

Effects of glucocorticoids on Fas gene expression in bovine blood neutrophils

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Abstract

Blood neutrophils are extremely short-lived cells that are programmed for rapid apoptosis after differentiation in bone marrow. Recently, glucocorticoids have been shown to prolong survival of human and rodent neutrophils, but the mechanisms and implications for leukocyte homeostasis and health are unclear. In this study, we investigated the effects of endogenous and exogenous glucocorticoids on Fas expression in bovine neutrophils because Fas is a major death receptor that stimulates apoptosis in circulating cells. Our study subjects were four periparturient dairy cows whose blood concentrations of cortisol peaked at calving, 15 dexamethasone-treated steers and three untreated steers whose neutrophils were exposed to dexamethasone *in vitro*. Fas mRNA abundance changes in collected neutrophils were monitored numerous times relative to the *in vivo* glucocorticoid challenges, and the relationships between these data and circulating neutrophil counts were estimated by correlation analyses. Fas mRNA and protein abundance, caspase 8 activity, and survival of neutrophils *in vitro* were also monitored in the presence and absence of dexamethasone. In the periparturient cows, Fas mRNA abundance in circulating neutrophils showed a sharp decrease between calving and 12 h postpartum. Based on PROC CORR analysis (SAS), this correlated negatively with blood neutrophil count ($r=-0.634$;

$P=0.0009$) and serum cortisol concentration ($r=-0.659$; $P<0.0001$), but showed no relationship with serum progesterone or estradiol concentrations ($P \geq 0.09$). Administration of dexamethasone to steers also caused a pronounced reduction in neutrophil Fas mRNA abundance that persisted for 12 h and correlated negatively with blood neutrophil count ($r=-0.748$; $P=0.0021$). *In vitro*, dexamethasone caused dose-dependent loss of GR proteins from the cytosol of neutrophils concurrently with Fas mRNA downregulation, which was inhibited by the glucocorticoid receptor (GR) antagonist, RU486. Dexamethasone treatment of cultured neutrophils also reduced surface Fas expression, spontaneous and sFasL-induced caspase 8 activity, and rate of apoptosis in the cells. Taken together, these *in vivo* and *in vitro* results suggest that glucocorticoids inhibit Fas expression in bovine blood neutrophils via GR activation, possibly contributing to the cells' increased longevity in culture and the pronounced neutrophilia observed in parturient cows and hormone-treated steers. We thus conclude that glucocorticoid-activated GR may change the homeostasis of circulating neutrophils, in part through its negative effects on Fas gene expression and downstream apoptosis signaling pathways.

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Introduction

Neutrophils are one of the most important immune cells regulating acute inflammation after trauma and infection. The best-known function of neutrophils is their bactericidal activity, which is accomplished by release of reactive oxygen species and lytic enzymes following the phagocytosis-induced respiratory burst after the cells migrate into inflamed and infected tissues. The onset of apoptosis shortly after phagocytosis impairs neutrophil function, in turn limiting damage to other healthy cells in the vicinity of infection foci (Whyte *et al.* 1993, Haslett 1999).

Unlike inflammatory neutrophils that live for 24–48 h in infected tissues, blood neutrophils have a short life span of 6–12 h in the circulation. This is because these cells are genetically programmed to undergo apoptosis as soon as they terminally differentiate in bone marrow (Kuijpers 2002). The tight regulation of blood neutrophil life span via apoptosis rather than necrosis is critical to avoid systemic inflammation and blood vessel damage (Sendo *et al.* 1996). However, the life span of blood neutrophils can be extended when circulating concentrations of cytokines and certain hormones are elevated. For example, colony-stimulating factors (G-CSF and GM-CSF), cytokines (interleukin (IL)-1 β , IL-2, IL-6, IL-8 and tumor necrosis

factor (TNF)- α), and glucocorticoids can delay apoptosis for up to 48 h in human and rodent neutrophils (Cox 1995, Liles *et al.* 1995, 1996, Meagher *et al.* 1996, Nittoh *et al.* 1998, Daffern *et al.* 1999, Webb *et al.* 2000). The main mechanism of prolonged neutrophil survival by cytokines appears to be activation of NF- κ B and its subsequent transactivation of numerous proinflammatory genes that are also antiapoptotic (Moulding *et al.* 1998, 2001, Dibbert *et al.* 1999). The cytokine-induced delay of apoptosis of neutrophils recruited into inflammatory foci may enhance phagocytosis of infectious pathogens (Coxon *et al.* 1999). However, the mechanisms explaining the prolonged survival of neutrophils mediated by glucocorticoids are not known. It is also not clear why glucocorticoids induce neutrophil survival, because the hormones also depress neutrophil migration and bactericidal activity (reviewed by Heasman *et al.* 2003).

Neutrophil apoptosis occurs through two main pathways (reviewed by Fanning *et al.* 1999), one that is mediated via exogenous death receptor signaling (Liles *et al.* 1996) and the other that occurs spontaneously through mitochondrial membrane changes under the influence of Bcl-2 family proteins (Lin *et al.* 1996). In the current study, we chose to examine the main exogenous death receptor of neutrophils, Fas. Fas (also called CD95/APO-1) is a type I membrane glycoprotein belonging to the TNF-receptor superfamily of molecules. Death signals are initiated in neutrophils when Fas becomes trimerized upon interaction with its ligand, Fas ligand (FasL). FasL activation of Fas recruits numerous death-domain-containing proteins to the cytoplasmic death domain of Fas, and these act as adaptor molecules for the ultimate recruitment and activation of caspase 8 to initiate apoptosis signaling via a variety of mechanisms (reviewed by Schulze-Osthoff *et al.* 1998, Sharma *et al.* 2000). Ultimately, caspase 8 activation results in cleavage of proteins involved in cytoskeletal maintenance and DNA repair, leading to membrane blebbing, nuclear condensation and collapse, and irreversible cell death (reviewed by Robertson 2000, Zimmermann *et al.* 2001). Fas and FasL are constitutively coexpressed in human blood neutrophils, rendering the cells highly sensitive to apoptosis (Liles *et al.* 1996).

Several studies report that glucocorticoids attenuate apoptosis in human and rat neutrophils (Cox 1995, Liles *et al.* 1995, 1996, Meagher *et al.* 1996, Nittoh *et al.* 1998). However, the mechanisms of survival induction by glucocorticoids are not known, and specific effects of glucocorticoids on Fas expression have not been reported. We hypothesized in the current study that glucocorticoids downregulate Fas expression in neutrophils by activating the cells' glucocorticoid receptors (GR). Our results show that glucocorticoids cause dose-dependent and GR-mediated downregulation of Fas gene expression in bovine blood neutrophils, with corresponding reductions in surface Fas expression, caspase 8 activity and rate of apoptosis *in vitro*, and increases in circulating cell numbers *in vivo*.

Materials and Methods

Animals and sample collections

Experiment 1: periparturient cows Four periparturient Holstein cows in first pregnancy were used to determine whether Fas mRNA expression in neutrophils changes during parturition in correlation with serum steroid concentrations and circulating neutrophil numbers. The cows were housed at Michigan State University's (MSU) Dairy Teaching and Research facility and cared for according to standard operating procedures there (MSU animal use approval no. 03/99-031-00). Blood samples were collected on days -8, -4, 0, 0.25, 0.5, 1, 1.5, 2 and 7 relative to parturition (on day 0), as described in a previous study (Weber *et al.* 2001). Blood designated for neutrophil isolations was collected in acid citrate dextrose (ACD) anticoagulant, while that used for serum steroid assays was collected into evacuated tubes containing no anticoagulant. All blood samples were placed on ice immediately after collection.

Experiment 2: dexamethasone-treated steers Fifteen Holstein steers (4–5 months of age) castrated at 1 month of age were used for an *in vivo* glucocorticoid challenge study. The animals were housed in individual pens at the MSU Dairy Teaching and Research facility, fitted with jugular catheters (Weber *et al.* 2004), and cared for according to standard operating procedures (MSU animal use approval no. 06/01-092-00). Steers were randomly allocated to five blood collection groups with three steers per group. One group served as untreated controls, and its blood samples were called '0 h'. Steers in the other four groups were treated with various doses of dexamethasone (Azium; Schering Plough, Animal Health, Kenilworth, NJ, USA), each dose administered intramuscularly at 0.10 mg/kg body weight. In the 3-h and 6-h groups, dexamethasone was administered once at 0 h, and blood samples were collected 3 and 6 h later respectively. In the 12-h group, dexamethasone was administered twice at 0 and 6 h, and blood samples were collected 12 h after the 0-h dose. In the 24-h group, dexamethasone was administered thrice, at 0, 6 and 12 h, and blood samples were collected 24 h after the 0-h dose. All blood samples were collected through the jugular catheters into ACD-containing tubes, which were placed on ice immediately.

Experiment 3: dexamethasone treatment of isolated neutrophils Neutrophils were isolated from the blood of three additional donor steers (MSU animal use approval no. 06/01-092-00) as needed for use in *in vitro* experiments. These animals were not treated with dexamethasone. Isolated blood neutrophils (see below) were cultured in basic medium (RPMI-1640 medium; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 25 mM HEPES, 1% BSA (Sigma) and 25 units/ml

penicillin–25 µg/ml streptomycin (Invitrogen), and treated with varying doses of dexamethasone (0, 10^{-9} , 10^{-7} or 10^{-5} M) as described below. Where indicated, additional cultures of identical cells were pretreated for 10 min with RU486 (10^{-4} M; Sigma) prior to addition of dexamethasone. Neutrophils intended for RNA and protein isolations were seeded into sterile, 50 ml, conical tubes (10 ml of 5×10^6 cells/ml) and incubated for 4 h (Forma Scientific Model 420; Thermo Electron Corporation, Marietta, OH, USA) at 39 °C (normal body temperature for cattle) and a shake speed of 90 r.p.m. After incubation, the cells were split into two aliquots and pelleted for lysis in either TRIzol Reagent (Invitrogen) or in a buffer containing protease inhibitors (see below). Neutrophils intended for apoptosis phenotyping were seeded into wells of culture plates, and treated as described below.

Assay protocols

Radioimmunoassay of serum steroids In experiment 1, sera were harvested (1000 µg at 4 °C for 20 min) from blood samples that were clotted overnight at 4 °C, and stored at –20 °C until assayed in duplicate for cortisol (µg/dl), progesterone (ng/ml) and estradiol (pg/ml) (Coat-A-Count RIA kits; Diagnostic Products Corporation, Los Angeles, CA, USA). As described in Weber *et al.* (2001), cortisol and progesterone assays were performed on single days (intra-assay CVs of <9%). Aliquots of sera for estradiol determination were extracted with diethyl ether before being assayed (intra-assay CV = 6.1%; inter-assay CV = 15.1%).

Neutrophil differentials in whole blood

Aliquots of whole blood collected from cows and steers in experiments 1 and 2 were subjected to flow cytometric analysis (FACScalibur Flow Cytometer and Cell Quest software; Becton Dickinson, San Jose, CA, USA) to determine the percentages of neutrophils (of total leukocytes) in each sample based on the cells' characteristic forward- and 90°-light scattering properties (Weber *et al.* 2001).

Purification of bovine blood neutrophils

Neutrophils were isolated from whole blood of all animals by Percoll density gradient centrifugation (Weber *et al.* 2001, 2004). Neutrophil purity was $\geq 95\%$ (based on flow cytometric analysis, described above), and viability was $\geq 97\%$ (based on propidium iodide staining of nuclei). The minor population of contaminating cells were eosinophils.

Extraction of neutrophil total RNA

Neutrophil samples from all three experiments that were lysed in TRIzol Reagent (1×10^7 cells/ml) were stored at –80 °C until total RNA isolations were performed

(according to the manufacturer's instructions). Total RNA concentration and purity were determined with a DU-650 spectrophotometer (Beckman Coulter, Scaumburg, IL, USA) and the 260 and 280 nm readings.

Northern and slot blotting

In experiment 1, Fas mRNA abundance in neutrophils from parturient cows was quantified by slot blot analysis. The Fas cDNA probe used was 517 base pairs (bp) in length and generated by PCR from bovine total leukocyte cDNA template. The forward primer was 5'- ATG GGC TAG AAG TGG AAC AAA AC-3' and the reverse primer was 5'- TTC TTC CCA TGA CTT TGA TAC C-3'. The PCR mixture contained 20 mM Tris-HCl, 50 mM KCl, 0.2 mM of each dNTP, 3.0 mM MgCl₂, 0.2 nM of each primer, 100 ng template cDNA and 2.0 units of *Taq* DNA polymerase (Invitrogen) in a final volume of 50 µl. PCR reactions were carried out in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA, USA) under the following conditions: hot start at 95 °C for 3 min; 35 cycles of PCR amplification at 95 °C for 30 s, 47 °C for 30 s and 72 °C for 30 s; and final extension at 72 °C for 10 min. The PCR amplification product was analyzed by agarose gel electrophoresis (1.8%) and visualized as a single bright band after ethidium bromide staining. This product was ligated into pGEM-T Easy (Promega) and the recombinant plasmid transformed into JM109 competent cells (Promega). The 517 bp insert was DNA sequenced and shown to be 97% homologous to the published sequence for bovine Fas (Yoo *et al.* 1996) (GenBank accession no. NM_174662). The cDNA insert was excised from plasmid DNA with EcoRI (New England Biolabs, Beverly, MA, USA), gel purified (Wizard Kit, Promega) and visualized as a single bright band on a 1.8% agarose gel stained with ethidium bromide for use in Northern blot assessment. This was done with 8 µg of neutrophil total RNA from one cow at 8 days prepartum (experiment 1) for electrophoresis, according to the protocol in Weber *et al.* (2001). Slot blot analysis (Weber *et al.* 2001) was then performed to quantify Fas mRNA abundance in all neutrophil samples of experiment 1. Briefly, 5 µg of each RNA sample was spotted on a nylon membrane, probed with ³²P-labeled Fas cDNA, stripped and reprobbed with ³²P-labeled β-actin cDNA. Slot intensities were determined by scanning densitometry (GS-710 Calibrated Imaging Densitometer and Multi-Analyst Software; BioRad), and Fas mRNA abundance was recorded as the density ratio Fas:β-actin for each slot.

Quantitative real-time reverse transcription polymerase chain reaction (Q-RT-PCR)

In experiments 2 and 3, Fas and (or) GRα mRNA abundance was quantified by Q-RT-PCR, as described by Madsen *et al.* (2004). An amount of 2 µg total RNA per

sample was used for cDNA synthesis (oligo(dT)_{12–18} primer (Invitrogen) and Superscript II RNaseH⁻-Reverse Transcriptase (Invitrogen)), and the Q-RT-PCR assay was performed in duplicate with 2.5 ng starting cDNA per sample and the SYBR Green PCR Master Mix system for detection (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Gene-specific primers for bovine Fas, GR α and β -actin were designed with Primer Express Software (Perkin Elmer Applied Biosystems) and synthesized at a commercial facility (Qiagen-Operon, Alameda, CA, USA). For Fas, the primers were as follows: forward, 5'-TGT AAA GTC AGC TTA TAC ACA GCA GAA GT-3'; reverse, 5'-GTG GGC TGC CGC CTA TG-3' (product length 104 bp, T_m=81 °C). For GR α , the primers were as follows: forward, 5'-TGT GGT TTA AAG AGG GCC AAG A-3'; reverse, 5'-TTC TAC GTT CCC ATC ACT GAA AAG-3' (product length=74 bp, T_m=78 °C). For β -actin, the primers were as follows: forward, 5'-CGC CAT GGA TGA TGA TAT TGC-3'; reverse, 5'-AAG CCG GCC TTG CAC AT -3' (product length=66 bp, T_m=84 °C). The resulting Fas and GR α mRNA abundance data were normalized against β -actin mRNA abundance, and the gene expression changes induced by the various treatments were determined by the 2^{- $\Delta\Delta$ C_T} method of Livak and Schmittgen (2001). In experiment 2, data from the 0-h samples were used as the calibrators in 2^{- $\Delta\Delta$ C_T} against which all other samples were compared. In experiment 3, untreated neutrophils at 0 h were used as the calibrators in the 2^{- $\Delta\Delta$ C_T} analyses.

Immunoblot analysis of glucocorticoid receptor proteins

Methods used to isolate neutrophil cytosolic fractions and perform the immunoblot assay are detailed in Weber *et al.* (2004). Lanes of 10% SDS-PAGE gels were loaded with 40 μ g cytosolic total protein from steer neutrophils treated *in vitro* with 0, 10⁻⁹, 10⁻⁷ or 10⁻⁵ M of dexamethasone (experiment 3). GR abundance in each sample was detected with an antirabbit GR polyclonal antibody (0.5 μ g/ml) (PA1-511A; Affinity Bioreagent, Golden, CO, USA) that recognizes both alpha (GR α) and beta (GR β) isoforms of GR. A goat antirabbit immunoglobulin (Ig)_{G1} horseradish peroxidase-conjugate (Pierce, Rockford, IL, USA) was used as the detection antibody. Immunoblots were developed with the SuperSignal West Pico chemiluminescent substrate system (Pierce). Membranes were stripped and reprobed with an anti- β -actin primary antibody (Abcam, Cambridge, UK), with goat antimouse IgG₁ horseradish peroxidase-conjugate (Bethyl, Montgomery, TX, USA) as the detection antibody, to check equal loading of samples across lanes.

Fluorescence-activated flow cytometry

Changes in neutrophil surface expression of Fas protein under *in vitro* treatment with dexamethasone were

monitored by immunostaining and fluorescence-activated flow cytometry (FACSCalibur Flow Cytometer; Becton Dickinson). Purified neutrophils from steers of experiment 3 were cultured in duplicate in basic medium that contained 1% fetal bovine serum (FBS), in the absence or presence 10⁻⁷ M dexamethasone, for 0, 4 or 8 h (in moist 5% CO₂ air at 39 °C). An anti-Fas polyclonal antibody (1:200 dilution; Stressgen, British Columbia, Canada) and PE-conjugated secondary antibody (1:400 dilution) (Molecular Probes, Eugene, OR, USA) were used for immunostaining, which was done according to our published method (Weber *et al.* 2004). Assay-negative controls included unstained cells and cells treated with only the secondary antibody. Data from 10 000 cells per sample were collected. Changes in surface Fas expression were determined from histogram plots of PE-fluorescence intensity, as the percentage (%) of Fas⁺ cells. These raw data were then used to calculate the percent change (% Δ) in Fas⁺ cells for dexamethasone-treated and -untreated cells at 4 and 8 h relative to untreated cells at 0 h.

Caspase 8 activity

Caspase 8 activity was determined with the ApoAlert Caspase-8 Colorimetric Assay Kit (BD Biosciences, Palo Alto, CA, USA), according to the manufacturer's recommended procedure. Briefly, 3 \times 10⁶ cells per well were seeded into 24-well plates and cultured in basic medium containing 1% FBS in the absence or presence of 10⁻⁷ M dexamethasone for 4 h. Then, cells were stimulated with 0, 10, 50 or 100 ng/ml of recombinant sFasL (Alexis, San Diego, CA, USA) for an additional 3 h. The assay control was cells pretreated for 30 min with a caspase 8 inhibitor (50 μ M of z-IETD-fmk; Calbiochem, La Jolla, CA, USA) before sFasL stimulation. After incubation, cells were centrifuged at 500 g for 5 min at 4 °C, and washed twice with cold PBS, pH 7.2. Cells were suspended in 50 μ l chilled cell lysis buffer and incubated on ice for 10 min. Cellular debris was separated by centrifugation (13 000 g, for 10 min at 4 °C). An equal amount of 2X Reaction Buffer/DTT mix containing the labeled Caspase 8 substrate (IETD-pNA; final concentration of 200 μ M), was added to the supernatant and incubated at 37 °C for 3 h. Substrate cleavage with pNA accumulation and accompanying color change was monitored by a microplate reader (405 nm) (Benchmark Microplate Reader, Bio-Rad). Protein determinations were made for each sample with the Bradford Assay Protein Detection Kit (Bio-Rad), and caspase 8 activity was recorded as optical density_{405 nm} per mg protein (OD/mg protein).

Annexin V-FITC and propidium iodide staining

Apoptosis status of cultured neutrophils from steers of experiment 3 was assessed by two-color fluorescence-activated flow cytometric analysis of cells stained at various

incubation times with Annexin V-FITC and propidium iodide (PI). Briefly, neutrophils were reconstituted at 1×10^7 cells/ml in basic culture medium containing 1% FBS, and 0.1 ml per well was seeded into 96-well cell culture plates (Fisher Scientific, Pittsburgh, PA, USA) and incubated in moist 5% CO₂ air at 39 °C for 7, 12 or 24 h. After incubation, cells were centrifuged at 500 g for 5 min at 4 °C, washed twice with cold PBS, pH 7.2, and stained with FITC-conjugated Annexin V and PI by the protocol contained in a commercial kit (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences Pharmingen, San Diego, CA, USA). Cells were then transferred to 5 ml polystyrene round-bottom tubes (Becton Dickinson), and two-color data were acquired for 10 000 cells per sample (FACSCaliber flow cytometer and Cell Quest software; Becton Dickinson). Quadrants were set on resulting density plots, effectively separating Annexin V⁻/PI⁻ non-apoptotic cells (lower left quadrant) from Annexin V⁺/PI⁻ early apoptotic cells (lower right quadrant), Annexin V⁺/PI⁺ late apoptotic cells (upper right quadrant) and Annexin V⁻/PI⁺ necrotic cells (upper left quadrant).

Statistical analysis

Before analyses, data sets were checked for normality of their distributions. All data were normally distributed and thus analyzed in their raw form, using the PROC MIXED function of SAS and including Tukey's adjustment for multiple comparisons (SAS Institute 1996). Stated differences between observations were declared when $P < 0.05$. Data presented in figures are time or treatment least-squares means \pm S.E.M. Pearson product moment correlations between neutrophil Fas mRNA abundance and blood cortisol concentrations, or percent neutrophils in experiments 1 and 2 were computed by the PROC CORR function of SAS (SAS Institute 1990).

Results

Experiment 1: periparturient cows

Figure 1A shows that our Fas cDNA probe recognized two distinct transcripts in bovine blood neutrophils (approximately 1.7 and 2.9 kb) that were similar in size to those reported in numerous human cell lines (Nambu *et al.* 1998, O'Donnell *et al.* 1999). We suspect that the 2.9 kb transcript represents a primary, unprocessed Fas mRNA, while the 1.7 kb transcript represents fully processed Fas mRNA. In a preliminary experiment, we confirmed that both transcripts were downregulated in neutrophils of a dexamethasone-treated steer (data not shown), and thus we proceeded with slot blot analysis of Fas mRNA abundance in neutrophils of the periparturient cows. This work showed that parturition caused a pronounced decrease ($P < 0.05$) in Fas total mRNA, which reached

nadir between days 0 and 0.5 and began to return to precalving levels by day 2 (Fig. 1B). The scatter plots in Fig. 2 show that Fas mRNA abundance was negatively correlated with serum cortisol concentrations ($r = -0.659$; $P < 0.0001$; Fig. 2A) and with percent neutrophils in blood of the periparturient cows ($r = -0.634$; $P = 0.0009$; Fig. 2B). Insignificant correlations were found between Fas mRNA abundance and the concentrations of serum estradiol ($r = 0.05$; $P = 0.78$) or progesterone ($r = -0.304$; $P = 0.09$) (data not shown).

Experiment 2: dexamethasone-treated steers

To test further the possible relationship between elevated blood glucocorticoid concentration and Fas gene expression in neutrophils, steers of experiment 2 were treated with various doses of dexamethasone and Fas mRNA abundance, and monitored by Q-RT-PCR. As shown in Fig. 3A, levels of Fas mRNA were decreased ($P < 0.05$) to nadir in the 6-h group ($79.3 \pm 2.3\%$) and the 12-h group ($86.4 \pm 2.8\%$) relative to levels observed in the 0-h group. Overall, Fas mRNA abundance was negatively correlated with percentage of neutrophils in blood of these steers ($r = -0.748$; $P = 0.002$; Fig. 3B).

Experiment 3: dexamethasone treatment of isolated neutrophils

Dexamethasone treatment *in vitro* caused dose-dependent downregulation of Fas mRNA abundance in neutrophils isolated from normal steers of experiment 3 (Fig. 4A). At the same time, dexamethasone treatment caused dose-dependent loss of cytosolic GR α proteins in the neutrophils (representative immunoblot shown in Fig. 4B) without affecting the abundance of GR α mRNA (data not shown). Cytosolic expression of GR β protein was not influenced by dexamethasone treatment (Fig. 4B). Inhibition of Fas gene expression by *in vitro* dexamethasone treatment was reversed when the neutrophils were pre-treated with RU486 (Fig. 5). Fas protein expressed on the surface of bovine neutrophils was also decreased by dexamethasone treatment *in vitro* (Fig. 6). Dexamethasone-induced decreases in Fas mRNA and protein appeared to be relevant because the steroid also reduced spontaneous and sFasL-induced caspase 8 activation (Fig. 7A and B) and the rate of apoptosis (representative two-color density dot plots shown in Fig. 8) in treated neutrophils.

Discussion

The most significant findings of this study are as follows: 1. Fas gene expression in circulating bovine neutrophils changed in relationship to changing blood concentrations of endogenous and exogenous glucocorticoids; 2. Fas mRNA abundance correlated with blood neutrophil counts during bovine parturition and after glucocorticoid

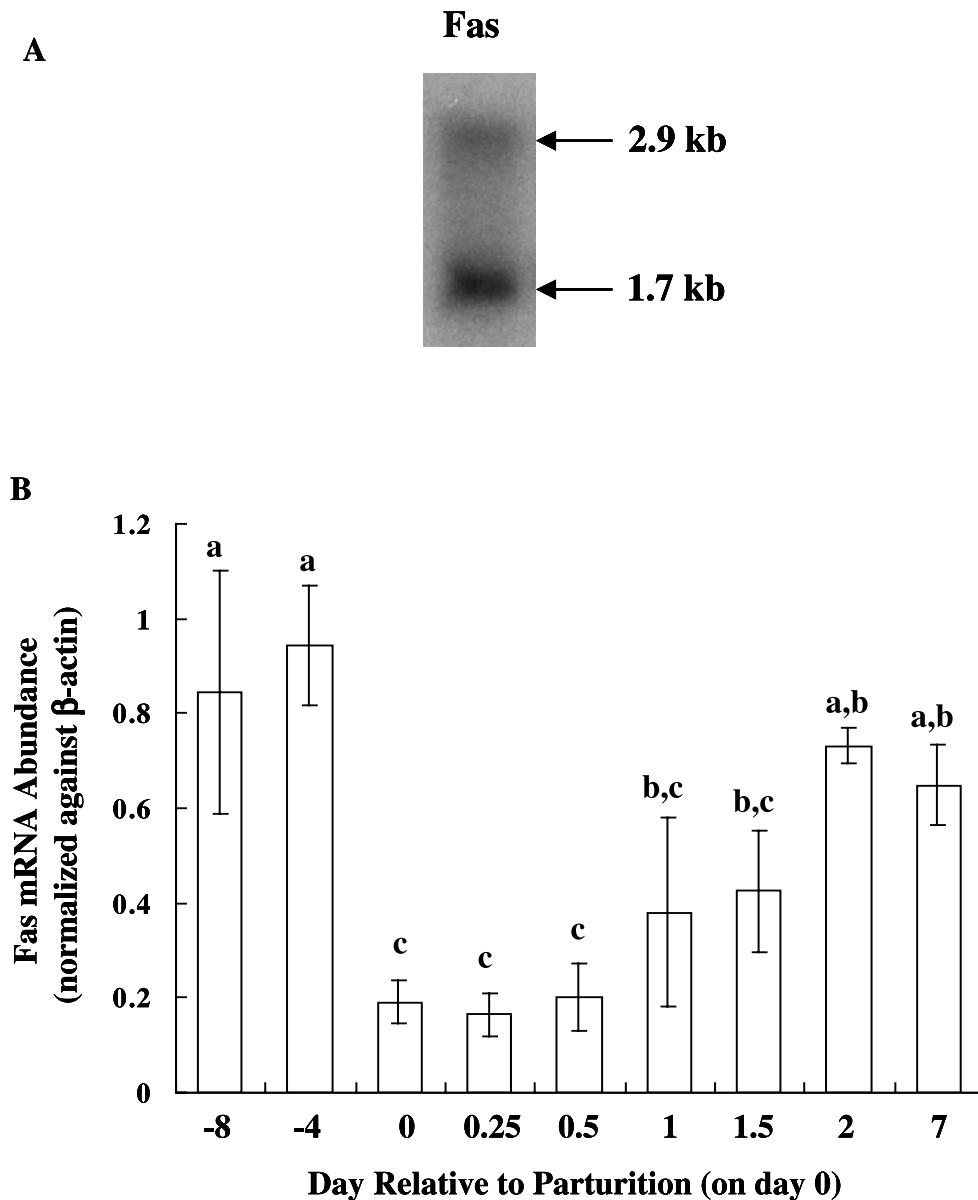


Figure 1 Fas mRNA abundance in blood neutrophils from periparturient cows. Fas mRNA abundance was decreased in bovine blood neutrophils for 1–5 days after parturition. (A) The 517 bp Fas cDNA probe developed for this study hybridized with two transcripts of 1.7 and 2.9 kb in bovine blood neutrophils. (B) Blood neutrophils were isolated from four periparturient cows on days –8, –4, 0, 0.25, 0.5, 1, 1.5, 2 and 7 relative to parturition (day 0). Fas mRNA abundance was analyzed by slot blot using the cDNA probe shown (A). The blots were stripped and reprobated with β -actin cDNA as the normalizing gene. Bars with different letters are significantly different at $P < 0.05$.

administration to cattle; 3. glucocorticoid-induced down-regulation of Fas gene and protein expression *in vitro* was associated with reduced activity of spontaneous and sFasL-induced caspase 8 activity and rate of apoptosis in cultured bovine neutrophils; 4. glucocorticoid-induced Fas down-regulation in bovine blood neutrophils involved hormone-activated GR α . Our results thus implicate inhibited Fas

expression and downstream apoptosis signaling as one possible mechanism to account for the reported ability of glucocorticoids to extend the survival of blood neutrophils (Cox 1995, Liles *et al.* 1995, 1996, Meagher *et al.* 1996, Nittoh *et al.* 1998, Daffern *et al.* 1999).

While GR exists in at least two isoforms (GR α and GR β), only GR α has known capacity for ligand binding

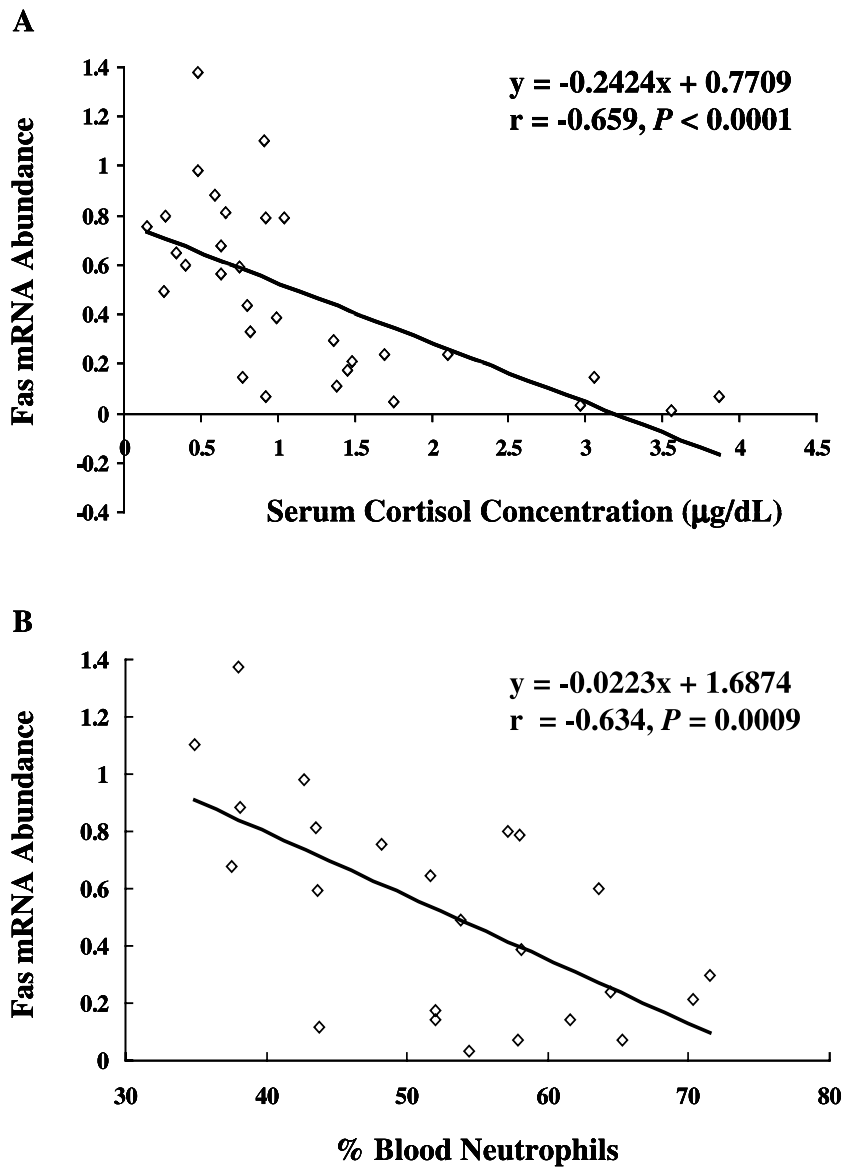


Figure 2 Fas mRNA abundance in blood neutrophils was negatively correlated with serum cortisol concentrations (A) and percentage of blood neutrophils (B) during parturition in dairy cows. Blood samples for these correlation analyses were from four parturient cows collected on days -8, -4, 0, 0.25, 0.5, 1, 1.5, 2 and 7 relative to parturition (day 0). Serum cortisol was determined by RIA. Percentage of neutrophils was determined flow cytometrically.

(Bamberger *et al.* 1996, Kino *et al.* 2001). Like the bovine neutrophils we studied here (Fig. 4B), human neutrophils are reported to express GR α and GR β (Strickland *et al.* 2001, Pujols *et al.* 2002). However, neutrophils are sensitive to glucocorticoids because of GR α expression (Weyts *et al.* 1998). In most resting cells, GR α is primarily located in the cytoplasm, where it complexes with heat-shock proteins to maintain a high-affinity hormone-binding state (Bamberger *et al.* 1996). Upon glucocorticoid binding,

GR α dissociates from this complex and translocates into nuclei, where it initiates transcription by binding to glucocorticoid-response elements (GRE) in promoters of inducible target genes, and by interacting positively with transcriptional coactivators of the genes (Bamberger *et al.* 1996, Collingwood *et al.* 1999, De Bosscher *et al.* 2003). However, GR α can also bind to negative GREs (nGRE), resulting in inhibition of gene transcription (Droulin *et al.* 1989). In addition to nGRE binding, GR α can halt

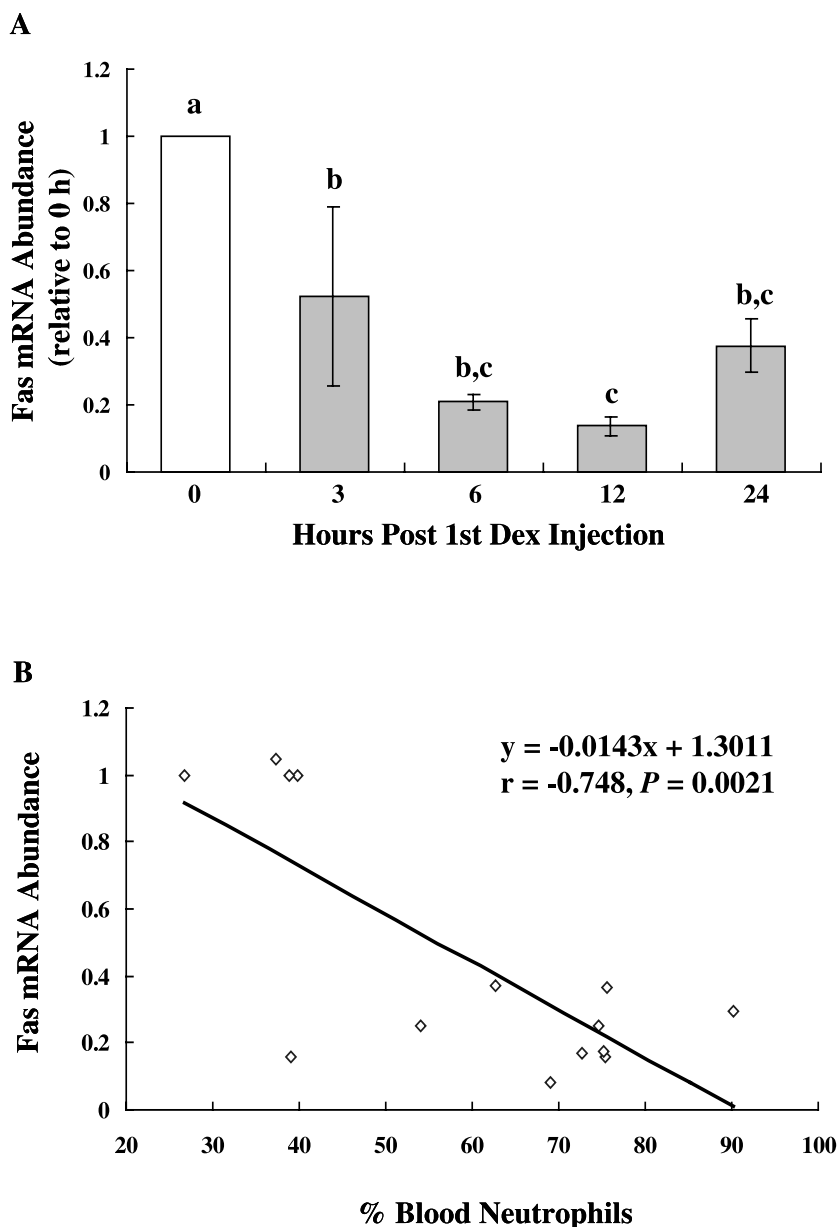


Figure 3 Fas mRNA abundance in blood neutrophils from dexamethasone-treated steers correlated negatively with percentage of circulating neutrophils. Dexamethasone (Dex) administered to steers caused a pronounced decrease in Fas mRNA abundance in blood neutrophils. The neutrophils were collected from 15 steers ($n=3$ per time group) at 0, 3, 6, 12 and 24 h after dexamethasone injection (0.10 mg/kg body weight). (A) Fas mRNA abundance was quantified by Q-RT-PCR. Shown are the mean (\pm S.E.M.) β -actin normalized Fas mRNA abundances for the 3-, 6-, 12- and 24-h samples (gray bars) relative to the 0-h sample (white bar). Bars with different letters are significantly different at $P<0.05$. (B) There was a significant negative correlation between Fas mRNA abundance and percentage of neutrophils in blood of the dexamethasone-treated steers.

transcription by interfering with the action of other transcription factors, such as AP-1 or NF- κ B (Scheinman *et al.* 1995, Bamberger *et al.* 1996, De Bosscher *et al.* 2003).

We observed that GR α was rapidly (4 h) lost in dose-dependent fashion from the cytosol of dexamethasone-treated bovine neutrophils (Fig. 4B), without concurrent loss of GR α mRNA (data not shown). Therefore, the

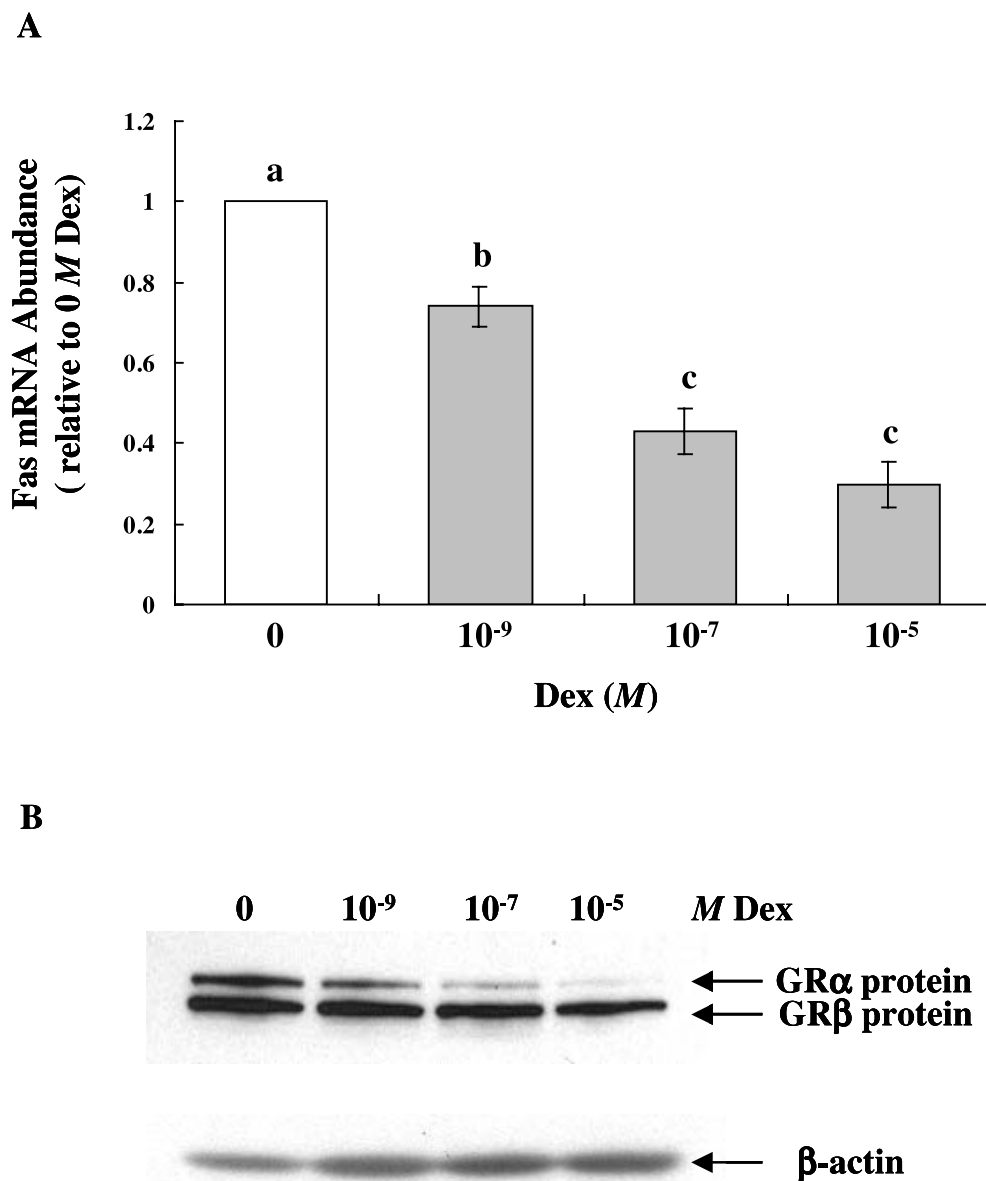


Figure 4 Dexamethasone effects on Fas mRNA abundance in bovine neutrophils were direct and associated with loss of glucocorticoid receptor α (GR α) from the cells' cytoplasm. Dexamethasone (Dex) caused a dose-dependent reduction of Fas mRNA abundance in isolated bovine blood neutrophils. The cells were cultured at 39 °C in RPMI-1640 medium containing 1% BSA and 0, 10^{-9} , 10^{-7} or 10^{-5} M Dex for 4 h. (A) Fas mRNA abundance was quantified by Q-RT-PCR. Shown are mean (\pm S.E.M.) β -actin normalized Fas mRNA abundances for the 10^{-9} , 10^{-7} or 10^{-5} M Dex samples (gray bars) relative to the 0 M Dex sample (white bar). Bars with different letters are significantly different at $P < 0.05$. (B) Dex caused dose-dependent loss of GR α protein from the cytosol of treated bovine neutrophils. Shown is a representative immunoblot for purified blood neutrophils of one steer cultured as in (A). The cells were harvested and processed for isolation of cytosolic proteins, and the cytosols probed with an anti-GR antibody that recognizes GR α (97 kDa) and GR β (94 kDa).

acute dexamethasone-induced decrease in GR α cytosolic proteins was probably not due to a limitation placed on GR α mRNA availability. Instead, ligand-activated GR α

may have translocated into the nucleus to influence Fas gene expression in the neutrophils (Fig. 4A). This possibility was substantiated by our GR antagonist

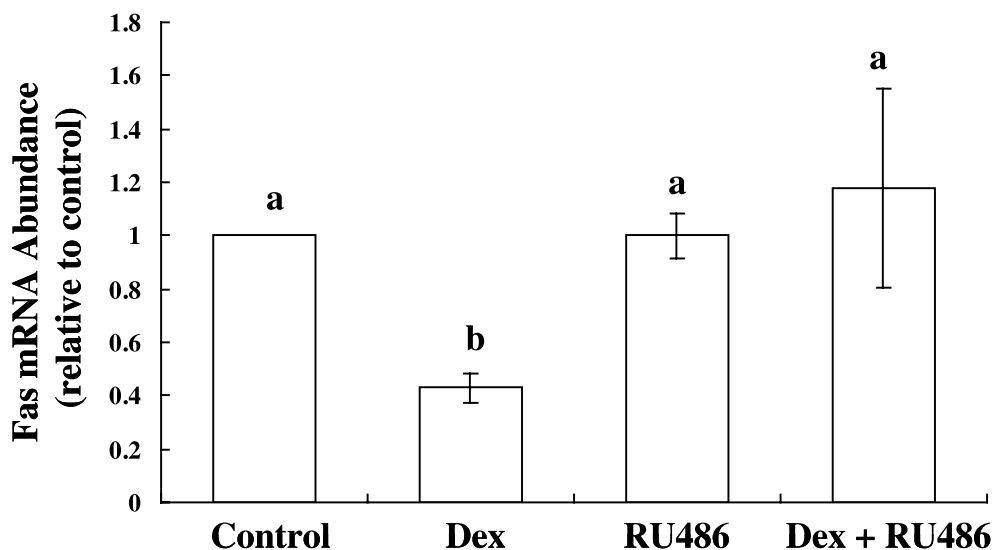


Figure 5 Dexamethasone (Dex)-induced downregulation of Fas mRNA abundance in isolated bovine blood neutrophils was inhibited by glucocorticoid receptor α (GR α) antagonism. Isolated blood neutrophils from three steers were cultured at 39 °C in basic culture medium for 10 min without (0 M) and with (10^{-4} M) RU486, prior to an additional incubation (4 h) in medium alone (0 M Dex) or in medium containing 10^{-7} M Dex. Fas mRNA abundance was quantified by Q-RT-PCR. Shown are mean (\pm S.E.M.) β -actin normalized Fas gene expressions for the Dex, RU486 and RU486 \pm Dex-treated cells relative to untreated cells (control). Bars with different letters are significantly different at $P < 0.05$.

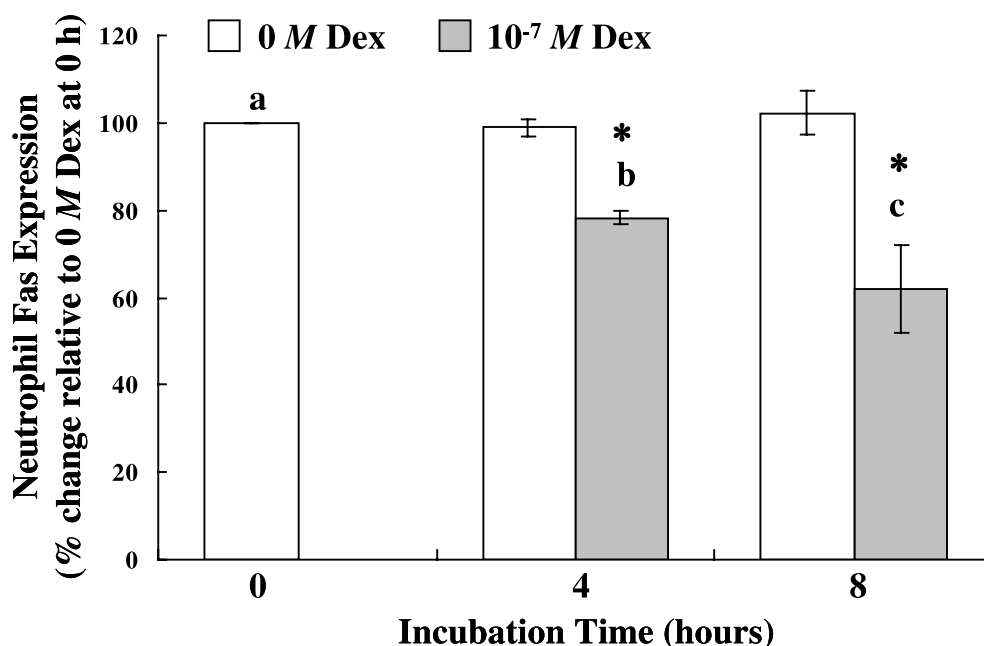


Figure 6 Dexamethasone (Dex) downregulated surface expression of Fas protein in cultured bovine neutrophils. Steer ($n=2$) neutrophils were cultured in duplicate at 39 °C in RPMI-1640 medium and 0 or 10^{-7} M Dex for 0, 4 or 8 h. Abundance of surface Fas protein was monitored by immunostaining (using an anti-Fas antibody and PE-conjugated secondary antibody) and fluorescence-activated flow cytometry. Data are mean (\pm S.E.M.) percentage of changes in percent Fas⁺ cells at 4 and 8 h relative to 0 h. Bars with different letters are significantly different at $P < 0.05$. The 4- and 8-h bars with asterisks above them indicate significant differences between control (white bars) and Dex-treated (gray bars) neutrophils ($P < 0.05$).

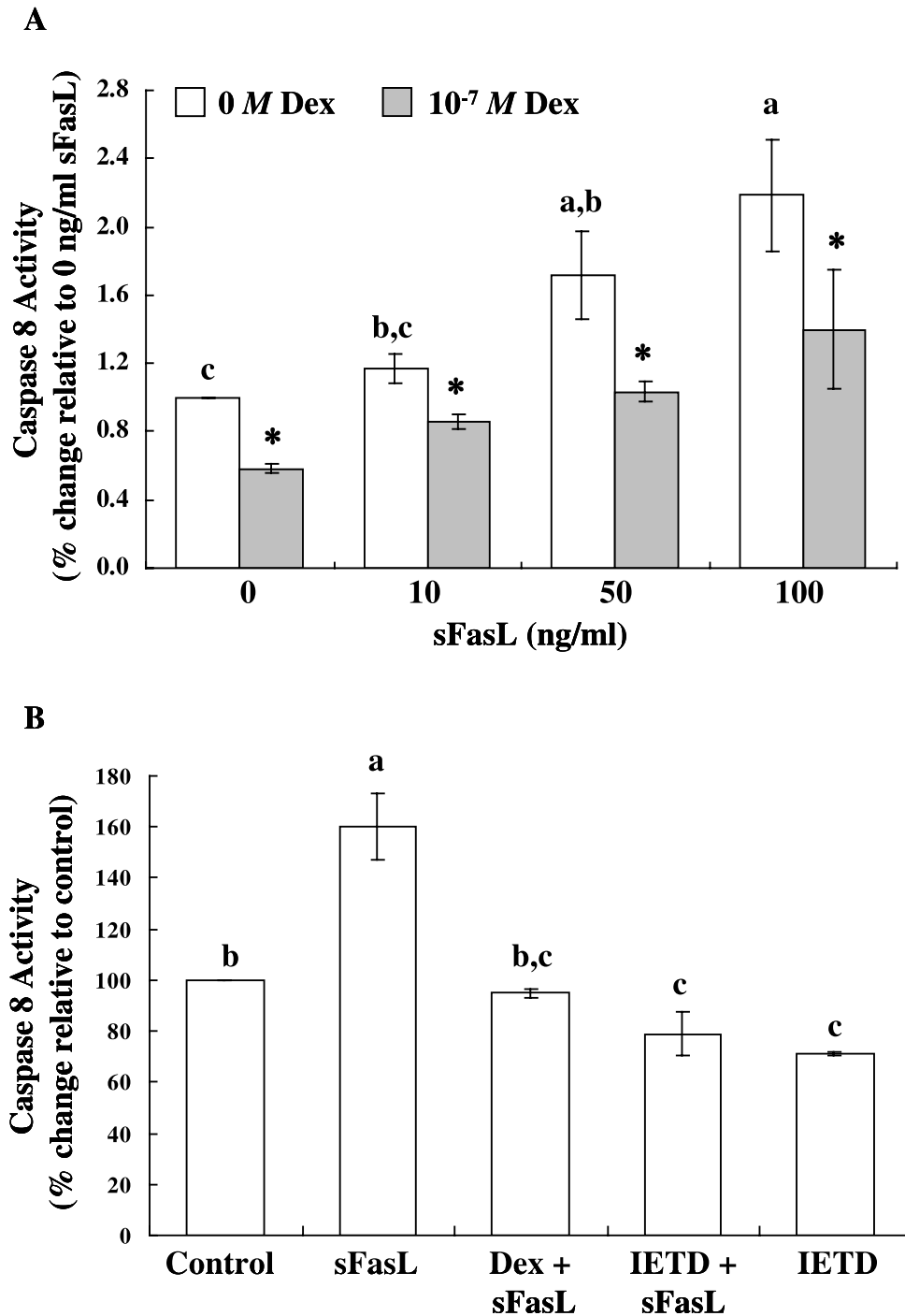


Figure 7 Dexamethasone (Dex) attenuates spontaneous and soluble FasL (sFasL)-induced caspase 8 activation. (A) Isolated neutrophils were cultured in duplicate at 39 °C in RPMI-1640 medium containing 1% fetal bovine serum. Cells were preincubated with or without Dex (10⁻⁷ M) for 4 h, and then incubated with various concentrations of sFasL (0, 10, 50 or 100 ng/ml) for an additional 3 h. (B) Neutrophils were preincubated with ± 10⁻⁷ M Dex for 4 h followed by sFasL (100 ng/ml) treatment for another 3 h. Where indicated, cells were also treated with the caspase 8 inhibitor, z-IETD-fmk (IETD, 50 μM), which was added for 30 min before the addition of sFasL. Caspase 8 activity was measured with a commercial kit. Shown in both plots are means (± S.E.M.) from two steers. Bars with different letters are significantly different at *P*<0.05.

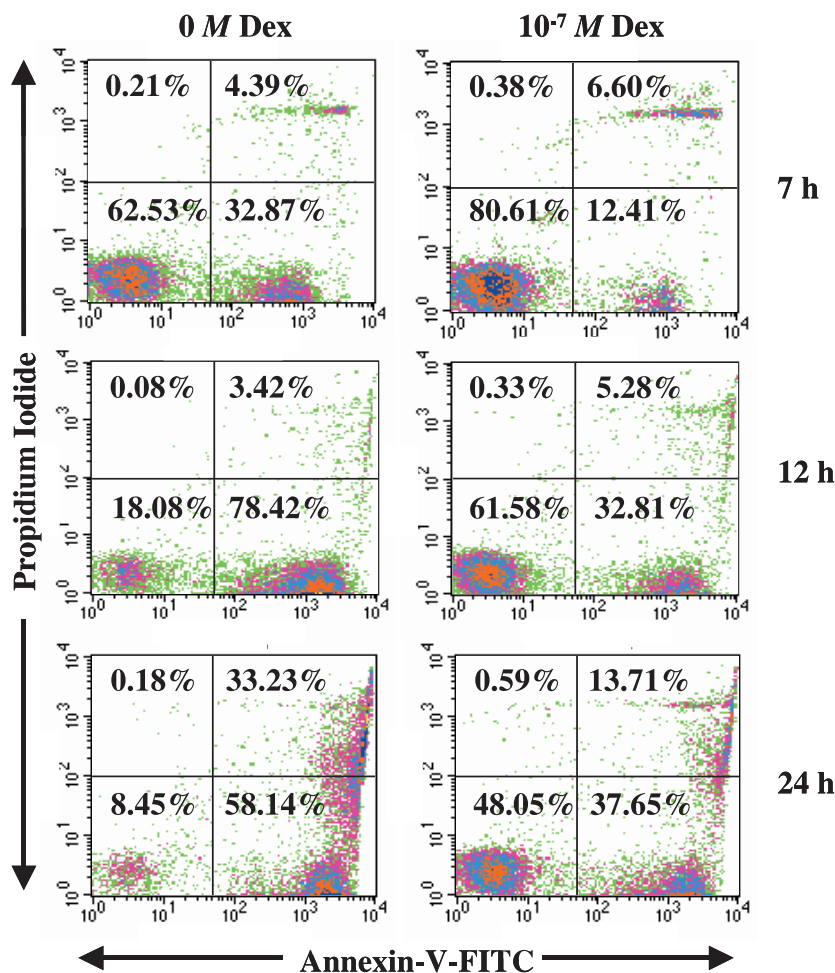


Figure 8 Dexamethasone (Dex) reduces the rate of spontaneous apoptosis in isolated bovine blood neutrophils. Neutrophils were cultured for 7, 12 or 24 h with (10^{-7} M) or without (0 M) Dex and subjected to Annexin V-FITC/propidium iodide staining with two-color flow cytometric analysis to monitor apoptosis. Shown are representative two-color density dot plots for one steer, with Annexin V-FITC staining on the x-axes and propidium iodide staining on the y-axes. As shown by changes over time in the percentage of cells falling into the lower and upper right quadrants of these plots, Dex reduced the rate of apoptosis in cultured bovine neutrophils.

experiment, where RU486 (a potent antagonist of $GR\alpha$) inhibited dexamethasone-induced Fas mRNA downregulation in isolated blood neutrophils (Fig. 5). $GR\beta$ had no apparent involvement in the Fas response to dexamethasone because its cytosolic abundance did not change during the same 4 h of dexamethasone treatment (Fig. 4B).

The precise mechanism of $GR\alpha$ regulation of Fas gene expression is not known. While the presence of nGRE in the promoter of the human Fas gene has not been documented, NF- κ B and AP-1 binding sites are present, and the NF- κ B and AP-1 transcription factors participate directly in transcriptional regulation of human Fas (Cheng *et al.* 1995, Chan *et al.* 1999, Lasham *et al.* 2000). If the

same is true in cattle, our *in vivo* and *in vitro* observations of glucocorticoid-induced downregulation of Fas gene expression in bovine neutrophils might be explained by $GR\alpha$ interference of NF- κ B and AP-1. It is also possible that $GR\alpha$ interacts with Fas transcripts to affect their stability, as it does for Bcl- $X_{L/S}$ mRNA (an apoptosis regulator belonging to the Bcl-2 family) (Chang *et al.* 1997). Yet another mechanism by which $GR\alpha$ may regulate Fas gene expression is through effects on new protein synthesis, as has been reported for FasL. In the FasL expression system, glucocorticoids inhibit activation-induced upregulation of FasL through induction of a separate glucocorticoid-induced gene, called the glucocorticoid-induced leucine zipper (GILZ)

(Mittelstadt & Ashwell 2001). We still do not know whether GR α regulation of Fas gene expression in bovine blood neutrophils is simple or complex.

Like a number of other proapoptotic proteins (such as Bad, Bax, Bak and Bik) (Moulding *et al.* 2001), surface Fas is constitutively expressed (Liles *et al.* 1996, Hsieh *et al.* 1997) and may have a relatively slow turnover rate in neutrophils. Thus, it may take longer for dexamethasone to change Fas protein expression than Fas mRNA abundance. This could explain why we observed relatively modest downregulation of surface Fas on neutrophils after 4 or 8 h of dexamethasone treatment *in vitro* (Fig. 6) compared with the pronounced decreases we observed in Fas mRNA abundance at 4 h of culture (Figs 4 and 5). However, even modest downregulation of surface Fas may be important biologically. A critical step in death signaling through Fas is activation of caspase 8 (Daigle & Simon 2001), which activates caspase 3 to cause subsequent proteolysis and increased enzymatic activity of key death-inducing proteins (Khawaja & Tatton 1999) that mediate plasma membrane instability, DNA fragmentation and cell death (Scaffidi *et al.* 1998, Schulze-Osthoff *et al.* 1998, Sharma *et al.* 2000, Zimmermann *et al.* 2001). Our *in vitro* experiments showed that dexamethasone attenuated spontaneous caspase 8 activity and dramatically slowed the rate of spontaneous neutrophil apoptosis (Figs 7 and 8). This suggested to us that Fas downregulation may have played a role in extending the longevity of glucocorticoid-treated neutrophils. However, the results of a recent study question the involvement of Fas signaling during spontaneous apoptosis of neutrophils in culture (Daigle & Simon 2001). Accordingly, we repeated our *in vitro* experiment using sFasL to induce caspase 8 activation through Fas signaling, and included a specific inhibitor of caspase 8 (z-IETD-fmk) as a negative control. We showed that dexamethasone was as effective as z-IETD-fmk in decreasing sFasL-induced caspase 8 activation in bovine blood neutrophils (Fig. 7). We thus propose that glucocorticoid-induced downregulation of Fas gene and protein expression were upstream events that impinged on this apoptosis signaling pathway, possibly influencing the ability of the death-inducing signaling complex to form, recruit and activate caspase 8. In substantiation of this proposition, alternations in Fas expression are known to change the sensitivity of a human myeloma cell line and eosinophils to FasL-mediated apoptosis (Luttmann *et al.* 1998, Shain *et al.* 2000). Thus, while it is possible that glucocorticoids also affect the expression or activity of other proteins involved in spontaneous apoptosis, our current results implicate Fas downregulation as one event that could explain the steroid's ability to reduce FasL-mediated caspase 8 activation and apoptosis in neutrophils. We did not directly measure caspase 8 activity or apoptotic status of blood neutrophils from our periparturient cows or dexamethasone-treated steers, but we did observe strong negative correlations between Fas gene expression and

circulating numbers of blood neutrophils in these animals (Figs 1–3). Thus, we hypothesize that Fas downregulation, reduced caspase 8 activity and extended cell survival also occurred in circulating neutrophils *in vivo* during the surge in parturient cortisol and after dexamethasone administration.

Conclusion

We conclude from the *in vivo* and *in vitro* results of this study that Fas gene expression in bovine blood neutrophils is regulated by GR α -mediated events when the cells are exposed to physiologic and pharmacologic levels of glucocorticoids, and that this leads to attenuated caspase 8 activity and extended longevity of the cells.

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