Research

Signaling mechanisms in tumor necrosis factor alpha-induced death of microvascular endothelial cells of the corpus luteum

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Abstract

The microvasculature of the corpus luteum (CL), which comprises greater than 50% of the total number of cells in the CL, is thought to be the first structure to undergo degeneration via apoptosis during luteolysis. These studies compared the apoptotic potential of various cytokines (tumor necrosis factor α, TNFα; interferon gamma, IFNγ; soluble Fas ligand, sFasL), a FAS activating antibody (FasAb), and the luteolytic hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on CL-derived endothelial (CLENDO) cells. Neither sFasL, FasAb nor $PGF_{2\alpha}$ had any effect on CLENDO cell viability. Utilizing morphological and biochemical parameters it was evident that TNFα and IFNγ initiated apoptosis in long-term cultures. However, TNFα was the most potent stimulus for CLENDO cell apoptosis at early time points. Unlike many other studies described in non-reproductive cell types, TNFα induced apoptosis of CLENDO cells occurs in the absence of inhibitors of protein synthesis. TNFα-induced death is typically associated with acute activation of distinct intracellular signaling pathways (*e.g.* MAPK and sphingomyelin pathways). Treatment with TNFα for 5–30 min activated MAPKs (ERK, p38, and JNK), and increased ceramide accumulation. Ceramide, a product of sphingomyelin hydrolysis, can serve as an upstream activator of members of the MAPK family independently in numerous cell types, and is a well-established pro-apoptotic second messenger. Like TNFα, treatment of CLENDO cells with exogenous ceramide significantly induced endothelial apoptosis. Ceramide also activated the JNK pathway, but had no effect on ERK and p38 MAPKs. Pretreatment of CLENDO cells with glutathione (GSH), an intracellular reducing agent and known inhibitor of reactive oxygen species (ROS) or TNFα-induced apoptosis, significantly attenuated TNFα-induced apoptosis. It is hypothesized that TNFα kills CLENDO cells through elevation of reactive oxygen species, and intracellular signals that promote apoptosis.

Background

Progesterone derived from the corpus luteum (CL) is required for the establishment and maintenance of a suitable uterine environment during early pregnancy [1,2]. Inappropriate luteal function (*i.e.* luteal insufficiency) may contribute to the high incidence of spontaneous abortions that occur early in mammalian pregnancies [3]. In most species, the luteolytic cascade, starting with loss of progesterone production and followed by tissue regression (*i.e.* apoptosis), is initiated by prostaglandin $F_{2\alpha}$

($PGF_{2\alpha}$) *in vivo* [4]. However, this process can only be partially recapitulated *in vitro* using primary cultures of steroidogenic cells. Gonadotropin-induced progesterone production is attenuated by $PGF_{2\alpha}$ treatment, but this prostanoid does not activate apoptosis of bovine steroidogenic cells *in vitro* [8]. These findings helped develop the concept that additional factors, such as immune cells [5–7] or cytokines [7,8], contribute to the luteolytic process. Indeed, tumor necrosis factor α (TNFα), interferon gamma (IFNγ) and/or interleukin-1 beta (IL-1β) have all been shown to reduce gonadotrophin-induced progesterone synthesis in a number of species [8–12], and TNFα and IFNγ are known to increase $PGF_{2\alpha}$ production by steroidogenic cells [13–15]. Steroidogenic cell apoptotic paradigms are activated by cytokines generated by several cell types within the CL, including immune cells and endothelial cells [7,11,16]. The synergistic actions of IFNγ and TNFα, for example, induce steroidogenic cell apoptosis [13,17], as does soluble Fas ligand (FasL) [18–26]. Furthermore, several acute intracellular signaling events either required for, or associated with, the functional and structural aspects of luteal regression have been mapped using *in vitro* steroidogenic cell cultures [8,27,28].

The microvasculature of the CL is thought to be the first structure to undergo degeneration via apoptosis during luteolysis [2,8]. In contrast to information available on steroidogenic cells of the CL, a gap exists in our understanding of the factors and their associated signaling pathways that initiate apoptosis in the vascular component of the CL. Elevated TNFα activity may play a role in the apoptosis of CL-derived microvascular endothelial (CLENDO) cells [29]. TNFα is known to initiate apoptosis in diverse cell types by activating its cognate receptor, TNF-RI, a cell surface receptor and member of the tumor necrosis receptor super family (TNFRSF). TNF-RI is also a death receptor based on the presence of a carboxy-terminal functional domain known as the death domain. The death domain serves as a binding domain for cytoplasmic adapter proteins, which contain a death domain and a death effector domain (*i.e.* Fas associated death domain, FADD)[30] ultimately activating members of the caspase family resulting in apoptosis. Activation of TNFRI initiates stress-activated pathways, which often are associated with the onset of apoptosis in a cell specific fashion.

Of interest to these studies are the three subfamilies within the mitogen-activated protein kinase (MAPK) superfamily including the extracellular signal-related kinases (ERKs), p38 and c-jun N-terminal kinases (JNKs) [31]. TNFα is one of many cytokines involved in luteal regression. Although the mechanism(s) by which cytokines induce apoptosis vary among cell types, it is presumed that it involves either an increase in reactive oxygen species (ROS) or activation of stress-related signaling pathways.

Interestingly it has been demonstrated that rapid production of ROS in endothelial cells exposed to TNFα is mediated by ceramide [32]. Ceramide is a central figure in the sphingomyelin pathway, which has also been linked to stress related signaling and apoptosis [33,34]. This report provides evidence that cytokines regulate the fate of CLENDO cells. In the first set of experiments, we identified those factors that initiate CLENDO cell death amongst a host of cytokines and lipid signaling molecules that mediate various aspects of luteal structural regression. In a second series of experiments, we evaluated the activation of several signal transduction pathways known to coordinate apoptosis in other cell types. A working knowledge of the collective signaling network activated in CLENDO cells by specific death stimuli is essential to complete our understanding of how apoptosis is controlled within the CL.

Materials and Methods
Cell culture and treatments
Purified bovine CL-derived microvascular endothelial cell populations were generated commercially by Cambrex Biosciences (BioWhittaker, Walkersville, MD) as described in detail [35]. In the present study, CLENDO cells from frozen aliquots (passage 3) were seeded (5,000 cells/cm^2) in 6- or 24-well plates or 10 cm culture dishes. The cells were cultured in EGM-2MV, as recommended by the supplier with progesterone (250 ng/ml) and 3% fetal bovine serum added. Once 90% confluency was reached, the medium was changed and the cultures were maintained in serum- and growth factor-free conditions (EBM-2 medium) for 24 h. Medium was then removed once again and fresh serum- and growth factor-free medium was applied. Cells were allowed to equilibrate for a minimum of 3 h prior to treatment. Treatment concentrations were based upon previously published *in vitro* studies of mixed luteal cells [13–15,17,29,36] and all experiments were completed a minimum of three times.

To identify putative luteolytic factors that initiate CLENDO cell apoptosis, cells were treated with TNFα (50 ng/ml; Upstate Biotechnology, Inc.; Lake Placid, NY), bovine IFNγ (200 IU/ml; generous gift from Dr. Dale Godson, Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada), sFasL (50 ng/ml; Oncogene, LaJolla, CA), mouse Fas activating antibody (Jo2; 500 ng/ml; Pharmingen, San Diego, CA), human Fas activating antibody (CH-11; 500 ng/ml; Upstate Biotechnology, Inc., Lake Placid, NY), $PGF_{2\alpha}$ (1 μM; Sigma Chemical Company; St. Louis, MO) or C2 ceramide (50 μM; BioMol; Plymouth Meeting, PA) for 24, 48 and 72 h. To confirm specificity of ceramide in triggering CLENDO cell death, cultures were treated with sphingosine-1-phosphate (S1P; 10 or 50 μM; BioMol; Plymouth Meeting, PA) for 48 h. Furthermore, to determine if S1P

attenuated ceramide- or TNFα-induced cell death, CLEN-DO cells were first pretreated with S1P for 30 min, followed by application of ceramide (50 μM) or TNFα (50 ng/ml). Finally, to evaluate the impact of glutathione (GSH), a known inhibitor of TNFα-induced ROS formation and apoptosis, on CLENDO cell survival, cells were pretreated with control media or GSH (10 mM) for 30 min, and then treated with or without TNFα (50 ng/ml) for 48 h.

For Western blot analysis, CLENDO cells were treated with TNFα (50 ng/ml) or C2 ceramide for 0, 2, 5, 10, 30, or 60 min. Cell lysates were collected and assayed for active MAPK family members as described below. For measurements of ceramide content in response to TNFα (50 ng/ml) treatment, pre-equilibrated cells were treated with TNFα (50 ng/ml) for 0, 2, 5, 10 or 30 min and lipids were extracted as described below.

Parameters of apoptosis
To identify apoptosis-inducing agents in CLENDO cells, culture medium was removed following treatments, cells were washed one time with ice cold PBS, and fixed for 10 min with 4% paraformaldehyde. The cells were rinsed one time with PBS and nuclei stained by placing 200 μl glycerol (80% in 1X PBS) containing 2 μg/ml Hoechst 33258 in each well. While pyknotic nuclei were visible in adherent cells in response to some treatments, we also observed a large number of dead, floating cells as confirmed by trypan blue staining. Thus, the average number of non-apoptotic cells in 10 fields was determined and presented as raw values. Biochemical analyses were employed to verify whether or not cell loss was the result of apoptosis.
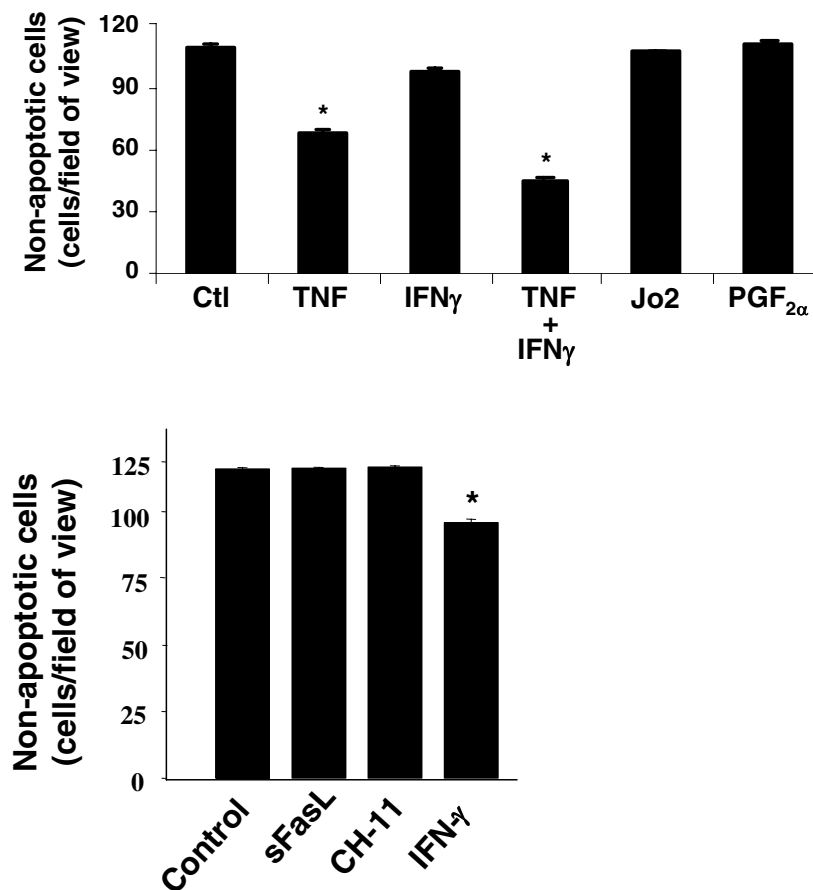
Oligonucleosomal DNA fragmentation was used as a biochemical marker of endothelial cell apoptosis in which cells were untreated for 48 h, treated with TNFα (50 ng/ml) for 12, 24 or 48, or treated with C2 ceramide (50 μM) for 12 h. Cells were scraped and separated from culture medium by centrifugation (1200 rpm, 10 min, 4°C) and DNA was isolated as described [37]. Following electrophoresis, fragmented DNA (5 μg) was visualized by ethidium bromide staining. To provide a second source of biochemical evidence that TNFα induced apoptosis, confluent cultures of CLENDO cells were incubated with a fluorogenic substrate, which detects caspase-3 activity (10 μM, DEVD sequence; Phi Phi Lux; OncoImmunin, Inc, College Park, MD) at 2 and 8 h after TNFα treatment for 30 min. These times were selected since no visible morphological signs (chromatin condensation or formation of apoptotic bodies) of apoptosis were evident until after 12 h. Following the incubation, cells were rinsed 3 times with medium and analyzed by fluorescent microscopy as described [26,38].

Western blot analysis
Protein lysates were collected for Western blot analysis as described elsewhere [17,35,39]. Briefly, following separation by SDS-PAGE, the proteins (20 μg/lane) were transferred (100 V, 1 h) to polyvinylidene difluoride membranes. Nonspecific binding was blocked with 5% fat free milk in TBST [50 mM Tris-HCL (pH7.5) 0.15 Na-Cl, 0.05% Tween-20] 1 h at room temperature. Membranes were then incubated with primary antibodies to various MAPK family members overnight at 4°C (phospho-ERK, 1:5000, Promega; Madison, WI; phospho-p38, 1:1000, New England Biolabs, Beverly, MA; phospho-JNK, 1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Previous studies have demonstrated that these antibodies recognize the phosphorylated (active) forms of extracellular-regulated kinase (ERK), p38, and Jun-N-terminal kinase (JNK) Membranes were then washed (3 × 5 min each) with TBST buffer and incubated with anti-rabbit IgG horseradish peroxidase conjugate or anti-mouse IgG horseradish peroxidase conjugate (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The membranes were washed with TBST as before and the bound antibody was detected using ECL reagents based on the manufacturer's recommendations (Amersham; Piscataway, NJ). X-ray films were scanned and band intensity was determined using a Kodak-1D software package. Since pJNK, pERK and panERK appear in two isoforms, both bands were scanned together. To verify equal protein loading membranes were then stripped and re-probed with the panERK antibody (1:1000; Transduction Laboratories; Lexington, KY) which recognizes the constitutively expressed ERKs in bovine CLENDO cells as previously described [35].

Evaluation of ceramide production in response to TNFα
The amount of ceramide generated by CLENDO cells in response to TNFα treatment (50 ng/ml) was determined in three independent sets of experiments via the diacyl glycerol kinase assay as described [26,40]. Each experiment contained an untreated control that was used for comparison with TNFα treated samples. Briefly, membrane lipids were extracted (1 ml methanol, 10 min at -80°C) from endothelial cells immediately following treatment and dried under nitrogen gas. Lipids were subjected to mild alkaline hydrolysis and dried under nitrogen gas. Ceramide in the lipid extracts were labeled with [^{32}P-ATP] and the resulting products resolved using thin layer chromatography. Graded levels of commercially purified ceramides were employed as a standard curve. The band in each lane corresponding to ceramide-1-phosphate was cut from the chromatography plate and counted in a scintillation counter. Changes in ceramide production in response to TNFα treatment were presented as a percentage of the untreated control value.

Figure 1
TNFα induces loss of viability of CLENDO cells *in vitro*. (Panel A) CLENDO cells were treated for 48 h with media alone (Ctl), TNFα (50 ng/ml), IFNγ (200 IU/ml), Jo2 (500 ng/ml), or PGF$_{2\alpha}$ (1 μM). Cells were stained with Hoechst 33258 and the numbers of non-apoptotic cells per field in each of 10 separate fields per dish were counted. (Panel B) CLENDO cells were treated for 72 h with media alone (control), soluble FasL (50 ng/ml), human Fas activating antibody (CH-11, 500 ng/ml) or bovine IFNγ (200 IU/ml). Cell viability was determined as described above. These data represent the mean ± SEM of at least three independent experiments. The "*" denotes significance (P < 0.05) compared to untreated control.

Statistics
Assignment to treatments was made at random. Raw data were subjected to least squares analysis of variance followed by Duncan's new multiple range test or the Student *t*-tests for paired comparisons. The results were expressed as the mean ± SEM and a value of P < 0.05 was considered significant.

Results
TNFα reduces viability of CLENDO cells
To determine which cytokines initiate apoptosis in CLENDO cells we treated the cells for 48 h with TNFα, IFNγ, or the combination of the two. TNFα treatment resulted in a significant reduction in CLENDO cell viability (34 %, P <

0.05), which was reduced (P < 0.05) an additional 23 % by co-treatment with IFNγ (Fig. 1a). Treatment with IFNγ alone showed no statistical difference in viability compared with the untreated control during the first 48 h of treatment. Although Fas activating antibody, Jo2, induces apoptosis in ovarian surface epithelial cells [23], Jo2 had no effect on CLENDO cell survival. Similarly, treatment of CLENDO cells with the luteolytic agent, PGF$_{2\alpha}$, for 48 h failed to induce apoptosis compared to the untreated control.

In a second group of experiments, we observed that CLENDO cells were resistant to apoptosis when treated for 72 h with sFasL or the human Fas activating antibody

CH-11. Interestingly, treatment with IFNγ for 72 h showed a modest increase (P < 0.05) in cell death compared to the untreated control group (Fig 1b).

Treatment with TNFα caused a linear decrease in the number of non-apoptotic CLENDO cells starting at 24 h and continuing through 72 h of treatment (Fig. 2a). CLENDO cells treated with TNFα for 12 h showed no significant difference in the number of non-apoptotic cells compared with the 72 h untreated control. However, some cells in the 12 h group showed early signs of apoptosis in that pyknosis and partitioning of nuclei were observed (not shown). To provide biochemical evidence that the form of cell death observed was apoptosis, assays for oligonucleosomal DNA fragmentation and caspase-3 activity were performed. Oligonucleosomal DNA fragmentation was evident 12 h after TNFα treatment and continued through 24 and 48 h (Fig. 2b). Caspase-3 activity (cleavage of Phi Phi Lux) was easily detected by 8 h after TNFα treatment, and was evident as early as 2 h (Fig 2c.).

Ceramide is generated in response to TNFα treatment and kills CLENDO cells
Previous studies have shown that TNFα, like other death receptors, stimulates the accumulation of the second messenger ceramide, and that this lipid signaling molecule can serve as an upstream activator of cell stress signaling pathways [41]. Measurements of total cellular ceramide in CLENDO cells revealed that ceramide levels were significantly elevated (P < 0.05) as early as 2 min following TNFα treatment, and remained elevated through 30 min of treatment (Fig. 3a.). Treatment of CLENDO cells with an exogenous source of ceramide (C2, 50 μM) caused a significant decrease in cell viability starting 24 h after treatment compared to untreated cells cultured for 72 h (Fig. 3b). Treatment of CLENDO cells for 48 h with S1P at either 10 μM or 50 μM concentrations did not induce CLENDO cell death (data not shown). Furthermore, pretreatment of CLENDO cells for 30 min with S1P did not prevent ceramide- or TNFα-induced apoptosis (data not shown). As with TNFα treatment, application of ceramide to CLENDO cells resulted in oligonucleosomal DNA fragmentation within 12 h, thus confirming apoptosis as the mechanism of cell death (see Fig. 2b, lane 6).

Acute activation of the mitogen-activated protein kinase superfamily by TNFα and ceramide
Substantial data support the notion that ceramide serves as an upstream signaling molecule in the activation of other signaling pathways such as the mitogen-activated protein kinase (MAPK) superfamily [33]. In the present study quantitative measures were performed on members of the MAPK superfamily using antibodies that recognize only the phosphorylated, and presumptively active, forms of ERK, JNK, and p38. Western blot analysis confirmed that all three members of the MAPK superfamily were transiently activated by treatment with TNFα (Fig. 4a). The levels of phospho-ERKs increased within 5 min of TNFα treatment, peaked by 10 min and decreased by 30 min (Fig. 4b). Phospho-p38 levels peaked at 5 min and showed an equally rapid decline in activity. Levels of phospho-JNK activity increased (P < 0.05) by 10 min and remained elevated throughout 30 min of incubation with TNFα (Fig. 4b).
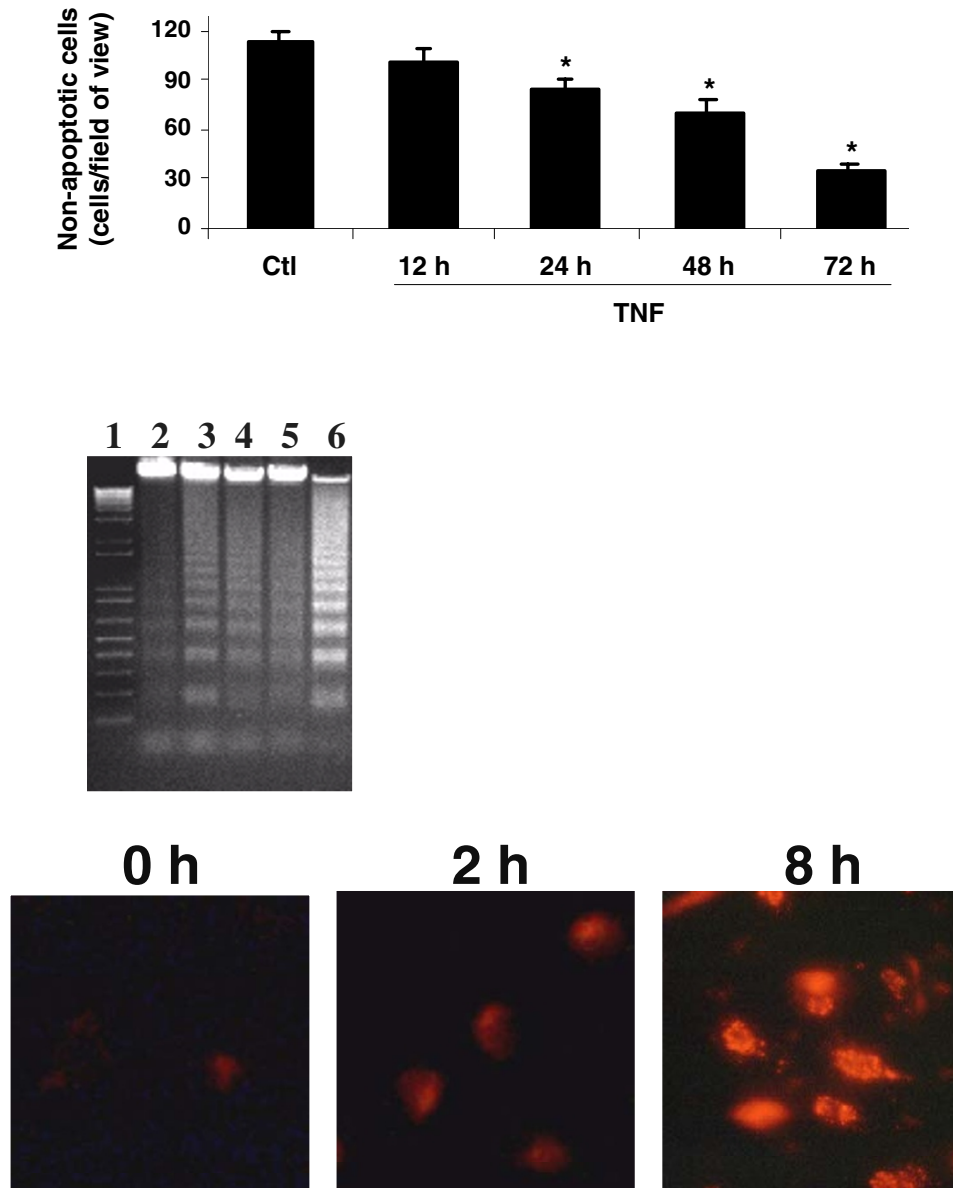
Since ceramide is linked to MAPK signaling pathways [33] we determined the effect of treatment of CLENDO cells with an exogenous source of ceramide. Treatment with 50 μM C2 ceramide failed to activate the ERK and p38 MAPK pathways. However, treatment with C2 ceramide did activate the JNK pathway similar to that observed with TNFα treatment (Fig. 4c). Phospho-JNK was elevated with in 5 minutes after C2 addition and remained elevated for at least 30 min.

Glutathione attenuates TNFα-induced cell death
Since TNFRSF induces cell death by causing an efflux of glutathione (GSH) in other cell types [42] we sought to determine if pretreatment of CLENDO cells with GSH would attenuate lethality associated with TNFα treatment. CLENDO cells pretreated for 30 min with GSH were significantly less susceptible (P < 0.05) to the lethal effects of TNFα over a 48 h period than cells treated with TNFα alone (Fig. 5). Treatment with GSH alone had no effect on the CLENDO cell viability.
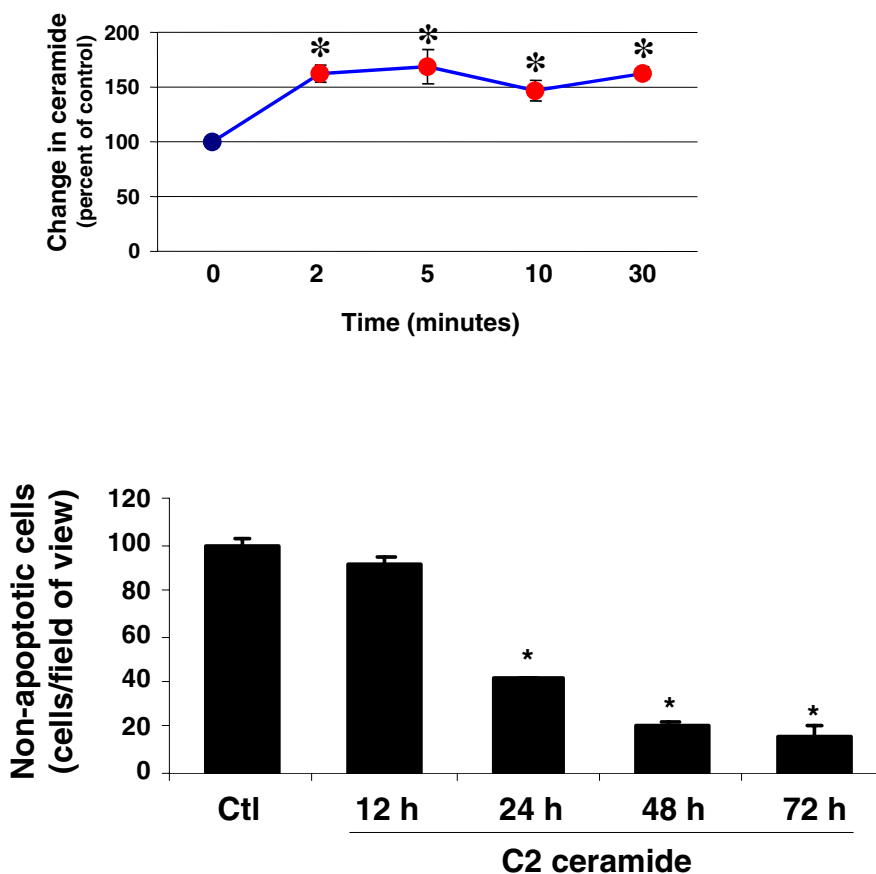
Discussion
Treatment of animals with $PGF_{2\alpha}$ or its analogs results in endothelial cell death *in vivo* [43], suggesting that $PGF_{2\alpha}$ may have a direct effect on CLENDO cells. However, the microvasculature of the CL is composed of a number of different luteal endothelial cell types; the most common of which does not express mRNA encoding the $PGF_{2\alpha}$ receptor FP [35,44]. This particular endothelial cell type is unresponsive to $PGF_{2\alpha}$ treatment [35] in that $PGF_{2\alpha}$ treatment does not increase the secretion of monocyte chemoattractant protein-1 [35] or stimulate well-recognized $PGF_{2\alpha}$-responsive intracellular signaling pathways [35,36]. Lehmann, *et al.* [45] recently characterized a predominant cytokeratin-negative endothelial cell type in the bovine CL in addition to a "rare" cytokeratin-positive endothelial cell type. These two cell populations may have different functional capacities in that they differ in their expression of FP [44,45], as well as their secreted cytokine profiles [45]. In the present studies $PGF_{2\alpha}$ was incapable of directly inducing apoptosis of CLENDO cells. Consequently, there is some question as to whether $PGF_{2\alpha}$ has any direct effect on CLENDO cell function, which directed

Figure 2
Apoptosis is the form of luteal microvascular endothelial cell death initiated by TNFα. (Panel A) Cells were treated TNFα (50 ng/ml) for 72 h and cell viability determined after Hoechst staining. The numbers of non-apoptotic cells per field in each of 10 separate fields of view per dish were counted. These data represent the mean ± SEM of at least three independent experiments. The "*" denotes significance (P < 0.05) compared to untreated control. (Panel B) Oligonucleosomal DNA fragmentation of CLENDO cells cultured for 48 h in serum and growth factor free conditions (control, lane 2); or for 12 h (lane 3), 24 h (lane 4), or 48 h (lane 5) with TNFα (50 ng/ml), and for 48 h with ceramide (50 μM, lane 6). Lane 1 shows a low molecular weight DNA ladder. (Panel C) Cleavage of Phi Phi Lux, demonstrating caspase-3 activity in CLENDO cells treated with TNFα (50 ng/ml) for 2 or 8 h.

Figure 3
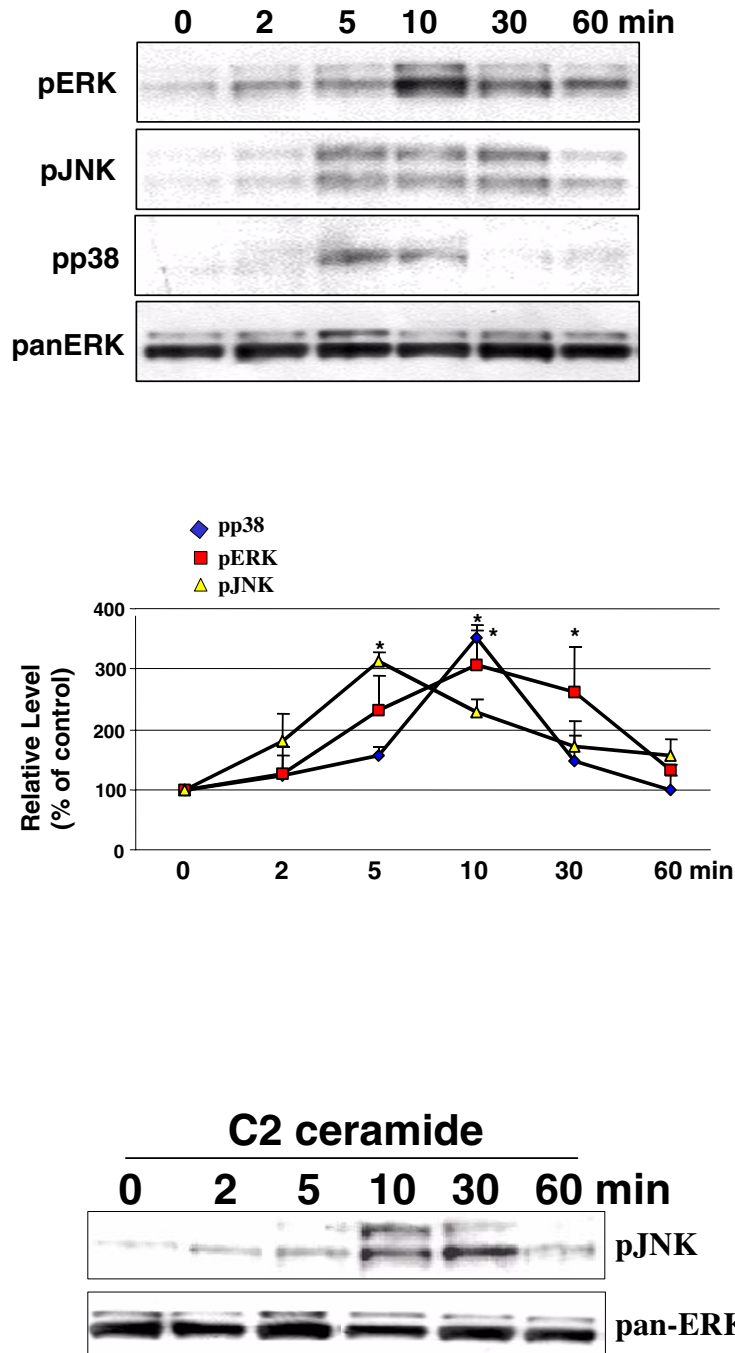TNFα activates the sphingomyelin signaling pathway in CLENDO cells. (Panel A) CLENDO cells were treated for up to 30 min with TNFα (50 ng/ml) and ceramide levels were determined as described in the Materials and Methods. (Panel B) The numbers of non-apoptotic cells per field in each of 10 separate fields per dish were counted in which cultures of CLENDO cells were treated with exogenous ceramide (C2, 50 μM) for up to 72 h under serum- and growth factor-free conditions. Ctl, untreated controls cultured in parallel for 72 h in serum free, growth factor free medium. * = P < 0.05. These data represent mean ± SEM of at least three independent experiments.

our attention toward other known luteolytic factors (*i.e.*, cytokines).

A primary objective of this study was to determine which cytokines initiate CLENDO cell death. Of the various cytokines tested (TNFα, IFNγ, or sFasL), TNFα was the only potent initiator of CLENDO cell death. Time course analysis suggested that the loss of viability was rapid (Fig 1a and 2a) and progressive, with only 30 % of the cells remaining non-apoptotic by 72 hr post-treatment. The rapid induction of apoptosis in response to TNFα is similar to the microvascular endothelial cell death which was observed by Friedman and colleagues [29] at higher concentrations of TNFα. One receptor for TNFα, TNF-RI is ex-

pressed throughout the bovine estrous cycle [46], but is more abundantly expressed by the endothelial cell component than in steroidogenic cells [29].
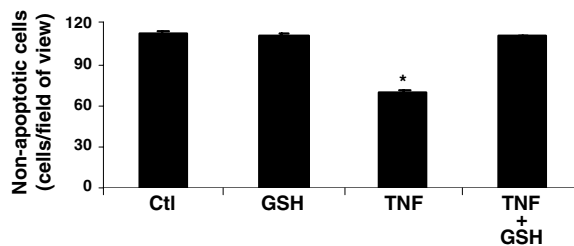
At physiological concentrations, TNFα induces cell death in most TNFα responsive cell types via apoptosis. This effect was evident in CLENDO cells by hallmark features of apoptosis including nuclear chromatin condensation, formation of apoptotic bodies and it was confirmed by an increase in caspase-3 activity and oligonucleosomal DNA fragmentation. Active caspase-3 is a functional requirement of spontaneous luteal regression in that caspase-3-deficient mice retain CL for a longer period of time than wild type littermates [47]. However, this murine study did

Figure 4
TNFα and the TNFα-induced second messenger ceramide activate members of the MAPK superfamily. (Panel A) Western blot of lysates from CLENDO cells treated for up to 60 min with TNFα (50 ng/ml) probed with antibody to phosphorylated ERK, p38, JNK and pan-ERK. Pan-ERK antibody, which detects all activated and inactivated ERK isoforms was used for normalization. (Panel B) Graphical representation of the percent change in expression of MAPK superfamily after densitometry of band developed in Western blots analysis as in Panel A and normalization to panERK. These data represent mean ± SEM of at least three independent experiments. * = P < 0.05. (Panel C) Treatment with ceramide, a product of TNFα treatment in CLENDO cells, for up to 60 min and Western blot detection of phosphorylated JNK isoforms. This Western blot is representative of similar results obtained in at least three separate experiments.

Figure 5
Glutathione (GSH) blocks TNFα-induced CLENDO cell death. CLENDO cells were pretreated with or without the reactive oxygen species scavenger GSH (10 mM, 30 min) prior to incubation for 48 h in the presence and absence of TNFα (50 ng/ml). CLENDO cell apoptosis was determined as described in the Material and Methods. The data represent mean ± SEM of at least three independent experiments where "*" denotes P < 0.05 compared to untreated control (Ctl).

not delineate which luteal cells were affected. Whether caspase-3 activity is a required component of the luteal endothelial cell apoptosis paradigm remains to be determined.

Unlike TNFα, other cytokines (IFNγ or sFasL) or Fas activating antibodies had little effect on the endothelial cell viability. It is not readily apparent why these cytokines failed to induce CLENDO cell death, but one explanation may be the lack of cognate receptors on the cell surface. Co-treatment of luteal endothelial cells with IFNγ augmented TNFα-induced death, a result that may be explained in two ways. First, it is possible that the signaling events initiated by the combined treatment of TNFα and IFNγ are more potent at activating endothelial cell death than TNFα alone. Alternatively, IFNγ may increase the expression of the death domain containing TNF-RI and thus increase the luteal endothelial cell death susceptibility upon exposure to TNFα. This later suggestion has been shown to be the case in luteal steroidogenic cells for another death receptor, in which Fas mRNA expression increased in response to IFNγ treatment [20,22].

Interestingly, we find that two different cytokines (*i.e.* sFasL and TNFα) can activate the sphingomyelin pathway in two distinct CL cell types. TNFα elevates ceramide in CLENDO cells, whereas sFasL elevates ceramide in primary luteal steroidogenic cells [26]. Ceramide treatment induces apoptosis in both cell types, suggesting that the sphingomyelin pathway may be a common mediator of cell death among distinct cell types within the CL.

Sphingomyelin content and luteal cell membrane fluidity decrease during luteolysis *in vivo* [48–51]. Ceramide, a product of sphingomyelin hydrolysis, is a potent stress-activated second messenger and is well established as a functional component of death receptor-mediated apoptosis [52–54]. Upon treatment of CLENDO cells with TNFα, we observed a rapid and sustained increase in the production of ceramide, suggesting that the sphingomyelin pathway is present and active in these cells. Exogenous ceramide induced CLENDO cell oligonucleosomal DNA fragmentation and reduced cell viability in a manner consistent with TNFα treatment. The exact role and functional significance of ceramide production in response to TNFα in CLENDO cell apoptosis are not presently known. However, in some cell types, ceramide production has been placed at the top of molecular ordering schemes for several signaling cascades as well as increase in reactive oxygen species. Substantial evidence exists to suggest that stress-induced apoptosis is mediated through activation of MAPK signaling pathways in non-ovarian cell types. While these pathways are activated in luteal steroidogenic cells by diverse cell stressors, including cytokines [55], no such detailed studies have been completed to date in the endothelial cell component of the CL. In the present study we provide evidence that TNFα and a by-product of TNFα signaling, ceramide, both have the capacity to activate members of the MAPK family. Activation of MAPK stress pathways can alter cell viability through multiple mechanisms. For example, TNFα can activate transcription factors that bind genomic DNA and initiate gene expression of pro-apoptotic genes [56,57].

A number of studies provide evidence that the luteolytic process may involve a rapid and sustained increase in ROS [58,59]. Other studies demonstrate that luteal regression is associated with a dramatic decrease in the mRNAs encoding enzymes responsible for regulating the intracellular redox state [60]. Death induced by TNFα is also reportedly mediated by an increase in ROS, an event correlated with a marked decrease in the vital intracellular ROS scavenger GSH. Activation of TNF-RI and other death domain containing receptors of the TNF receptor superfamily have been shown to cause an efflux of GSH, which contributes to the apoptotic process [42,61]. Provision of GSH in the present study reduced the deleterious effects of TNFα on CLENDO cells. We suggest that one mechanism by which TNFα induces CLENDO cell death is through the formation of ROS, a result that may stem from depletion of GSH stores in CLENDO cells. Provision

of exogenous GSH may restore the redox state disrupted by TNFα treatment. These findings lend support to the notion that TNFα contributes to luteolysis by initiating formation of endothelial cell-derived ROS.

In this study, we have corroborated findings of Friedman *et al* [29] that TNFα is a potent apoptotic stimulus of primary luteal microvascular endothelial cells and extend these findings by characterizing the activation of several stress-activated signal transduction pathways in response to TNFα treatment of cultured endothelial cells. Whether these acute signaling events are linked to, and functionally required for, down stream apoptotic mechanisms remain to be determined. Furthermore, the possibility that alterations in GSH metabolism or cellular flux, and thus the redox state, within CLENDOs result from exposure to TNFα needs to be tested. These experiments as well as future studies designed to identify the enzymatic pathway(s) and in which cellular compartment(s) ceramide is generated in luteal microvascular endothelial cells in response to TNFα treatment should help determine which, if any, of the acutely activated signal transduction pathways contribute to luteal endothelial cell apoptosis. In conclusion, TNFα activates several stress-activated signaling pathways that may facilitate GSH efflux, and thus increase ROS and subsequent cell death.

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