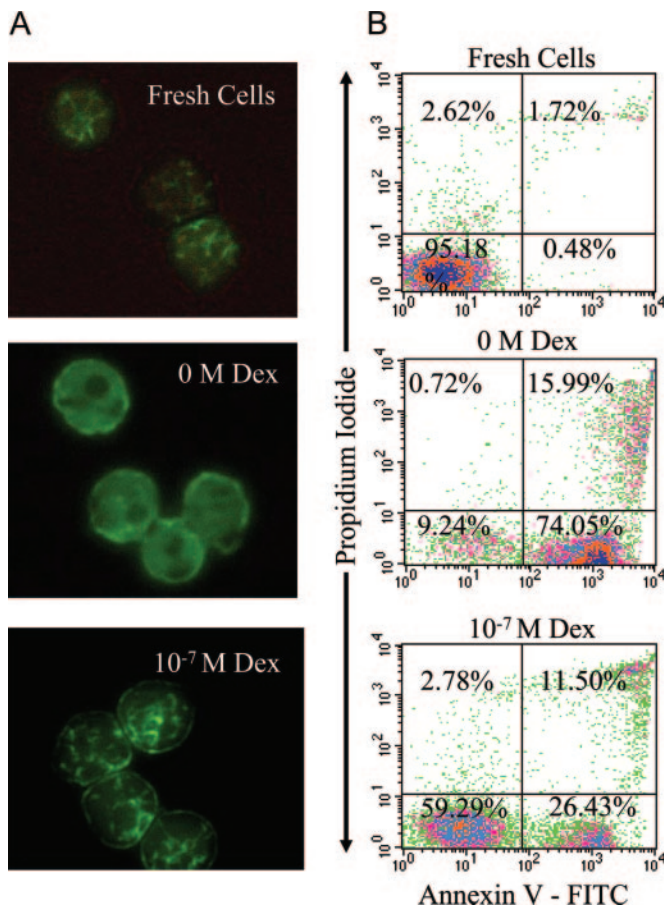


FIG. 2. Glucocorticoid treatment of neutrophils during *ex vivo* aging delayed their spontaneous apoptosis in conjunction with maintenance of mitochondrial membrane stability. Freshly isolated neutrophils (*top panels*) contained intact, tubular mitochondria, visualized as focused, *bright green staining* with MitoTracker (A) and showed no evidence of apoptosis based on annexin V-FITC binding (B). Neutrophils cultured in basic medium for 12 h (*middle panels*) contained disrupted mitochondria, visualized as diffuse intracellular staining with MitoTracker (A), and were highly apoptotic based on annexin V-FITC binding (74.05% of neutrophils; B). Inclusion of dexamethasone (Dex) for the 12 h incubation (*bottom panels*) maintained the stability of mitochondrial membranes (A) and dramatically reduced neutrophil apoptosis (26.43% annexin V-FITC-positive cells in B). Figures are representative of neutrophils from $n = 3$ steers.

rate of apoptosis (Fig. 2, A and B, *bottom panels*). Thus, the biologically effective dose of dexamethasone that delayed spontaneous apoptosis in bovine neutrophils likely did so in part by delaying mitochondrial membrane decay.

Dexamethasone treatment delayed activation of caspase-9 in neutrophils aged *ex vivo*

Maintenance of mitochondria outer membranes occurred in conjunction with decreased caspase-9 activation in the dexamethasone-treated neutrophils (Fig. 3A). Significantly less caspase-9 activity was observed after 6 and 9 h in dexamethasone-treated neutrophils relative to untreated cells. Pretreatment of the neutrophils with RU486 removed the inhibiting effect of dexamethasone on caspase-9 activation (Fig. 3B). In addition, dexamethasone-mediated decreases in caspase-9 activity were similar to decreases observed in neu-

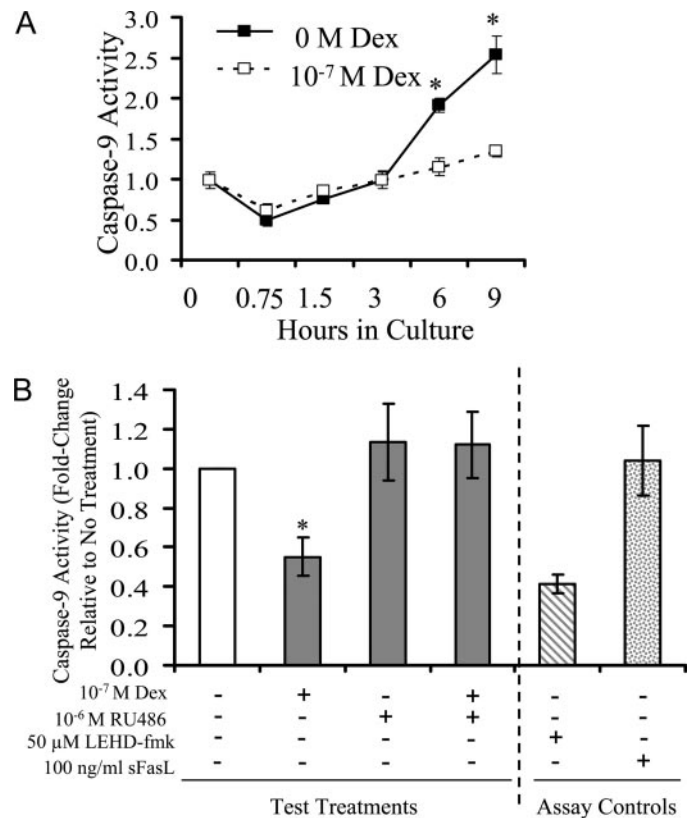


FIG. 3. Glucocorticoid treatment delayed the activation of caspase-9 in neutrophils aged *ex vivo* over 9 h. A, Dexamethasone (Dex; 10⁻⁷ M) significantly inhibited caspase-9 activation at 6 and 9 h of treatment [specific activity = micromole-free pNA/(milligrams per milliliter protein × assay incubation hour)]. B, Caspase-9 activation in dexamethasone (Dex)-treated neutrophils aged for 9 h *ex vivo* (shown as fold change relative to no treatment) was not different from that observed in neutrophils treated with the caspase-9 inhibitor LEHD-fmk. No treatment and RU486 pretreatment resulted in high caspase-9 activities that were similar to that observed in positive control cells (neutrophils treated with sFasL). Cells from $n = 4$ steers. *, $P < 0.05$ relative to no treatment.

trophils treated with LEHD-fmk, a known caspase-9 inhibitor (Fig. 3B). Activation of caspase-9 in untreated and RU486-treated neutrophils was similar to levels induced by sFasL, a known activator of caspase-9 (Fig. 3B). These results, in conjunction with the mitochondria morphology data from aging neutrophils, indicate that actions of glucocorticoids on neutrophil spontaneous apoptosis are targeted at the preservation of mitochondrial membrane integrity and corresponding down regulation of the caspase-9 death-inducing pathway.

Dexamethasone treatment altered neutrophil abundance of A1 and Bak

Because A1 and Bak coregulate the rate of mitochondrial membrane demise and downstream activation of caspase-9 and dexamethasone delayed these events, we next tested whether this glucocorticoid affects neutrophil abundance of A1 and Bak. First, isolated neutrophils were incubated up to 4 h in the presence or absence of dexamethasone ± RU486 pretreatment and analyzed for A1 and Bak mRNA by Q-RT-

PCR. Representative standard curves used for the quantification of A1 and Bak mRNA are depicted in Fig. 4, A and C (linearity $R^2 > 98.00\%$). Dexamethasone was observed to have an acute but short lived up-regulating effect on neutrophil A1 mRNA abundance, which peaked at 2.4-fold above that of untreated cells at 1 h ($P < 0.05$; Fig. 4B), decreased to 1.4-fold above by 2 h ($P = 0.05$; not shown), and was absent by 4 h (not shown). Pretreatment with RU486 did not fully reverse the effect of dexamethasone on A1 mRNA up-regulation at 1 h (Fig. 4B) or 2 h (not shown), although A1 mRNA levels in the RU486-treated cells were not significantly different from levels in untreated cells (Fig. 4B). In contrast to its acute effects on A1, it took 4 h for dexamethasone to affect Bak and the effect was a modest (~2-fold) down-regulation of Bak mRNA abundance in the treated *vs.* untreated cells (Fig. 4D). RU486 pretreatment of the neutrophils caused total reversal of the dexamethasone-induced down-regulation of Bak (Fig. 4D). These results suggest that dexamethasone has direct regulatory effects on A1 and Bak gene expression in bovine blood neutrophils, which for Bak is clearly mediated by steroid-activated glucocorticoid receptors. However, the role of glucocorticoid receptor activation in mediating A1 mRNA abundance changes during dexamethasone treatment was less clear.

Subsequently Western blotting was used to evaluate *in vitro* (3, 6, and 9 h in culture) and *in vivo* (0 h *vs.* 9 h after administration) effects of dexamethasone on neutrophil A1 and Bak protein abundance. Both proteins were abundant in neutrophil cytosolic fractions (Figs. 5A and 6A) with relatively low (almost undetectable) levels found in organelle fractions that contained mitochondrial membranes (not shown). *In vitro*, an overall effect of treatment group was detected for A1 ($P < 0.05$) and was observed at all time points (representative blot from 9-h samples in Fig. 5A). Averaged over the time points, dexamethasone caused a 3.3-fold in-

crease ($P = 0.025$; Fig. 5B) in A1 protein abundance, which was completely inhibited by RU486 pretreatment. Thus, at the protein level, dexamethasone-induced up-regulation of A1 abundance was mediated via glucocorticoid receptor activation. In contrast to its effects on Bak mRNA abundance, dexamethasone did not affect Bak protein abundance in cultured neutrophils (Fig. 5, A and C). However, we detected a 5.4-fold down-regulation ($P < 0.01$) of Bak protein abundance in circulating neutrophils 9 h after dexamethasone administration *in vivo* (Fig. 6, A and C), with a concurrent 2.4-fold up-regulation ($P < 0.01$) of A1 protein abundance (Fig. 6, A and B). This shift in pro- and antiapoptotic proteins occurred in conjunction with a pronounced leukocytosis (not shown), which we documented in previous studies to result from steroid-induced mature neutrophilia (26–29). Thus, by virtue of its ability to increase the A1 to Bak expression ratio in neutrophils and delay downstream mitochondrial membrane decay, caspase-9 activation, and apoptosis, dexamethasone may induce temporary neutrophilia *in vivo* by extending the life of circulating cells. *In vitro*, direct effects of dexamethasone on neutrophil survival appeared to target primarily A1 because Bak protein abundance was not altered, further suggesting that additional glucocorticoid-responsive factor(s) present *in vivo* but not in our minimal culture system may be required to effect changes in the Bak expression system of bovine blood neutrophils.

Discussion

Glucocorticoid-induced delay in spontaneous apoptosis has been documented in human (22, 23), rodent (24), and carp neutrophils (25), but the processes involved are relatively unknown. To date, antiapoptotic mechanisms explored have included glucocorticoid effects on the activity of reactive oxygen species (ROS) (41) and regulation of Fas gene ex-

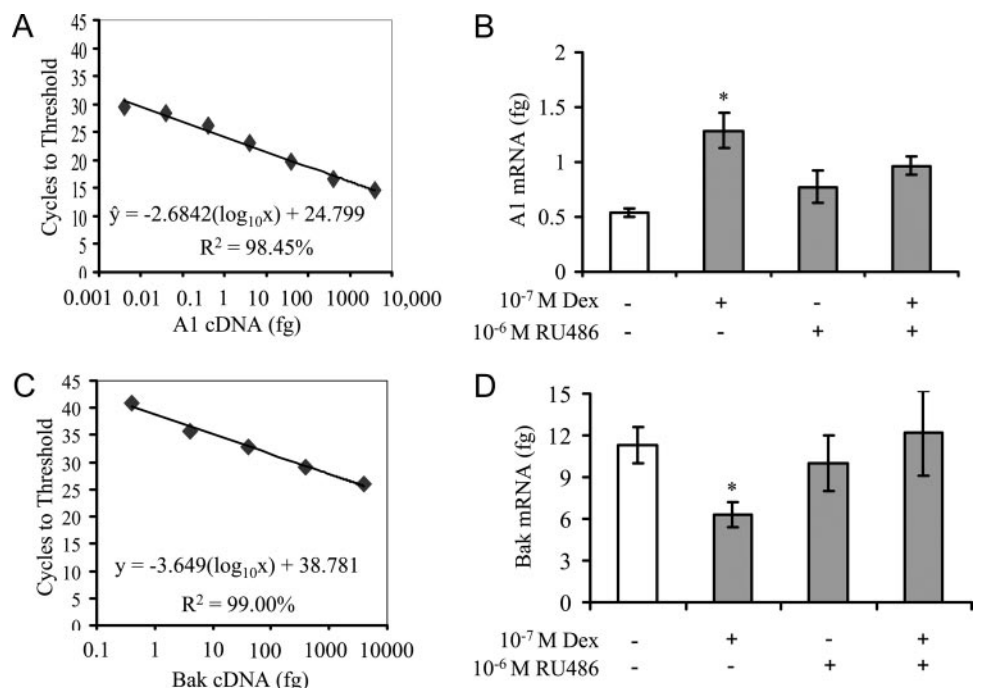


FIG. 4. Abundance of A1 and Bak mRNA was altered by dexamethasone treatment. A, The A1 Q-RT-PCR standard curve made with the A1-284 bp amplicon. B, A1 mRNA abundance was increased ($P < 0.05$) after 1 h of dexamethasone (Dex) treatment. Pretreatment with RU486 partially blocked the effect of dexamethasone on A1 mRNA abundance. C, The Bak Q-RT-PCR standard curve made with the Bak-306 bp amplicon. D, Bak mRNA abundance was decreased ($P < 0.05$) after 4 h of dexamethasone (Dex) treatment. Pretreatment with RU486 eliminated this effect. B and D, Neutrophils from $n = 4$ steers. *, $P < 0.05$ relative to no treatment.

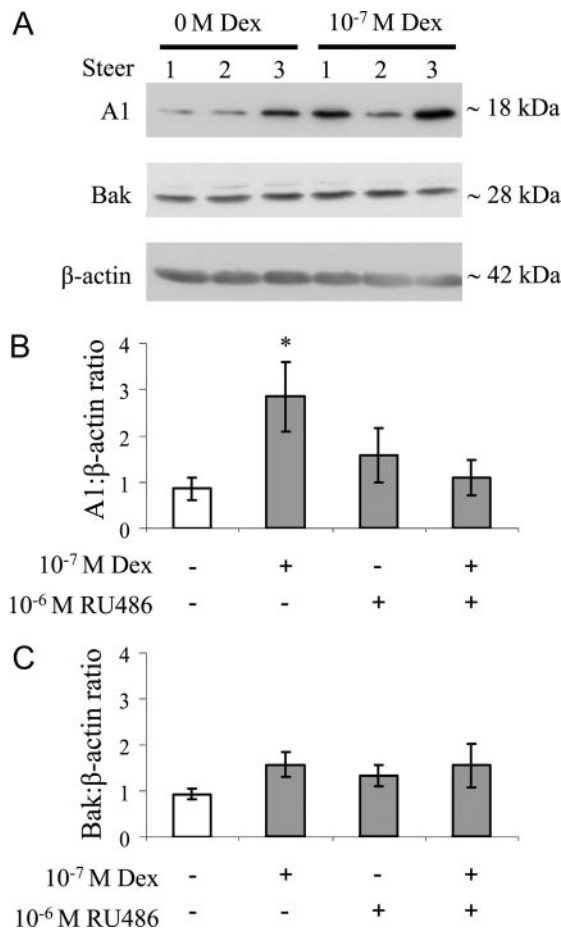


FIG. 5. Cytosolic abundance of A1 in neutrophils was altered by dexamethasone (Dex) treatment over 9 h of *ex vivo* aging, whereas Bak abundance was unchanged. A, Western blots demonstrating that A1 protein was increased and Bak protein unchanged in neutrophils treated for 9 h with dexamethasone (compared with untreated cells). β -Actin was used as the lane-loading control. In B and C, bands visualized by chemiluminescence were analyzed by scanning densitometry and A1 and Bak protein abundance recorded as density ratios to β -actin. B, A1 protein was significantly increased in dexamethasone-treated neutrophils regardless of time in culture (up to 9 h). Shown is the mean A1 abundance over all culture times. Pretreatment with RU486 removed the effect of dexamethasone on A1 protein abundance. C, Dexamethasone treatment did not affect Bak abundance at any time point tested (not shown) or across all time points. Differences in protein abundance between treatments assessed in $n = 5$ steers. *, $P < 0.05$ relative to no treatment.

pression (26). Results of the current study show for the first time that an additional action of glucocorticoid on neutrophil apoptosis delay may be its targeting of the cells' mitochondrial membrane stability system that contributes to spontaneous apoptosis regulation. Key findings to support this observation were that dexamethasone delayed mitochondrial membrane demise (Fig. 2) and caspase-9 activation (Fig. 3) in bovine neutrophils aged *ex vivo*. Because Bcl-2 proteins are critical regulators of mitochondrial membrane stability and downstream activity of caspase-9 in neutrophils from other species (3, 15), it was of interest to determine whether glucocorticoid-mediated changes in family members A1 and Bak also occurred. We had previously observed a significant increase in A1 and decrease in Bak mRNA abundance in

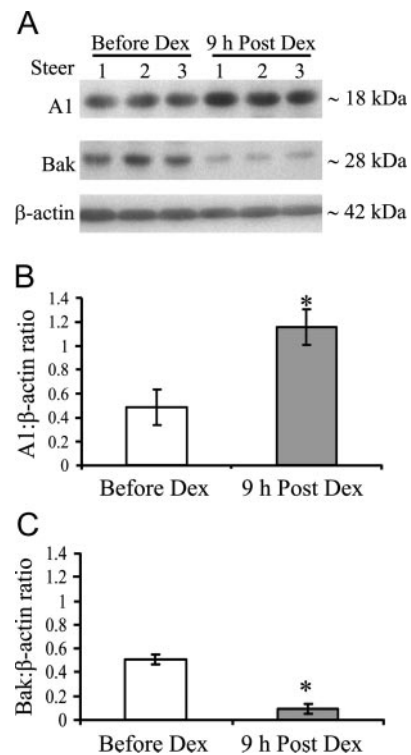


FIG. 6. Dexamethasone (Dex) administration into three steers altered the abundance of A1 and Bak proteins in circulating neutrophils. The Western blots in A show that A1 protein was increased, whereas Bak protein was decreased in neutrophils at 9 h after dexamethasone administration, compared with before steroid administration. β -Actin was used as the lane-loading control. In B and C, bands visualized by chemiluminescence were analyzed by scanning densitometry and A1 and Bak protein abundance recorded as density ratios to β -actin. B, A1 protein exhibited a significant 2.4-fold increase in neutrophils after dexamethasone administration. C, Bak exhibited a significant 5.4-fold decrease in the same neutrophils. *, $P < 0.05$ relative to before dexamethasone administration.

circulating bovine neutrophils sampled during the cortisol surge at parturition (34).

In this study, neutrophils treated with dexamethasone and aged *ex vivo* had significantly increased A1 protein abundance, compared with untreated cells (Fig. 5, A and B), which may have resulted from corresponding increases in A1 mRNA abundance (Fig. 4B). Pretreatment with RU486, a potent glucocorticoid receptor antagonist, removed the effect of dexamethasone on A1 protein increase showing that the steroid's actions on A1 were direct and mediated through the cell's glucocorticoid receptors. Dexamethasone administration into steers also resulted in increased A1 protein abundance in circulating neutrophils (Fig. 6, A and B). *In vivo* A1 increases occurred in conjunction with neutrophil driven leukocytosis that is typical in steroid-treated animals (26–29). Previous work of others demonstrated that changes in A1 abundance occur when neutrophils are exposed to a variety of proinflammatory factors, including G-CSF (16), GM-CSF, TNF α , IFN γ (5), and LPS (16, 17). Our results extend these findings to include glucocorticoids as an acute and direct inducer of A1 abundance in bovine neutrophils.

Our results of effects of dexamethasone on neutrophil Bak expression were not as clear-cut as for A1. For example,

whereas Bak mRNA abundance was significantly reduced by dexamethasone treatment *in vitro* and this glucocorticoid effect was also inhibited by RU486 (Fig. 4D), corresponding decreases in Bak protein abundance were not detectable in the dexamethasone-treated cells (Fig. 5, A and C). In contrast, clear decreases in Bak protein were observed in circulating neutrophils after dexamethasone administration *in vivo* (Fig. 6, A and C). The inconsistency between our *in vitro* and *in vivo* Bak protein results may be related to the minimal culture system used for the *in vitro* experiments. For example, Bak protein down-regulation in response to the steroid may require additional glucocorticoid-induced (or suppressed) factors from other cell types that are present *in vivo* but not *in vitro*. We speculate that such factor(s) may help regulate Bak protein synthesis, half-life, and (or) subcellular location in neutrophils. One such factor may be G-CSF, which is massively increased in blood of humans treated with dexamethasone (42) and well known to delay neutrophil apoptosis (14, 19). The lack of Bak protein decrease *in vitro* also may have resulted from cell activation during the neutrophil isolation procedure and (or) due to the nonphysiological environment of our minimal cell culture system. However, we feel that these are unlikely explanations as neutrophil activation typically results in delayed apoptosis, which was not observed in our control (0 M dexamethasone, 0 M RU486) cultures.

Alternatively, differences between our *in vitro* and *in vivo* Bak protein results may have been related to the neutrophil-driven leukocytosis that occurs in steroid-treated animals. Glucocorticoid-induced neutrophilia derives largely from demargination of mature neutrophils tethered to blood vessel endothelia, although increased bone marrow release of mature neutrophils (some band but mostly segmented cells) also contributes to the increase in circulating neutrophil counts (28, 35). Thus, the population of circulating neutrophils sampled 9 h after dexamethasone administration may be somewhat younger on average than the blood cells sampled from animals to which the steroid had not been administered, possibly explaining differences in Bak expression and (or) sensitivity of the Bak system to glucocorticoid challenge. Furthermore, the effective dose of dexamethasone available to circulating neutrophils *in vivo* may be different from that used in the *in vitro* experiments.

Estimated blood dexamethasone concentrations after im administration are approximately 1000-fold higher than what was used in our *in vitro* studies (43). With the numerous other cells and tissues found *in vivo*, it is unlikely that neutrophils would absorb all available dexamethasone. This large dose would also affect surrounding cells, potentially stimulating release of factors capable of altering Bak abundance. Thus, whereas we have shown that glucocorticoid directly regulates Bak mRNA abundance in bovine neutrophils, other as-yet-unidentified factors must be required to translate the mRNA abundance change into altered Bak protein abundance and (or) availability *in vitro*.

Other considerations to help explain our observations are that A1 abundance changes may be relatively more important to the apoptotic status of bovine blood neutrophils than changes in Bak (6, 8). With conflicting reports of whether A1 and Bak interact (11, 44), it would seem relevant in future studies to determine whether glucocorticoid-mediated in-

creases in A1 abundance creates competition with existing Bak proteins for the formation of A1/Bak heterodimers and thereby limit the availability of Bak/Bak homodimers for mitochondrial membrane pore formation. It is also possible that interactions between hormone-activated glucocorticoid receptors, A1, and Bak in neutrophil apoptosis control involve association between some or all of these molecules with the BH3-only Bcl-2 family member Bid. Bid undergoes cleavage to truncated Bid (tBid) during Fas-induced apoptosis (13) as well as spontaneous or ROS-induced apoptosis (14, 45). A1 associates with tBid to inhibit its activation of Bak (12). Thus, the increases in A1 we observed in glucocorticoid-treated neutrophils may have delayed the cells' apoptosis by decreasing interactions between tBid and Bak. It is perhaps not coincidental, and may be critical to apoptosis regulation, that glucocorticoids also inhibit expression of ROS-generating genes (35), ROS production (46), and Fas expression in treated neutrophils (26). Therefore, glucocorticoid control of A1 may act as not only a key regulatory point in the spontaneous death of neutrophils through the mitochondrial pathway but also a link between the spontaneous and death receptor-induced apoptosis pathways. Regardless, it does appear that the relative ratio of pro- and antiapoptotic Bcl-2 family members is a pivotal control point in neutrophil apoptosis regulation (10), and, given the A1 and Bak abundance results of the current study, the role of glucocorticoids in altering the ratio of these Bcl-2 proteins and subsequent effects on the inflammatory behaviors of neutrophils warrant further investigation.

In summary, our observations suggest that glucocorticoid-induced changes in A1 and Bak abundance may be part of the mechanism by which this steroid preserves mitochondrial membrane integrity and delays spontaneous apoptosis in bovine blood neutrophils. Understanding the molecules affected during glucocorticoid regulation of neutrophil apoptosis may provide insight into the seemingly contradictory actions of this steroid to delay apoptosis and concurrently provide potent antiinflammatory signals to neutrophils. In the case of Bak, it may be necessary for glucocorticoids to influence or act in conjunction with other factors or cells present in a whole animal system to transform the *in vitro* mRNA abundance changes we observed into changes in Bak protein abundance. Whether changes in A1 and Bak alone or in conjunction with glucocorticoid-mediated decreases in death receptor signaling were responsible for observed stability of mitochondria membranes and inhibited caspase-9 activation in glucocorticoid-treated neutrophils has yet to be determined. In either scenario, results of this study implicate likely roles for A1 and Bak expression, and subsequent mitochondrial membrane stability, in glucocorticoid-mediated delay of neutrophil spontaneous apoptosis.

Acknowledgments

We thank Dr. Paul Coussens for critical review of the manuscript; Dr. Patty Weber, Ms. Ling-Chu Chang, and Ms. Kelly Buckham for their assistance with animal handling and blood collections; Ms. Chang for assistance with generation of standard curves for real-time PCR; and Mr. Bob Kreft and his staff at the Michigan State University-Dairy Teaching and Research Facility for their excellent care of the steers used in this study.

Received February 3, 2006. Accepted April 27, 2006.

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This work was supported by funds from the Michigan Agricultural Experiment Station Project MICL01836 and MIC01691 (for J.L.B.'s participation in U.S. Department of Agriculture Multistate Research Project NC-1010) and USDS-IFAFS Grant 2001-52100-11211.

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S.A.M.-B., G.J.M.R., and J.L.B. have nothing to declare.

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