THE ROLE OF OXIDATIVE STRESS AND NUCLEAR FACTOR-KAPPA B IN THE CONTROL OF APOPTOSIS AND ATRESIA IN DOMINANT BOVINE FOLLICLES

By
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DEDICATION

For my family.
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ABSTRACT

Apoptosis of granulosa cells is an early feature of atresia in bovine follicles. However, the mechanisms initiating apoptosis of granulosa cells have not been well characterized. In many cell types, apoptosis can be induced by oxidative stress and prevented by increased expression of endogenous oxidative stress response proteins. The transcription factor nuclear factor-kappa B (NF-κB) regulates genes involved in preventing oxidative stress and has been characterized as a mediator of cell survival. Studies have suggested that NF-κB activity may be regulated by estradiol-17β, a major determinant of follicular health. Therefore, these studies tested the hypotheses that oxidative stress is involved in the atresia of non-ovulatory dominant bovine follicles and NF-κB suppresses apoptosis in the healthy dominant follicle. Because the onset of atresia during the first wave of follicular development following ovulation has previously been characterized in cattle, it provides an excellent model to study the relationship between apoptosis, oxidative stress response proteins, NF-κB, and estradiol production. Decreased production of estradiol was accompanied by increased numbers of non-viable granulosa cells in dominant follicles between Days 4 and 6 of the first follicular wave in cattle. Unexpectedly, expression of genes encoding oxidative stress response proteins in granulosa cells increased on Day 8 of the follicular wave, but did not translate into increased expression of the corresponding proteins or greater enzyme activity. The decline in estradiol production observed on Day 6 of the 1st follicular wave is not due to lack of androgen substrate or down-regulated expression of the aromatase gene, but instead appears to be
the direct result of decreased activity of the aromatase enzyme within granulosa cells. Follicular NF-κB activation was associated with estradiol production. However, direct inhibition of NF-κB activity suppressed apoptosis of granulosa cells \textit{in vitro}. Therefore, whether NF-κB is playing a pro- or anti-apoptotic role in granulosa cells of bovine follicles remains to be elucidated. These experiments begin to elucidate the signals that trigger the sequence of events leading to apoptosis and atresia of bovine follicles and provide the framework for future experiments designed to further clarify the complex interplay of signals that determine a follicle’s fate.
Ovarian follicular development

The ovarian follicle is the basic unit of structure and function in the ovary. The formation of a resting pool of follicles takes place during fetal life, beginning with the migration of primordial germ cells into the gonadal ridges. In the developing ovary, the primordial germ cells transform into oogonia and mitotically divide until around embryonic day 80-110. At this time the peak number of germ cells is observed [2.1 million in cattle; Erickson, 1966; Hirshfield, 1991]. No more germ cells will be produced during the female's lifespan. The cessation of mitosis is accompanied by the transformation of oogonia into oocytes and the beginning of meiosis, which is arrested during the 1st meiotic division and doesn't resume until ovulation [Hirshfield, 1991; Hsueh et al., 1994].

The oocytes at the earliest stage of development are part of the primordial follicle. The primordial follicle is made up of the oocyte, surrounded by a single layer of flattened (squamous) pre-granulosa cells. In the largest cross sectional diameter (25 μm) 5 pre-granulosa cells will be found [Rajakoski, 1960] surrounded by a basement membrane. The primordial follicle is quiescent, growth-arrested, and not steroidogenic. Upon receiving a signal to grow, primordial follicles transform from the resting pool into primary follicles. The primary follicle has a diameter of ~60μm, and consists of the
oocyte surrounded by a single layer of cuboidal granulosa cells. Between the oocyte and granulosa cells is an acellular layer known as the zona pellucida. Initiation of follicular growth involves the transformation of primordial follicles from a quiescent, growth-arrested state to a growth-committed state. Once this transition has occurred, follicles are committed to grow until they either ovulate or degenerate by a process known as atresia [Tsafiri et al., 1994]. The signals causing primordial follicles to leave the resting pool are not well understood, but are thought to be under the influence of factors such as kit ligand [Parrott et al., 1997; Parrot et al., 2000], basic fibroblast growth factor [Lavranos et al., 1994; Rodgers et al., 1996; Roberts et al., 1999; Nilsson et al., 2001], bone morphogenetic proteins [Dube et al., 1998; Laitinen et al., 1998; Lee et al., 2001; Otsuka et al., 2000] leukemia inhibitory factor [Nilsson et al 2002], and growth differentiation factor-9 [McGrath et al., 1995; Dong et al., 1996; Carabatsos et al., 1998; Laitinen et al., 1998; Aaltonen et al., 1999; Bodensteiner et al., 1999; Elvin et al., 1999; Hayashi et al., 1999; Jaatinen et al., 1999].

Primary follicles develop into secondary follicles, which are characterized by the presence of multiple layers of granulosa cells and formation of the theca interna, a vascular layer of cells distinct from the granulosa cells located outside of the basement membrane [Peters et al., 1975; Crane et al., 1980; Hirshfield, 1991]. As the follicle continues to grow, the layers of granulosa cells increase and eventually a fluid filled space begins to develop within the follicle, at which time the follicle is called a tertiary follicle. When the follicle reaches a diameter of ~0.2-0.4 mm, a distinct antrum is present, and the follicle is referred to as a Graafian or antral follicle [Lussier et al., 1987].
While these early stages of follicular growth occur independently of gonadotrophic stimulation [Baker et al., 1981], further growth requires stimulation by follicle-stimulating hormone [FSH; Gougeon, 1982; Gong et al., 1996]. Once a follicle reaches a diameter of 7-9 mm, luteinizing hormone (LH) stimulation is required for further growth [Gong et al., 1996]. Antral follicles can continue to grow to a diameter of 14-20 mm in the cow [Fortune et al., 1988; Savio et al., 1988; Ginther et al., 1989].

Figure 1.1. Primordial to antral follicular changes during follicular development.
**Follicular waves**

Bovine follicular development occurs in a pattern of waves throughout the 21-day estrous cycle. In 1984, ultrasound was first used to monitor sizes of follicles during the estrous cycle of heifers, with the diameter recorded as the width of the nonechogenic fluid-filled antrum of a follicle [Pierson et al., 1984; Pierson et al., 1988]. It was determined that growth of antral follicles occurs in a pattern of waves, with typically 2 [Pierson et al., 1988] or 3 [Fortune et al., 1988; Savio et al., 1988; Sirois et al., 1988] per each 21-day estrous cycle. The wave-like pattern of follicular growth is common in many domestic species (Table 1.1). More recently, it is a phenomenon that has also been documented in humans [Baerwald et al., 2003].

There doesn’t appear to be any clear breed or age-specific predictors of whether a cow will exhibit two or three waves in an estrous cycle, although lactating Holstein dairy cows tend to have two follicle waves per cycle [Taylor et al., 1991; Townson et al., 2002], and beef and dairy heifers tend to have either two or three waves per cycle [Savio et al., 1988; Sirois et al., 1988; Ginther et al., 1989]. There is an indication that cattle that have two follicle waves per cycle tend to have shorter cycles, to ovulate larger and older follicles and to be less fertile than cattle with three waves per cycle [Townson et al., 2002].
Table 1.1 Examples of species exhibiting follicular growth in a pattern of waves.*

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of follicle waves per cycle</th>
<th>Maximum follicle diameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-ovulatory (mm)</td>
<td>Ovulatory (mm)</td>
</tr>
<tr>
<td>Cattle</td>
<td>2 or 3</td>
<td>10-15</td>
<td>12-20</td>
</tr>
<tr>
<td>Sheep</td>
<td>2-4</td>
<td>5-7</td>
<td>6-7</td>
</tr>
<tr>
<td>Goat</td>
<td>3 or 4</td>
<td>5-9</td>
<td>6-9</td>
</tr>
<tr>
<td>Horse</td>
<td>1 or 2</td>
<td>30-45</td>
<td>40-55</td>
</tr>
<tr>
<td>Pig</td>
<td>0</td>
<td>6-7</td>
<td>7-10</td>
</tr>
<tr>
<td>Buffalo</td>
<td>2 or 3</td>
<td>10-16</td>
<td>13-18</td>
</tr>
<tr>
<td>Dog</td>
<td>?</td>
<td>1-2</td>
<td>7-11</td>
</tr>
<tr>
<td>Cat</td>
<td>1 every 8-24 days</td>
<td>?</td>
<td>3-4</td>
</tr>
<tr>
<td>Llama</td>
<td>1 every 11-20 days</td>
<td>9-16</td>
<td>9-12</td>
</tr>
<tr>
<td>Camel</td>
<td>1 every 17-19 days</td>
<td>17-24</td>
<td>9-19</td>
</tr>
<tr>
<td>Chicken</td>
<td>0</td>
<td>6-8</td>
<td>Up to 40</td>
</tr>
<tr>
<td>Human</td>
<td>2 or 3</td>
<td>8-10</td>
<td>23</td>
</tr>
</tbody>
</table>

*Adapted from Evans [2003].
In cattle the beginning, or emergence, of a follicular wave (Day 1) is defined as the first day 2 or more growing follicles ≥4 mm in diameter are detected [Ginther et al., 1989]. When 2 waves occur during the estrous cycle, the days of emergence are typically on days 2 and 11 of the cycle. If an animal exhibits 3 waves during the estrous cycle, they typically initiate on days 2, 9, and 16 of the cycle [Sirois et al., 1988]. During each wave, between 2-6 follicles are recruited to continue growing from a pool of small growing follicles. This recruitment is stimulated by a slight elevation of plasma follicle-stimulating hormone (FSH), and in fact, block of this secondary FSH surge results in a delay in the emergence of the first wave [Dobson, 1978; Adams et al., 1992; Turzillo et al., 1990].

From the group of growing follicles, several are selected to continue growing until one follicle deviates, growing at a faster rate than others of the cohort. This follicle is established as the dominant follicle of the wave once it reaches a diameter 1-2 mm greater than others of the cohort [Ginther et al., 1997a]. A defining characteristic of the dominant follicle is its greater capacity to produce estradiol-17β, which requires the cooperative actions of the theca interna and the granulosa cells [Rodgers, 1990]. As soon as the dominant follicle is detected as having a slightly larger size than its subordinates, higher concentrations of estradiol-17β are found in the follicular fluid [Bodensteiner et al., 1996, Evans et al., 1997; Ginther et al., 1997b]. The remaining follicles are referred to as subordinate follicles and will cease growing and undergo atresia. Only the dominant follicle of the final wave of each estrous cycle will ovulate. The other dominant follicle(s), which always includes that of the 1st wave following ovulation, will
undergo atresia (Fig 1.2). The loss of dominance is defined functionally as occurring on the day of emergence of the next follicular wave, usually around Day 10 of the follicular wave [Xu et al., 1995a,b]. However, the onset of atresia is not clear but appears to occur much earlier than the loss of dominance [Xu et al., 1995a,b]. While changes, such as cessation of estradiol production, are observed as early as day 6 of the wave [Xu et al., 1995a,b] that would seem to indicate the initiation of atresia, follicles are capable of ovulation as late as day 8 of the follicular wave [Fortune et al., 1991].

Figure 1.2 Pattern of follicular development in cattle. Shown is a 3-wave per estrous cycle pattern of growth.
Atresia

At any point in time during this developmental process, follicles are lost to a deteriorative process known as atresia. Although the bovine ovary contains approximately 150,000 follicles at birth (less than 5% of the peak number of germ cells), very few are successfully ovulated and greater than 99.9% undergo atresia [Erickson, 1966; Byskov 1978]. In cattle, atresia is heaviest just before the final stages of follicular development [at a diameter greater than 8.6 mm; Fortune, 1994; Tilly et al., 1991].

During atresia, the basement membrane breaks down and the theca interna is absorbed back into the ovarian stroma. However, the granulosa cells within the basement membrane undergo a controlled form of cell death known as apoptosis. Apoptosis of granulosa cells is one of the earliest features of atresia in bovine follicles [Van Wezel et al., 1999; Yang et al., 2000a]. However, the mechanisms initiating apoptosis of granulosa cells have not been well characterized. A number of mechanisms have been proposed to induce apoptosis in granulosa cell, including binding of specific ligands to their receptors, such as tumor necrosis factor-α and fas [Kaipia et al., 1996; Porter et al., 2000], inhibition of cell-cell contact [Trolice et al., 1996], presence or absence of specific growth factors [Quirk et al., 2000], and altered levels of hormones such as estrogens and androgens [Billig et al., 1993].

In addition to these mechanisms, follicular apoptosis may be induced by oxidative stress [Tilly et al., 1995]. Rat granulosa cells cultured without serum spontaneous undergo apoptosis after 24 h. However, the addition of the oxidative stress response
protein, SOD, inhibits the apoptosis by 44% compared to controls, suggesting the spontaneous apoptosis was at least in part due to oxidative stress. Furthermore, MnSOD mRNA expression increased in antral follicles stimulated with eCG. This provides evidence that gonadotropins may promote granulosa cell survival in developing antral follicles by activation of an oxidative stress response.
Apoptosis

Apoptosis is a distinct form of programmed cell death in which a cell actively participates in its own destruction, permitting the safe disposal of cells at the point in time when they have fulfilled their intended physiological function. As opposed to necrosis, a form of accidental cell death as a result of pathological cellular injury typically occurring to large tissue areas, apoptosis occurs in scattered single cells, is energy dependent, and requires coordinated expression of a number of different genes [Alison et al., 1995; Cotter et al., 1990].

Apoptosis can be divided into initiation, execution, and apoptotic stages. During initiation, activation of initiator caspases occurs. Caspases are cysteine proteases that cleave their substrate proteins specifically behind an aspartate residue. They are constitutively expressed in an inactive proenzyme form and are activated following cleavage at specific aspartate residues. The initiator caspases are divided into 3 subfamilies: ICE-like caspases (caspases 1, 4, 5), CPP32-like caspases (caspases 3,6,7,8,9,10), and ICH-1 (caspase 2). It is the CPP32-like caspases that are thought to participate in the apoptosis cascade [Kidd, 1998; Miller, 1997]. Activation of initiator caspases leads to the first proteolytic events of apoptosis, including proteins of the cytoskeleton which leads to characteristic blebbing of the cell surface, shrinking of the cell, and formation of apoptotic bodies containing organelles and chromatin [McCarthy et al., 1997]. Also during the initiation stage, due to unknown reasons, phosphatidylserine
residues flip from the inner to the outer leaflet of the plasma membrane. This is thought to possibly be a signal to attract macrophages in some cell types [Marin et al., 1995].

Initiator caspases cleave and thus activate a second subpopulation of caspases, the execution caspases, leading to the execution phase and irreversible progression of the apoptotic cascade [Mignotte et al., 1998]. Activation of the execution caspases is strongly influenced by the bcl-2 family of proteins, which either promote (bad, bak, bax, bcl-xS, bid, bik, hyk, mtd) or inhibit (A1, bcl-2, bcl-w, bcl-xL, bfl-1, mcl-1, NR13) cleavage of execution caspases by initiator caspases. Members of the bcl-2 family associate to form homo- and/or heterodimers [Mignotte et al., 1998]. A shift in equilibrium toward apoptotic-promoting vs. antiapoptotic bcl-2 proteins makes the mitochondrial membrane permeable to release cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm [Amarente-Mendes et al., 1998; Kluck et al., 1997; Yang et al., 1997; Jurgenmeier et al., 1998]. The release of cytochrome c and AIF is associated with the activation of initiator caspases [Kuwana et al., 1998].

Execution caspases, either directly or indirectly, cleave a broad array of proteins necessary for cell survival, such as those involved in DNA maintenance and repair and organization of intermediate filaments [Tewari et al., 1995; Caulin et al., 1997]. Execution caspases activate endonucleases, which cleave DNA at specific internucleosomal intervals and give the DNA a ladder appearance when run on an agarose gel [Liu et al., 1997].

During the final stage of apoptotic events, there is a down-regulation of transcription, fragmentation and condensation of DNA, and alteration of nuclear and
cellular shape. The cytoplasm shrinks and later fragments due to dehydration, but organelles appear normal. The formation of apoptotic bodies, which can be phagocytosed by neighboring cells, allows the disposal of cellular contents without eliciting an immune or inflammatory response [Kerr et al., 1995; Schwartzman et al., 1993].

The first evidence of apoptosis in bovine follicles came in 1993, with the observation of oligonucleosome formation ("DNA laddering effect") of genomic DNA from atretic follicles [Imig et al., 1993]. The occurrence of oligonucleosome formation in isolated granulosa cells of small, medium, and large antral bovine follicles classified as atretic was demonstrated in 2000 [Yang et al., 2000a]. In this experiment, the researchers observed a higher prevalence of apoptosis in antral (closer to the fluid-filled antrum) compared to mural (closer to the basement membrane) granulosa cells. They also noted that apoptosis was a phenomenon constrained primarily to the granulosa cell, as apoptotic thecal cells were only found sparsely, an observation conserved in other species [Hughes et al., 1991; Chun et al., 1994]. While this study clearly demonstrated that the death of bovine granulosa cells occurs by apoptosis, the follicles used in this study were collected without knowledge of the timing of the follicular wave or estrous cycle. Later in 2000, the same researchers characterized apoptosis in granulosa cells in dominant bovine follicles in which atresia was induced by progesterone administration [Yang et al., 2000b]. Again, evidence of oligonucleosome formation in granulosa cells of atretic follicles was given. Furthermore the ratio of Bcl-2 to Bax protein was reduced in atretic dominant follicles, suggesting the involvement of the Bcl-2 family in granulosa cell
apoptosis. *In vitro* studies of Fas-mediated apoptosis in the bovine follicle have detected phosphatidylserine on the outside of granulosa cell membranes, an early marker of apoptosis [Vickers et al., 2000; Quirk et al., 2000; Hu, 2001]. However, none of these studies have addressed apoptosis that occurs during the natural atresia of the non-ovulatory dominant follicle.
Oxidative stress

Reactive oxygen species (ROS), such as hydroxyl radicals (\(^{*}{\text{OH}}\)), superoxide anion (\(O^{*}_{2}\)) and hydrogen peroxide (\(H_2O_2\)) are byproducts of normal aerobic metabolism (primarily electron transport). Intracellular accumulation of these highly reactive molecules, known as oxidative stress, can damage cells by causing nucleic acid strand breaks, lipid peroxidation, protein degradation and ultimately, cell death [Yu, 1994]. In cortical neurons [Geller et al., 1991], rat hepatocytes [Shiba et al., 1999], and mouse fibrosarcoma cells [Kuroda et al., 2000], it has been shown that apoptosis can be initiated by ROS. It has been suggested that steroidogenically active cells, such as granulosa cells of antral follicles, require high levels of energy production and thus generate large amounts of ROS [Tilly, 1996; Rapoport, 1995]. Therefore, it is possible that oxidative stress is involved in the mechanisms that trigger apoptosis in healthy, steroidogenic antral follicles.

The primary endogenous defense against oxidative stress is a series of enzymes that work in a cooperative manner to scavenge ROS. Four oxidative stress response proteins, glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/ZnSOD) and catalase (CAT), metabolize ROS and thus protect cells from oxidative stress. MnSOD, found in the mitochondria, and Cu/ZnSOD, found in the cytosol, catalyze the dismutation of the superoxide anion (\(O^{*}_{2}\)) to hydrogen peroxide [\(H_2O_2\); Yu, 1994; McCord et al., 1997; Mates, 2000]. GSHPx,
found primarily in the cytosol, and CAT, contained within peroxisomes, are responsible for detoxifying hydrogen peroxide to water [Yu, 1994; Mates, 2000; Riley, 1991].

Increased expression of oxidative stress response proteins prevented apoptosis in mouse fibrosarcoma cells [Kuroda et al., 2000], liver [Laukkanen et al., 2001], and cultured cortical neurons [Pong et al., 2001]. Oxidative stress response proteins may also provide a mechanism for follicles to avoid atresia by attenuating or preventing oxidative stress-induced apoptosis. In support of this idea, incubation of rat granulosa cells with SOD or catalase caused a dose-dependent inhibition of apoptosis [Tilly et al., 1995]. Information on the role of oxidative stress response proteins in the ruminant follicle is limited. In sheep and goats, an inverse relationship was found between intrafollicular MnSOD activity and concentrations of estradiol-17β [Singh et al., 1998]. In a preliminary study in the cow, expression of mRNA encoding MnSOD and Cu/ZnSOD in granulosa cells of dominant follicles was greater during the mid-luteal phase compared to the preovulatory phase [Bruemmer et al., 1996]. These results provide evidence that oxidative stress response genes are expressed in the bovine ovary and may be regulated by endocrine factors.
Nuclear factor-kappa B

The ubiquitous transcription factor nuclear factor-kappa B (NF-κB) has been implicated as an important regulator of apoptosis. In the unactivated state, NF-κB is sequestered as heterodimers or homodimers of a family of structurally related proteins in the cytosol bound to inhibitory IκB proteins [Fig. 1.4; Karin et al., 2000; Bauerle et al., 1996]. To date, there are five known proteins in the NF-κB family in mammalian cells: p65, c-Rel, RelB, p50, and p52. Each contains a conserved N-terminal region called the Rel-homology domain (RHD) which contains the DNA-binding and dimerization domains and the nuclear localization signal [May et al., 1998]. The interaction between NF-κB and the inhibitory protein IκB masks the nuclear localization sequence of NF-κB and interferes with sequences important for DNA binding [Chen et al., 1996; Cramer et al., 1997; Ghosh et al., 1995]. The IκB family includes seven known members: IκB-α, IκB-β, IκB-γ, IκB-ε, Bcl-3, p100, and p105. Each of these proteins contains multiple copies of a 30-33 amino acid sequence called ankyrin repeats that interact with the RHD region of NF-κB [May et al., 1998]. In response to one of over 150 extracellular signals, IκB kinase (IKK) is activated, leading to the phosphorylation of specific serine residues on IκB proteins [Karin et al., 2000; Ghosh et al., 1998]. Phosphorylation of IκB targets it for ubiquitination by a specific ubiquitin ligase, E3RS^{IκB}, and degradation by the 26 S proteosome [Winston et al., 1999; Yaron at al., 1998; Spencer et al., 1999]. This unmask the nuclear localization signal of NF-κB, leading to its translocation from the
cytosol to the nucleus where it serves as a critical regulator of the inducible expression of many genes [Fig. 1.4; Karin et al., 2000; Read et al., 2000].

The targeted genes of NF-κB can generally be grouped into 3 classes: those involved in immune response, stress response, or cell survival and development. Innate immunity is the first line of defense against infection. The Toll-like receptors (TLRs) are activated by infection and in turn lead to the activation of the NF-κB pathway. Many of the genes up-regulated in response are those encoding inflammatory cytokines, chemokines, immune receptors, and cell surface molecules [Li et al., 2002]. NF-κB is also activated in response to various stresses: physiological stress such as ischemia/reperfusion, liver regeneration, and hemorrhagic shock; physical stresses such as irradiation and oxidative stress; or environmental stress such as heavy metals and chemotherapeutic agents [Li et al., 2002]. In response, the expression of inducible nitric oxide synthase [Amoah-Apraku et al., 1995], and the cytokines IL-1, TNF-α, interferon-γ, and IL-6 can be increased [Valen et al., 2001]. In addition, NF-κB regulates expression of the oxidative stress response gene, MnSOD [Kiningham et al., 2001]. In fact, stimulation of MnSOD by tumor necrosis factor is dependent on NF-κB activation and necessary to prevent tumor necrosis factor-mediated apoptosis [Sugino et al., 2002; Guo et al., 2003].

In addition to its well-studied role in immune and stress responses, NF-κB has also been implicated as an important regulator of cell proliferation, differentiation and apoptosis [Baeuerle et al., 1996]. Initially NF-κB was considered a pro-apoptotic factor, primarily due to its rapid activation in response to apoptotic signals and its involvement
in the expression of some apoptotic genes, including TNF-\(\alpha\), c-myc, and fas ligand, in some cell types [Hsu et al., 1999; Matsui et al., 1998]. However, more recent work supports the role of NF-\(\kappa\)B as an anti-apoptotic factor. Many gene knockout studies where various members of the NF-\(\kappa\)B family or upstream kinases were disrupted have resulted in lethality due to overwhelming apoptosis [Chen et al., 2001]. Studies of NF-\(\kappa\)B in the ovary have been limited. However, there is evidence that NF-\(\kappa\)B could be involved in the attenuation of apoptosis in the follicle. Through the increased expression of antiapoptotic proteins X-linked inhibitor of apoptosis protein (Xiap) and Flice-like inhibitory protein (FLIP), NF-\(\kappa\)B has been shown to be responsible for preventing tumor necrosis factor \(\alpha\)-induced apoptosis in rat granulosa cells [Xiao et al., 2001; Xiao et al., 2002]. The same researchers have demonstrated that treatment of rat granulosa cells with FSH activated NF-\(\kappa\)B, stimulated XIAP expression, and inhibited apoptosis; and that this FSH-stimulated growth of follicles in vitro was suppressed by inhibition of NF-\(\kappa\)B activation [Wang et al., 2002].

Recent studies in cardiac myocytes have indicated that estradiol-17\(\beta\) may modulate NF-\(\kappa\)B activation [Caulin-Glaser et al., 1996; Harnish et al., 2000; Pelzer et al., 2000; Pelzer et al., 2001]. Since production of estradiol-17\(\beta\) is a hallmark of healthy follicles, we hypothesize that estradiol-17\(\beta\) stimulates follicular activation of NF-\(\kappa\)B, thus inhibiting apoptosis in healthy dominant follicles.
Figure 1.3 The signaling pathway of NF-κB activation
Specific aims

Reproductive efficiency in dairy cattle in the United States is declining [Lucy, 2001]. A better understanding of the events controlling follicular development and atresia can lead to improved pregnancy rates in dairy and beef cattle, and increased profits. Because of similar cycle length and patterns of follicular development, studies conducted using the bovine ovary also are relevant to human reproduction, where ovulatory disorders account for 40% of infertility cases in women 15-44 years of age [Abma et al., 1997]. Atresia is known to be accompanied by apoptosis. However, the factors controlling the initiation of apoptosis in dominant bovine follicles have not been identified. Previous studies have demonstrated that oxidative stress may have a role in initiating apoptosis. The transcription factor NF-κB is capable of regulating genes involved in preventing oxidative stress and has been characterized as a mediator of cell survival. Recent studies have suggested that NF-κB activity may be regulated by estradiol-17β, a major determinant of follicular health. The experiments described are designed to determine if oxidative stress is involved in the initiation of apoptosis and atresia of dominant bovine follicles, and if the transcription factor NF-κB is involved in the attenuation of apoptosis in healthy dominant follicles. Therefore, the studies described here will test the hypotheses that: *Oxidative stress is involved in the atresia of non-ovulatory dominant bovine follicles and NF-κB is involved in preventing apoptosis in the healthy dominant follicle.*
Specific Aim 1: Determined if expression of oxidative stress response proteins in dominant bovine follicles is related to follicular health. Oxidative stress could be involved in the atresia of non-ovulatory follicles. Increased expression of oxidative stress response proteins may protect follicular cells against apoptosis induced by oxidative stress, and thus contribute to the successful development of the pre-ovulatory follicle. In these preliminary experiments, the presence of oxidative stress response proteins in follicular cells was confirmed and it was determined that expression varies among follicles of differing health status.

Specific Aim 2: Characterized expression of oxidative stress response genes, proteins, and activity, along with the initiation of apoptosis in dominant bovine follicles at 3 times during the first wave of follicular development. Ultrasonography was used to collected follicles of known stages of development. The highly characterized pattern of follicular development and atresia during the 1st wave following ovulation provided an excellent model to determine when apoptosis is initiated during atresia and if the prevention of oxidative stress is involved in attenuation of apoptosis.

Specific Aim 3: Characterized follicular steroidogenic capacities during the first follicular wave and their relation to granulosa cell apoptosis. The hallmark of a healthy follicle is high levels of estradiol-17β, which has been suggested to have an anti-apoptotic role. Because apoptosis is an intracellular event, characterization of the steroidogenic capacities of follicular cells is key to understanding how estradiol-17β may
be involved in preventing apoptosis. Steroidogenic cells from dominant follicles of the 1<sup>st</sup> wave following ovulation were characterized for their ability to produce, and the relation of this ability to the onset of apoptosis examined. Because androstenedione is a necessary substrate for estradiol-17β production, its synthesis by the theca interna was also characterized.

**Specific Aim 4:** Characterized the role of activated NF-κB in apoptosis of dominant follicles of the first wave in relation to steroidogenic capacity, in vivo and in vitro. Estradiol-17β may modulate NF-κB activation. Since production of estradiol-17β is a hallmark of healthy follicles, we hypothesized that estradiol-17β stimulates follicular activation of NF-κB, thus inhibiting apoptosis in healthy dominant follicles. The regulation of NF-κB by estradiol-17β was examined, along with the initiation of apoptosis when NF-κB activity is altered.
CHAPTER 2

EXPRESSION OF OXIDATIVE STRESS RESPONSE GENES AND PROTEINS IN THECA INTERNA AND GRANULOSA CELLS OF DOMINANT BOVINE FOLLICLES

Abstract

Apoptosis of follicles occurs by apoptosis. However, the signals initiating follicular apoptosis are not well understood. In many cell types, apoptosis is caused by oxidative stress due to accumulation of reactive oxygen species (ROS). The endogenous antioxidant enzymes glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/ZnSOD) and catalase (CAT) metabolize ROS and protect cells from oxidative stress. Therefore, regulated expression of these oxidative stress response proteins may provide a mechanism for follicles to avoid atresia. The purposes of this study were to 1) determine if oxidative stress response genes and proteins are expressed in theca interna and granulosa cells of dominant bovine follicles, and 2) test the hypothesis that expression of these genes is increased in preovulatory follicles. In experiment 1, follicles were collected at slaughter and classified as atretic or healthy based on follicular fluid estradiol concentrations. In experiment 2, beginning on the day of estrus (Day 0), follicular development was monitored daily in Holstein cows using transrectal ultrasonography. Dominant follicles of the first wave were collected on Day 9 (control), or 24 h or 48 h after administration of 25 mg PGF2α on Day 8 (n = 5 per group). Follicular fluid was collected for analysis of estradiol-17β (E2). In both experiments, theca interna and granulosa cells were isolated
and frozen. Steady-state levels of mRNA encoding oxidative stress response genes were determined by ribonuclease protection assay, and amounts of protein were measured by Western blot analysis. In experiment 1, levels of follicular fluid estradiol were higher \((P<0.05)\) in healthy compared to atretic follicles. In granulosa cells, genes encoding the oxidative stress response proteins GSHPx and MnSOD were more highly \((P<0.05)\) expressed in healthy compared to atretic dominant bovine follicles. In theca interna, amounts of mRNA encoding GSHPx and MnSOD and MnSOD protein did not differ between atretic and healthy follicles. In experiment 2, the mean concentration of estradiol in follicular fluid of 24 h follicles was higher \((P<0.05)\) than control or 48 h. There were no differences in levels of oxidative stress response genes among control, 24 h, and 48 h follicles. These results demonstrate that genes encoding oxidative stress response proteins are differentially expressed in healthy and atretic follicles, and support the idea that endogenous antioxidant enzymes protect follicles from atresia. However, increased expression of these enzymes does not appear to be involved in preovulatory follicular development.
Introduction

Bovine follicular development occurs in a pattern of waves throughout the 21-day estrous cycle. The beginning, or emergence, of a wave is defined as the first day 2 or more growing follicles ≥4 mm in diameter are detected [Ginther et al., 1989]. During each wave, between 2-6 follicles are recruited to continue growing from a pool of small follicles. From the group of growing follicles, several are selected to continue growing until one follicle deviates, growing at a faster rate than others of the cohort. This follicle is established as the dominant follicle of the wave once it reaches a diameter 1-2 mm greater than others of the cohort. A defining characteristic of the healthy dominant follicle is its greater capacity to produce estradiol-17β, which requires the cooperative actions of the theca interna and the granulosa cells. As soon as the dominant follicle is detected as having a slightly larger size than its subordinates, higher concentrations of estradiol-17β are found in the follicular fluid [Bodensteiner et al., 1996, Evans et al., 1997; Ginther et al., 1997]. The remaining follicles are referred to as subordinate follicles and will cease growing and undergo the deteriorative process of atresia. Only the dominant follicle of the final wave of each estrous cycle will ovulate. Most estrous cycles are characterized by either 2 [Pierson et al., 1988] or 3 [Fortune et al., 1988; Savio et al., 1988; Sirois et al., 1988] waves of follicular development. However, regardless of the number of waves per estrous cycle, all follicles except the pre-ovulatory follicle will undergo atresia.

Atresia of bovine follicles occurs by apoptosis. However, the signals that initiate follicular apoptosis and atresia in non-ovulatory follicles and rescue the pre-ovulatory
follicle are not well understood. In cortical neurons [Geller, 2001], rat hepatocytes [Shiba, 1999], and mouse fibrosarcoma cells [Kuroda et al., 2000], apoptosis can be initiated by reactive oxygen species (ROS). ROS, such as hydroxyl radicals (•OH), superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) are byproducts of normal aerobic metabolism (primarily electron transport). Intracellular accumulation of these highly reactive molecules, known as oxidative stress, can damage cells by causing nucleic acid strand breaks, lipid peroxidation, protein degradation and ultimately, cell death (Yu 1994). It has been suggested that steroidogenically active cells, such as granulosa cells of antral follicles, require high levels of energy production and thus generate large amounts of ROS [Rapoport et al., 1995; Tilly, 1996]. Therefore, it is possible that oxidative stress is involved in the mechanisms that trigger apoptosis in healthy, steroidogenic dominant follicles.

The primary endogenous defense against oxidative stress is a series of enzymes that work in a cooperative manner to scavenge ROS. Four oxidative stress response proteins, glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/ZnSOD) and catalase (CAT), metabolize ROS and thus protect cells from oxidative stress. MnSOD and Cu/ZnSOD, found in the mitochondria and cytosol, respectively, catalyze the dismutation of the superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂) [Yu, 1994; McCord et al., 1997; Mates, 2000]. GSHPx, found primarily in the cytosol, and CAT, contained within peroxisomes, are responsible for detoxifying hydrogen peroxide to water [Yu, 1994; Mates, 2000; Riley et al., 1991]. Increased expression of oxidative stress response proteins can prevent
apoptosis in mouse fibrosarcoma cells [Kuroda et al., 2000], liver [Laukkonen et al., 2001], and cultured cortical neurons [Pong et al., 2001]. Oxidative stress response proteins may also provide a mechanism for follicles to avoid atresia by attenuating or preventing oxidative stress-induced apoptosis. In support of this idea, incubation of rat granulosa cells with SOD or CAT caused a dose-dependent inhibition of apoptosis [Tilly et al., 1995]. Information on the role of oxidative stress response proteins in the ruminant follicle is limited. In a preliminary study in the cow, expression of mRNA encoding MnSOD and Cu/ZnSOD in granulosa cells of dominant follicles was greater during the mid-luteal phase compared to the preovulatory phase [Bruemmer et al., 1996]. These results provide evidence that oxidative stress response genes are expressed in the bovine follicle and may be regulated by endocrine factors.

We hypothesized that decreased expression of oxidative stress response proteins leads to oxidative stress and thus plays a role in atresia of non-ovulatory dominant follicles, while increased expression of oxidative stress response proteins may protect follicular cells against apoptosis induced by oxidative stress, and thus contribute to the successful development of the pre-ovulatory follicle. In this experiment the presence of oxidative stress response proteins in follicular cells was confirmed and it was determined if expression varies among follicles of differing health status. If oxidative stress plays a role in atresia of bovine follicles, then oxidative stress response proteins should be expressed at lower levels in atretic compared to healthy follicles. Therefore, the purposes of this experiment were 1) to determine if expression of oxidative stress response proteins in dominant bovine follicles is related to follicular health, and 2) to test the hypothesis
that increased expression of oxidative stress response proteins protects follicular cells against apoptosis, and thus contributes to the successful development of the pre-ovulatory follicle by providing a mechanism by which pre-ovulatory follicles avoid atresia.
Materials and methods

Experiment1
Collection of follicles

Ovaries were collected from beef heifers and cows at a local slaughterhouse. Ovaries were placed in dissection medium (1X MEM with Earle’s salts and 25 mM HEPES, without L-glutamine; Life Technologies, Rockville, MD), and transported on ice 4 miles to the laboratory. Dominant follicles ≥ 10 mm in diameter were dissected from the ovarian stroma and follicular fluid aspirated. Follicular diameter was measured using calipers and follicular fluid was aspirated and stored at -20°C. The collapsed follicle was cut into 4 pieces. Theca interna, along with the basement membrane and granulosa cells, was separated from the theca externa and remaining stroma using fine forceps. Using an angled, finely pulled Pasteur pipette, granulosa cells were scraped from the basement membrane and theca interna. Granulosa cells were collected in dissection medium, centrifuged 15 min at 800 x g, resuspended in 1 ml fresh dissection medium, and counted with a hemocytometer. The remaining cells were repelleted and stored, along with theca interna, at -80°C.

Estradiol assay

Follicles were classified as atretic (n=3) or healthy (n=4) based on concentrations of estradiol-17β in follicular fluid as determined using the Double Antibody Estradiol radioimmunoassay (Diagnostic Products Corp.) according to the manufacturer’s recommendations as previously validated in our laboratory [Sanders et al., 2002]. The
estradiol antiserum exhibits very low cross-reactivity with related steroids (androstenedione, 0.004%; estriol, 0.235%; progesterone and testosterone, not detectable). Follicular fluid was not extracted prior to assay. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 1.5 pg/ml. The intra- and inter-assay CV were 5.91 and 9.5%, respectively.

**Template Generation and cRNA Probe Synthesis**

Complementary DNA encoding bovine GSHPx and bovine MnSOD were kindly provided by Dr. Bo Rueda at Massachusetts General Hospital. Both of these cDNAs were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA derived from bovine corpus luteum. The 456-bp GSHPx cDNA (corresponding to bp 166-622 of GenBank accession no. X13684) and the 394-bp MnSOD cDNA (corresponding to bp 200-594 of GenBank accession no. L22092) were ligated into the pGEM®4Z Vector (Promega Life Sciences, Madison, WI). A linear antisense template encoding 18S ribosomal RNA (pTRI RNA 18S) was purchased from Ambion, Inc (Austin, TX). The identity and orientation of all cDNAs were verified by dideoxy sequencing [Sanger et al., 1977].

To provide a shorter transcription product that could be used simultaneously with other cRNA probes for ribonuclease protection assay, the template for MnSOD was linearized at sites within the cDNA insert using PstI. All other cDNAs were linearized at PstI sites within the vector. Antisense [³²P] UTP-labeled cRNA probes were transcribed from linearized cDNA templates using T7 polymerase and the MAXiscript™ In Vitro
Transcription Kit (Ambion, Inc.) according to the manufacturer’s recommendations. Because of the high abundance of 18S ribosomal RNA, the 18S antisense riboprobe was generated at 1/10 the specific activity of the other riboprobes. Antisense cRNA probes were purified on a denaturing acrylamide gel and used for hybridization within 1 day.

Ribonuclease Protection Assays

Total RNA was extracted from granulosa cells and theca interna by the guanidinium isothiocyanate-phenol-chloroform extraction procedure [Chomczynski et al., 1987] using TRIzol® Reagent (Life Technologies). Optical densities at 260 and 280 nm were measured to determine the quantity and purity of RNA samples. Ribonuclease protection assays were carried out using the RPA III™ Ribonuclease Protection Assay Kit (Ambion, Inc.) according to the manufacturer’s recommendations. Assays for mRNA encoding GSHPx, MnSOD, and 18S ribosomal RNA were performed using 5 μg of RNA isolated from granulosa cells or theca interna. All hybridizations were carried out at 50°C for 15 h, followed by incubation with a 1:50 dilution of RNase A/T1 cocktail (Ambion, Inc.) and resolution of protected fragments on a 5% acrylamide/8M urea gel. Following electrophoresis at 260 V for 2 h, gels were transferred to filter paper, covered with plastic wrap, and placed in an InstantImager Electronic Autoradiography System (Parkard Instrument Company, Meriden, CT) for 15 min to quantify size and abundance of beta emission as counts per min (CPM), of all protected fragments. Gels were also exposed with 1 intensifying screen overnight to Hyperfilm MP autoradiography film (Amersham Pharmacia Biotech Inc., Piscataway, NJ) at -80°C.
Western Analysis

After RNA isolation, DNA was precipitated from the interphase and organic phase with ethanol. Following sedimentation of the DNA by centrifugation, protein was isolated from the phenol-ethanol supernatant [Chromczynski 1993]. Protein was quantified by the Bradford method using BSA as the protein standard [Bradford 1976]. Protein from theca interna (25 µg) was added to an equal volume of SDS sample buffer (1.25 M Tris-base, 30% glycerol, 0.2% SDS, 0.02% 2-ME, 0.0001% bromphenol blue, pH 6.8). Samples were heated for 5 min at 100°C prior to electrophoresis through a 15% acrylamide gel for 45 min at 150 V. Proteins were transferred at 90 mA overnight to nitrocellulose membranes at 4°C. Ponceau S staining was used to confirm equal transfer of proteins. Following 3 washes in TTBS, membranes were incubated 1 h with 5% nonfat dry milk (NFDM) in TTBS. Rabbit anti-human MnSOD (StressGen Biotechnologies Corp., Victoria, BC, Canada) was diluted 1:1000 in TTBS containing 5% NFDM and incubated 1 h with the nitrocellulose membranes. The membranes were washed 3 times in TTBS and incubated 1 h with incubation with anti-sheep or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Chemicon International Inc., Temecula, CA) and detected by chemiluminescence and autoradiography using x-ray film. Amounts of β-tubulin protein (mouse anti-β-tubulin antibody purchased from Chemicon International Inc.) were also measured using a 1:1000 dilution to normalize for loading differences among samples.
Experiment 2

Animals

All animal protocols were approved by the University of Arizona Animal Care and Use Committee and were within guidelines established by the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Lactating Holstein cows were housed in dry lots, fed high quality alfalfa hay and flaked corn with mineral supplement, and provided with constant access to water and shade. Experiments were conducted during October, November, and December.

Ultrasonography and Collection of Follicles

Cows were observed twice daily for estrous behavior. Beginning on the day of estrus, blood samples were collected by coccygeal venipuncture and ultrasonographic examinations of ovaries were performed daily as previously described [Turzillo et al., 1990] using a real-time B-mode linear array ultrasound scanner equipped with a 7.5 MHz intrarectal probe (Aloka SSD-550V Zug, Switzerland). Examinations were recorded on videotape (digital Handycam, Hi8™ Recording Tape, Sony Electronics Inc., Park Ridge, NJ). Ovulation was identified by the disappearance of a large follicle preceded by estrus. Following ovulation, diameters and positions of all follicles ≥ 4 mm in diameter were analyzed. The ovary bearing the dominant follicle of the first wave was collected surgically on Day 9 of the estrous cycle (“non-ovulatory” dominant follicles; n=4). To obtain preovulatory follicles, cows were treated with 25 mg PGF$_{2\alpha}$ on Day 8 of the estrous cycle, and the ovary bearing the dominant follicle was collected 24 (n=4) or 48 h
(n=4) later. Ovaries were placed in dissection medium (1X MEM with Earle’s salts and 25 mM HEPES, without L-glutamine; Life Technologies) and transported 4 miles on ice to the laboratory. Ovaries were processed as described above, with collection of follicular fluid, theca interna, and granulosa cells. Follicular fluid estradiol was measured by RIA as described above.

**Progesterone Assay**

Concentrations of progesterone were measured in serum using the Coat-A-Count Progesterone radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) according to the manufacturer’s recommendations as previously validated in our laboratory [Sanders et al., 2002]. The progesterone antiserum exhibits extremely low cross-reactivity with related steroids (androstenedione and estradiol, not detectable; pregnenolone and testosterone, 0.1%). Serum was not extracted prior to assay. Sensitivity of the assay, calculated as 2 standard deviations below the mean cpm at maximum binding, was 0.02 ng/ml. The intra-assay CV was 15.2%.

**Template Generation and cRNA Probe Synthesis**

Synthesis of cRNA probes were carried out as described above with the addition of 2 templates: a 255-bp cDNA encoding Cu/ZnSOD and a 159-bp cDNA encoding CAT were generated in our laboratory by RT-PCR using bovine liver RNA. The Cu/ZnSOD cDNA (corresponding to bp 166-369 of GenBank accession no. X54799) and the CAT cDNA (corresponding to bp 154-240 of the human cDNA, GenBank accession no. XM_030347) were ligated into the pGEM®-T Easy Vector (Promega Life Sciences).
The identity and orientation of all cDNAs were verified by dideoxy sequencing [Sanger et al., 1977]. For generation of cRNA probes, cDNAs encoding Cu/ZnSOD and CAT were linearized at *PstI* sites within the vector.

**Ribonuclease Protection Assays**

RNA was isolated from theca interna and granulosa cells and ribonuclease protection assays were carried out as described above.

**Statistical Analyses**

One-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS [SAS User's Guide, 1987] was used to analyze differences among groups. Means were separated by least significant differences. When necessary, data were log-transformed to achieve normality and homogeneity of variances.
Results

Experiment 1

Classification of follicles

To provide 2 distinct groups, follicles were classified as atretic if the concentration of estradiol in follicular fluid was <1 ng/ml. Healthy follicles had follicular fluid estradiol concentrations >1000 ng/ml (Table 2.1). The mean diameter of the follicles did not differ between the 2 groups (Table 2.1).

Levels of mRNA encoding oxidative stress response proteins

Levels of GSHPx and MnSOD mRNA in theca interna were not different between atretic and healthy follicles (Figure 2.1). However, granulosa cell expression of mRNA encoding GSHPx and MnSOD was higher ($P < 0.02$) in healthy compared to atretic dominant follicles (Fig. 2.2).

Levels of MnSOD protein

In theca interna levels of MnSOD protein were not different between atretic and healthy dominant follicles (Fig. 2.3).
Experiment 2

Serum progesterone and follicular characteristics

In all cows, circulating concentrations of progesterone were undetectable on the day of estrus and increased until Day 8 of the estrous cycle, indicating normal luteal function following ovulation. At 24 and 48 h after PGF2α injection on Day 8, serum progesterone levels were lower ($P < 0.05$) on Day 9 of the estrous cycle compared to control cows (Fig. 2.4). Follicular diameter of dominant follicles following dissection remained constant among control, 24 h, and 48 h cows (Table 2.2). The numerical differences in numbers of granulosa cells recovered are most likely due to variation between personnel performing the cell counts, as cell counts for all control and 24 h cows were performed by one person, while the remaining 24 h and all 48 h cell counts were performed by another. Concentrations of estradiol in follicular fluid were higher ($P < 0.01$) in 24 h cows compared to control and 48 h cows (Table 2.2).

Levels of mRNA encoding oxidative stress response proteins

In theca interna, relative amounts of genes encoding GSHPx, MnSOD, and Cu/ZnSOD did not change among control, 24 h, and 48 h follicles (Fig. 2.5). Similarly, in granulosa cells relative expression of GSHPx, MnSOD, Cu/ZnSOD, and CAT mRNA did not change (Fig. 2.6).
Table 2.1 Characteristics of experiment 1 follicles classified as atretic or healthy based on concentrations of estradiol in follicular fluid.

<table>
<thead>
<tr>
<th>Follicle classification</th>
<th>Follicular diameter (mm) (^\text{a})</th>
<th>Follicular Fluid estradiol-17(\beta) (ng/ml) (^\text{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atretic</td>
<td>12.0 ± 1.3(^b)</td>
<td>0.113 ± 0.037(^b)</td>
</tr>
<tr>
<td>Healthy</td>
<td>17.5 ± 2.3(^b)</td>
<td>1472 ± 215(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Data are means ± SEM.

\(^b,c\) Values within each column without common superscripts differ (\(P < 0.05\)).
Figure 2.1 Theca interna expression of mean (± SEM) levels of mRNA encoding GSHPx and MnSOD in atretic (n=3) and healthy (n=4) dominant follicles. Levels of GSHPx and MnSOD mRNA in theca interna were not different between atretic and healthy follicles.
Figure 2.2 Granulosa cell expression of mean (± SEM) levels of mRNA encoding GSHPx and MnSOD in atretic (n=3) and healthy (n=4) dominant follicles. For each gene, asterisks indicate greater ($P < 0.02$) levels of expression in healthy vs. atretic follicles.
Figure 2.3 Mean (± SEM) levels of MnSOD protein in theca interna of atretic (n=3) and healthy (n=4) dominant follicles. Levels of MnSOD protein were not different between atretic and healthy dominant follicles.
Figure 2.4 Mean (± SEM) serum progesterone (P₄) concentrations on Days 0-10 of the estrous cycle. In 24 and 48 h cows, PGF₂α was administered on Day 8 to induce luteolysis and allow dominant follicles of the first follicular wave to become preovulatory. The asterisk indicates significant difference (P < 0.05) in serum progesterone concentrations on Day 9 between control cows and 24h and 48h cows given PGF₂α on Day 8 to induce luteolysis.
Table 2.2 Characteristics of experiment 2 dominant follicles collected during the first follicular wave on Day 9 of the estrous cycle (control), 24 h after PGF2α injection (24 h), or 48 h after PGF2α injection (48 h). PGF2α was administered on Day 8.

<table>
<thead>
<tr>
<th>Group</th>
<th>Follicular diameter (mm)</th>
<th>Granulosa cells recovered (x 10⁶)</th>
<th>Follicular Fluid estradiol-17β (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.8 ± 1.4ᵇ</td>
<td>90.4 ± 37.8</td>
<td>279 ± 92ᵇ</td>
</tr>
<tr>
<td>24 h</td>
<td>16.5 ± 1.3ᵇ</td>
<td>52.9 ± 23.6</td>
<td>1233 ± 388ᶜ</td>
</tr>
<tr>
<td>48 h</td>
<td>18.8 ± 1.8ᵇ</td>
<td>29.1 ± 3.7</td>
<td>266 ± 40ᵇ</td>
</tr>
</tbody>
</table>

ᵃ Data are means ± SEM.
ᵇ,ᶜ Values within each column without common superscripts differ (P < 0.05).
Figure 2.5 Amounts of GSHPx, MnSOD, and Cu/ZnSOD mRNA in theca interna of dominant follicles collected during the first follicular wave on Day 9 of the estrous cycle (control), 24 h after PGF2α injection (24 h), or 48 h after PGF2α injection (48h). PGF2α was administered on Day 8. Data are mean cpm ± SEM. There were no significant differences among treatments for any oxidative stress response gene.
Figure 2.6 Amounts of GSHPx, MnSOD, Cu/ZnSOD, and CAT mRNA in granulosa cells of dominant follicles collected during the first follicular wave on Day 9 of the estrous cycle (control), 24 h after PGF2α injection (24 h), or 48 h after PGF2α injection (48h). PGF2α was administered on Day 8. Data are mean cpm ± SEM. There were no significant differences among treatments for any oxidative stress response gene.
Discussion

During bovine follicular development, only a limited number of follicles (<1%) are selected for ovulation [Mariana et al., 1991]. The vast majority undergoes atresia at various stages of development. However, the mechanisms that contribute to atresia of all non-ovulatory follicles have not been elucidated. The purpose of this study was to gather preliminary evidence towards understanding the role of oxidative stress in apoptosis and atresia of dominant bovine follicles at various stages of follicular health.

In sheep and goats, an inverse relationship was found between intrafollicular MnSOD activity and concentrations of estradiol-17β [Singh et al., 1998]. In the current study, genes encoding the oxidative stress response proteins GSHPx and MnSOD were more highly expressed in granulosa cells of healthy (highly estrogenic) compared to atretic dominant bovine follicles. Future studies should directly address the enzyme activity of the oxidative stress response proteins to determine if this is a species-specific phenomenon. Higher expression of oxidative stress response proteins in granulosa cells of healthy bovine follicles may allow these follicles to avoid apoptosis caused by oxidative stress.

In theca interna, there were no differences in expression of genes encoding GSHPx and MnSOD, or MnSOD protein, between atretic and healthy follicles. It is possible that theca cells are less susceptible than granulosa cells to apoptosis mediated by oxidative stress. Within the bovine follicle, apoptosis is a phenomenon that has been observed primarily in granulosa cells. Only the occasional theca interna cell is observed
during atresia with the characteristic apoptotic phenotype [Yang et al., 2000]. Assuming oxidative stress is attributing to apoptosis in the bovine dominant follicle, this may account for the apparent lack of differential expression of oxidative stress response genes and proteins between atretic and healthy follicles in theca interna.

There were no differences in levels of oxidative stress response genes in either theca interna or granulosa cells of non-ovulatory compared to preovulatory dominant follicles. While protein levels and enzyme activity were not measured in this experiment, these results indicate that survival of the preovulatory follicle is not dependent upon an up-regulation of oxidative stress response proteins. However, it should be emphasized that the non-ovulatory (control) follicles in this experiment should not be considered atretic as demonstrated by their high follicular fluid estradiol concentrations. Future experiments should focus on measuring oxidative stress response proteins along with the initiation of apoptosis in dominant follicles in the early stages of atresia to further address the role of oxidative stress in atresia.

In conclusion, this preliminary study provides evidence that expression of the oxidative stress response genes decreases in atretic dominant bovine follicles. This supports the hypothesis that oxidative stress is contributing to the apoptosis that occurs as follicles undergo atresia. Future research will focus on the temporal relationship between apoptosis in granulosa cells and expression of proteins responsible for apoptosis to clarify their role in preventing oxidative stress in the bovine follicle.
CHAPTER 3

REGULATION OF APOPTOSIS DURING ATRESIA OF DOMINANT BOVINE FOLLICLES OF THE FIRST FOLLICULAR WAVE FOLLOWING OVULATION*

*This chapter has been submitted for publication in Reproduction:


Abstract

During atresia of bovine follicles, granulosa cells undergo the controlled form of cell death, apoptosis. However, the mechanisms that regulate apoptosis in granulosa cells are not well understood. The purpose of this study was to characterize changes in expression of genes and activity of enzymes that may regulate apoptotic death of granulosa cells in dominant bovine follicles during the first wave of follicular development. Dominant follicles were collected from Holstein heifers on Day 4, 6, or 8 of the first follicular wave (n = 5/Day). Regulation of apoptosis in granulosa cells was examined by 1) annexin V and propidium iodide staining; 2) measurement of relative levels mRNA encoding Bcl-2, Bcl-xL, and Bax; and 3) activity of caspase-3, -8, and 9. In many cell types, apoptosis can be induced by oxidative stress and prevented by increased expression of endogenous oxidative stress response proteins. Therefore,
steady-state levels of mRNA encoding 4 oxidative stress response proteins were determined by ribonuclease protection assay. Concentrations of estradiol in follicular fluid decreased ($P< 0.05$) between Days 4 and 6. Compared to Day 4, the incidence of apoptotic and non-viable granulosa cells tended to increase ($P \leq 0.06$) on Day 6, and numbers of non-viable cells were higher ($P < 0.02$) on Day 8. The ratios of relative levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to Bax were higher ($P < 0.05$) on Day 6 compared to Days 4 and 8, while activities of caspases-3, -8, and -9 in granulosa cells did not change among the 3 days. Amounts of GSHPx, MnSOD, and Cu/ZnSOD mRNA in granulosa cells were higher ($P< 0.05$) on Day 8 compared to Day 6. In theca interna, amounts of Cu/ZnSOD mRNA decreased between Days 4 and 6. Based on decreased production of estradiol and increased numbers of apoptotic and non-viable granulosa cells, we conclude that atresia of the dominant follicle is initiated between Days 4 and 6 of the first follicular wave. However, apoptosis of granulosa cells does not appear to be initiated by changes in expression of oxidative stress response proteins.
Introduction

Although the bovine ovary contains approximately 150,000 follicles at birth [Erickson, 1966], very few follicles are successfully ovulated and greater than 99.9% undergo atresia [Byskov, 1978]. It has been estimated that the incidence of atresia in bovine follicles is greatest after antrum formation, just before the final stages of follicular development [Fortune, 1994]. Apoptosis of granulosa cells is an early feature of atresia in bovine follicles [Jolly et al., 1994; Van Wezel et al., 1999; Yang et al., 2000]. However, the mechanisms initiating apoptosis of granulosa cells have not been well characterized.

The Bcl-2 family of proteins includes members which either promote (Bax) or inhibit (Bcl-2, Bcl-xL) apoptosis. These proteins associate to form homo- and/or heterodimers [Mignotte et al., 1998]. A shift in equilibrium toward proapoptotic (Bax) vs. antiapoptotic (Bcl-2, Bcl-xL) Bcl-2 proteins increases permeability of the mitochondrial membrane, releasing cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm [Amarente-Mendes et al., 1998; Kluck et al., 1997; Yang et al., 1997; Jurgenmeier et al., 1998]. Therefore, the ratio of Bcl-2 and Bcl-xL to Bax expression is important in determining susceptibility to apoptosis [Oltvai et al., 1993]. Yang et al. [2000b] demonstrated that an elevated ratio of Bax to Bcl-2 protein expression occurs during atresia of bovine dominant follicles induced by in vivo injection of progesterone. However, the roles of Bcl-2, Bcl-xL, and Bax during natural follicular development and atresia remain to be elucidated.
The Bcl-2 proteins affect apoptosis by either promoting or inhibiting cleavage of execution caspases. Caspases are cysteine proteases that cleave their substrate proteins specifically at an aspartate residue. They are constitutively expressed in an inactive proenzyme form and are activated following cleavage at specific aspartate residues. Activation of the execution caspases leads to irreversible progression of the apoptotic cascade [Mignotte et al., 1998]. To our knowledge, the activity of execution caspases, particularly caspases-3, -8, and -9, and their role in apoptosis of bovine granulosa cells has not been studied.

In cortical neurons [Geller, 2001], rat hepatocytes [Shiba, 1999], and mouse fibrosarcoma cells [Kuroda et al., 2000], apoptosis can be initiated by reactive oxygen species (ROS), which are byproducts of normal aerobic metabolism (primarily electron transport). Intracellular accumulation of ROS, known as oxidative stress, can damage cells by causing nucleic acid strand breaks, lipid peroxidation, protein degradation and ultimately, cell death [Yu, 1994]. It has been suggested that steroidogenically active cells, such as granulosa cells of antral follicles, require high levels of energy production and thus generate large amounts of ROS [Rapoport et al., 1995; Tilly, 1996]. Therefore, it is possible that oxidative stress is involved in the mechanisms that trigger apoptosis in healthy, steroidogenic antral follicles. The primary endogenous defense against oxidative stress is a series of enzymes that work in a cooperative manner to scavenge ROS. Four oxidative stress response proteins, glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (Cu/ZnSOD) and catalase (CAT), metabolize ROS and thus protect cells from oxidative stress. MnSOD
and Cu/ZnSOD, found in the mitochondria and cytosol, respectively, catalyze the
dismutation of the superoxide anion ($O^{2-}$) to hydrogen peroxide ($H_2O_2$) [Yu, 1994;
McCord et al., 1997; Mates, 2000]. GSHPx, found primarily in the cytosol, and CAT,
contained within peroxisomes, are responsible for detoxifying hydrogen peroxide to
water [Yu, 1994; Mates, 2000; Riley et al. 1991]. In vitro, increased expression of
oxidative stress response proteins can prevent apoptosis [Kuroda et al., 2000; Laukkanen
et al., 2001; Pong et al., 2001]. In the bovine corpus luteum, a decline in expression of
oxidative stress response genes was observed during structural regression [Rueda et al.,
1995]. In the follicle, oxidative stress response proteins may provide a mechanism to
avoid atresia by attenuating or preventing oxidative stress-induced apoptosis. In support
of this idea, incubation of rat granulosa cells with SOD or catalase caused a dose-
dependent inhibition of apoptosis [Tilly et al., 1995]. Information on the role of
oxidative stress response proteins in the ruminant follicle is limited. In sheep and goats,
an inverse relationship was found between intrafollicular MnSOD activity and
concentrations of estradiol-17β [Singh et al., 1998]. In a preliminary study in the cow,
expression of mRNA encoding MnSOD and Cu/ZnSOD in granulosa cells of dominant
follicles was greater during the mid-luteal phase compared to the preovulatory phase
[Bruemmer et al., 1996]. These results provide evidence that oxidative stress response
genes are expressed in the bovine follicle and may be regulated by endocrine factors.

The bovine estrous cycle is characterized by 2 or 3 waves of follicular
development [Savio et al., 1988; Sirlois et al., 1988; Ginther et al., 1989]. Previous
studies have characterized the onset of atresia in non-ovulatory bovine follicles during the
first wave of following ovulation [Xu et al., 1995a,b]. Therefore, the first follicular wave in cattle provides an excellent model to study the temporal relationship between apoptosis and expression of genes that regulate apoptotic cell death. The purpose of this study was to investigate possible mechanisms that lead to atresia in dominant bovine follicles during the first wave of follicular development, namely changes in expression of genes and activity of enzymes that regulate apoptotic death of granulosa cells. We hypothesized that apoptosis of granulosa cells would be associated with increased expression of Bax relative to anti-apoptotic Bcl-2 genes; increased caspase activity; and decreased expression of oxidative stress response proteins.
Materials and Methods

Animals

All animal protocols were approved by the University of Arizona Use and Care Committee and were within guidelines established by the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [1999]. Sexually mature Holstein heifers were housed in dry lots, fed high quality alfalfa hay and flaked corn with mineral supplement, and provided with constant access to water and shade. Experiments were conducted during October, November, and December.

Ultrasonography and Collection of Follicles

Heifers were observed twice daily for estrous behavior. Beginning on the day of estrus, blood samples were collected by coccygeal venipuncture and ultrasonographic examinations of ovaries were performed daily as previously described [Turzillo et al., 1990] using a real-time B-mode linear array ultrasound scanner equipped with a 7.5 MHz intrarectal probe (Aloka SSD-550V Zug, Switzerland). Examinations were recorded on videotape (digital Handycam, Hi8™ Recording Tape, Sony Electronics Inc., Park Ridge, NJ). Ovulation was identified by the disappearance of a large follicle preceded by estrus. Following ovulation, diameters and positions of all follicles ≥ 4 mm in diameter were analyzed. Day 1 of the follicular wave was defined as the day 2 or more follicles ≥ 4 mm in diameter were first observed. These follicles continued to grow until 1 follicle deviated from the cohort to become dominant while other follicles in the wave regressed.
The ovary bearing the dominant follicle was surgically removed via flank incision on Day 4, 6, or 8 of the first follicular wave (n=5 per day). These days were chosen based on previous work [Xu et al., 1995a,b] showing that functional regression of the dominant follicle occurs between Days 4 and 8 of the first wave and is marked by decreases in both steroidogenic capacity and expression of genes encoding key steroidogenic enzymes. Ovaries were placed in dissection medium (1X MEM with Earle's salts and 25 mM HEPES, without L-glutamine; Life Technologies, Rockville, MD), and transported immediately on ice to the laboratory. The dominant follicle was dissected from the ovarian stroma. Follicular diameter was measured using calipers and follicular fluid was aspirated and stored at -20°C. The collapsed follicle was cut into 4 pieces. Theca interna, along with the basement membrane and granulosa cells, was separated from the theca externa and remaining stroma using fine forceps. Using an angled, finely pulled Pasteur pipette, granulosa cells were scraped from the basement membrane and theca interna. Granulosa cells were collected in dissection medium, centrifuged 15 min at 800 x g, resuspended in 1 ml fresh dissection medium, and counted with a hemocytometer. A small portion of granulosa cells from each follicle (100,000) was set aside for annexin and propidium iodide (PI) staining while the remaining cells were repelleted and stored, along with theca interna, at -80°C.

**Hormone Assays**

Concentrations of progesterone were measured in serum using the Coat-A-Count Progesterone radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA)
according to the manufacturer’s recommendations as previously validated in our laboratory [Sanders et al., 2002]. The progesterone antiserum exhibits extremely low cross-reactivity with related steroids (androstenedione and estradiol, not detectable; pregnenolone and testosterone, 0.1%). Serum was not extracted prior to assay. Sensitivity of the assay, calculated as 2 standard deviations below the mean cpm at maximum binding, was 0.02 ng/ml. The intra-assay CV was 15.2%.

Concentrations of estradiol-17β (estradiol) were measured in follicular fluid using the Double Antibody Estradiol radioimmunoassay (Diagnostic Products Corp.) according to the manufacturer’s recommendations as previously validated in our laboratory [Sanders et al., 2002]. The estradiol antiserum exhibits very low cross-reactivity with related steroids (androstenedione, 0.004%; estriol, 0.235%; progesterone and testosterone, not detectable). Follicular fluid was not extracted prior to assay. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 1.5 pg/ml. The intra- and inter-assay CV were 5.91 and 9.5%, respectively.

**Annexin V and Propidium Iodide (PI) Staining**

Early-stage apoptosis of granulosa cells was assessed by staining with annexin V using the Vybrant™ Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations with modifications. Annexin V binds phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane early in apoptosis [van England et al., 1998]. To distinguish intact,
apoptotic cells from cells lacking intact plasma membranes (non-viable), PI is used to stain double-stranded nucleic acids. Granulosa cells (approximately 100,000) were washed in cold PBS, centrifuged at 800 x g for 15 min, and resuspended in 100 μl of 1X Annexin-Binding Buffer. Alexa Fluor 488 annexin V (5 μl) and PI (1 μl of a 100 μg/ml working solution) were added and cells were incubated at room temperature for 15 min. Cells were then spread on a glass slide, coverslipped, and viewed with an Olympus BX50WI fluorescence microscope using a FITC filter. At least 800 granulosa cells per follicle were observed. Numbers of cells that stained for annexin V exclusively or annexin V and PI were recorded.

Quantitative RT-PCR

Fluorescent real-time quantitative RT-PCR was used to determine differences in mRNA expression of Bax, Bcl-xL, and Bcl-2. Total RNA was extracted from granulosa cells and theca interna by the guanidinium isothiocyanate-phenol-chloroform extraction procedure [Chomczynski et al., 1987] using TRIzol® Reagent (Life Technologies). Optical densities at 260 and 280 nm were measured to determine the quantity and purity of RNA samples. Two μg of total RNA were subjected to reverse transcription using SuperScript™ III RNase Reverse Transcriptase (Invitrogen) following the manufacturer’s protocol using 3 μg of random hexamers. Primers (Table 3.1) for quantitative RT-PCR were made using PrimerQuest℠ software (Integrated DNA Technologies, Coralville, IA) with the following manufacturer’s restrictions.
Each PCR reaction (total volume of 10 µl) consisted of 16 ng of reverse transcription product, 0.4 µl of 25 µM MgCl$_2$, 1.0 µl of each 5.0 µM forward and reverse primer, 5.0 µl of SYBR Green Master Mix (Qiagen), and RNase free water. Thermal cycling conditions were as follows: 15 min at 95°C to activate Hotstart Taq, 40 cycles of 15 sec at 95°C for denaturing, 15 sec at 58°C for annealing, 20 sec at 72°C for extension, followed by a ramp from 72°C to 99°C over 15 min to determine the melting curve.

Ribosomal 18S rRNA was used as a housekeeping gene to normalize samples for variation in RNA loading. To verify that 18S rRNA was a valid housekeeping gene, the ability to detect fold changes in amounts of RNA loaded in a parallel fashion with the target probe (Bax, Bcl-2, or Bcl-xL) was tested. Decreasing amounts of 18S rRNA (500, 100, 50, 10, 5, or 1 pg) demonstrated parallel fold changes in amplification compared to decreasing amounts of each target (Bax, Bcl-2, or Bcl-xL) mRNA (500, 100, 50, 10, 5, or 1 ng).

Quantification of gene expression was made by setting an arbitrary threshold in the geometric portion of the RT-PCR amplification plot after examining the log view. Relative quantification of Bax, Bcl-2, or Bcl-xL mRNA expression was done using the comparative cycle threshold (Ct) method [ABI Prism Sequence Detection System User Bulletin No. 2, 1997; Livak et al., 2001]. Briefly, the ΔCt was determined by subtracting the 18S Ct from the target unknown (Bax, Bcl-2, or Bcl-xL) Ct value. For each target mRNA, the ΔΔCt was determined by subtracting the highest ΔCt from all other ΔCt values within each experiment. Fold changes in mRNA expression of Bax, Bcl-2, or Bcl-
xL were then calculated as $2^{-\Delta \Delta Ct}$. Each samples was run in triplicate. Data were expressed as ratio of relative expression of Bcl-2: Bax or Bcl-xL: Bax.

Template Generation and cRNA Probe Synthesis

Complementary DNA encoding bovine GSHPx and bovine MnSOD were kindly provided by Dr. Bo Rueda at Massachusetts General Hospital. Both of these cDNAs were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA derived from bovine corpus luteum. The 456-bp GSHPx cDNA (corresponding to bp 166-622 of GenBank accession no. X13684) and the 394-bp MnSOD cDNA (corresponding to bp 200-594 of GenBank accession no. L22092) were ligated into the pGEM®-4Z Vector (Promega Life Sciences, Madison, WI). A 255-bp cDNA encoding Cu/ZnSOD and a 159-bp cDNA encoding CAT were generated in our laboratory by RT-PCR using bovine liver RNA. The Cu/ZnSOD cDNA (corresponding to bp 166-369 of GenBank accession no. X54799) and the CAT cDNA (corresponding to bp 154-240 of the human cDNA, GenBank accession no. XM_030347) were ligated into the pGEM®-T Easy Vector (Promega Life Sciences). A linear antisense template encoding 18S ribosomal RNA (pTRI RNA 18S) was purchased from Ambion, Inc. (Austin, TX). The identity and orientation of all cDNAs were verified by dideoxy sequencing [Sanger et al., 1977].

To provide a shorter transcription product that could be used simultaneously with other cRNA probes for ribonuclease protection assay, the template for MnSOD was linearized at sites within the cDNA insert using PstI. All other cDNAs were linearized at
Psrl sites within the vector. Antisense [32P] UTP-labeled cRNA probes were transcribed from linearized cDNA templates using T7 polymerase and the MAXiscript™ In Vitro Transcription Kit (Ambion, Inc.) according to the manufacturer's recommendations. Because of the high abundance of 18S ribosomal RNA, the 18S antisense riboprobe was generated at 1/10 the specific activity of the other riboprobes. Antisense cRNA probes were purified on a denaturing acrylamide gel and used for hybridization within 1 day.

Ribonuclease Protection Assays

Ribonuclease protection assays were carried out using the RPA III™ Ribonuclease Protection Assay Kit (Ambion, Inc.) according to the manufacturer's recommendations. Assays for mRNA encoding GSHPx, MnSOD, CuZnSOD, CAT, and 18S ribosomal RNA were performed using 5 µg of RNA isolated from granulosa cells or theca interna. All hybridizations were carried out at 50°C for 15 h, followed by incubation with a 1:50 dilution of RNase A/T1 cocktail (Ambion, Inc.) and resolution of protected fragments on a 5% acrylamide/8M urea gel. Following electrophoresis at 260 V for 2 h, gels were transferred to filter paper, covered with plastic wrap, and placed in an InstantImager Electronic Autoradiography System (Parkard Instrument Company, Meriden, CT) for 15 min to quantify size and abundance of beta emission, as counts per min (CPM), of all protected fragments. Gels were also exposed with 1 intensifying screen overnight to Hyperfilm MP autoradiography film (Amersham Pharmacia Biotech Inc., Piscataway, NJ) at −80°C.
Western Analysis

After RNA isolation, DNA was precipitated from the interphase and organic phase with ethanol. Following sedimentation of the DNA by centrifugation, protein was isolated from the phenol-ethanol supernatant [Chromczynski 1993]. Protein was quantified by the Bradford method using bovine serum albumin (BSA) as the protein standard [Bradford 1976]. Granulosa cell (30 μg) and theca interna (25 μg) protein were added to an equal volume of SDS sample buffer (1.25 M Tris-base, 30% glycerol, 0.2% SDS, 0.02% 2-ME, 0.0001% bromphenol blue, pH 6.8). Samples were heated for 5 min at 100°C prior to electrophoresis through a 15% acrylamide gel for 45 min at 150 V. Proteins were transferred at 90 mA overnight to nitrocellulose membranes at 4°C. Ponceau S staining was used to confirm consistent transfer of proteins. Following 3 washes in TTBS, membranes were incubated 1 h with 5% nonfat dry milk (NFDM) in TTBS. One of 4 primary antibodies was then applied. Sheep anti-bovine GSHPx (Biogenesis Inc., Kingston, NH), rabbit anti-human MnSOD (StressGen Biotechnologies Corp., Victoria, BC Canada), rabbit anti-bovine Cu/ZnSOD, and rabbit anti-bovine CAT (both from Chemicon International, Inc., Temecula, CA) were diluted 1:1000 in TTBS containing 5% NFDM and incubated 1 h with the nitrocellulose membranes. The membranes were washed 3 times in TTBS and incubated 1 h with incubation with anti-sheep or anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Chemicon) and detected by chemiluminescence and autoradiography using x-ray film.
Enzyme Assays

Follicles were collected and granulosa cells isolated as described above on Day 4, 6, or 8 of the first follicular wave (n=5 per day). Granulosa cells were isolated and divided for isolation of functional protein by 1 of 2 methods. For GSHPx, SOD, and CAT enzyme activity assays, cells were resuspended in 200 µl buffer (PBS, 100 mM EDTA, pH 7.4) on ice and sonicated at 40% power for 5 sec. Cell lysates were centrifuged (10,000 x g, 10 min, 4°C) 3 times, retaining the supernatant each time, and stored at -80°C. For caspase activity assays, protein was isolated using reagents in the BD ApoAlert™ Caspase Assay Plate kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's recommendations. Briefly, granulosa cells were resuspended in ice-cold 1X Cell Lysis buffer at a concentration of 2 x 10^5 cells per 50 µl buffer and incubated on ice for 10 min. Cells were centrifuged at 12,000 x g, 5 min, 4°C and the supernatant was transferred to a new tube and stored at -80°C. Protein isolated by both methods was quantitated by the bicinchoninate (BCA) method using the Pierce protein assay kit (Pierce Biotechnology, Rockford, IL).

GSHPx activity in granulosa cells was determined using the Glutathione Peroxidase Assay Kit (Cayman Chemical Company, Ann Arbor, MI). This assay measures GSHPx indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced by reduction of hydroperoxide by GSHPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. When GSHPx activity is limiting, the rate of decrease in the A₃₄₀ is directly proportional to the GSHPx activity of
the sample. Each sample was assayed in triplicate according to the manufacturer's recommendations. Briefly, 10 μg lysate in 20 μl Sample Buffer were added to 100 μl Assay Buffer and 50 μl co-substrate mixture in a 96-well microplate. Reactions were initiated by the addition of 20 μl cumene hydroperoxide and the absorbance was read immediately at 340 nm in a microplate reader. The absorbance was read every minute for 9 min and the change in absorbance (ΔA_{340}) per minute determined. The rate of ΔA_{340}/min of the background (determined by reading the absorbance of Assay Buffer, co-substrate mixture, and cumene hydroperoxide) was subtracted from the rate of each sample. One unit of GSHPx activity is defined as the amount of enzyme that will cause oxidation of 1.0 nmol of NADPH to NADP⁺ per minute at 25 °C, with the NADPH extinction coefficient being 0.00373 μM⁻¹. Therefore, the following equation was used to determine GSHPx activity:

\[
\text{GSHPx activity} = \frac{\Delta A_{340}/\text{min}}{0.00373 \mu M^{-1}} x \frac{0.19 \text{ ml}}{0.02 \text{ ml}} x \text{Sample dilution} = \text{nmol/min/ml}
\]

Total SOD activity of granulosa cells was determined using the SOD Assay Kit-WST according to the manufacturer's recommendations. (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). This assay relies on WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction with O²⁻, a reaction that is inhibited by SOD. Standards for the inhibition curve were obtained by diluting SOD (Sigma Corp., St. Louis, MO) to 200, 100, 50, 20, 10, 5, 1, 0.1, 0.05, 0.01, and 0.001 U/ml. All standards, samples (5 μg), and controls (B1, B2, B3) were assayed in triplicate. In a 96-
well microplate, 20 μl of sample solution or water (B1 and B3) were mixed with 200 μl of WST Working Solution. For B2 and B3, 20 μl of Dilution Buffer were added. B2 also received 20 μl of a pooled granulosa cell lysate (5 μg). Enzyme Working Solution (20 μl) was added to each standard and sample well, along with B1 wells. The plate was incubated at 37°C for 20 min and the absorbance read at 450 nm using a microplate reader. SOD activity (inhibition rate %) was determined using the following equation:

\[
\text{SOD activity (inhibition rate %)} = \frac{\left( (A_{B1} - A_{B3}) - (A_{\text{sample}} - A_{B2}) \right)}{(A_{B1} - A_{B3})} \times 100
\]

CAT activity of granulosa cells was determined using the Amplex Red Catalase Assay Kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer’s recommendations. In the assay, CAT reacts with H₂O₂ to produce water and oxygen. The Amplex Red reagent reacts with any unreacted H₂O₂ in the presence of horseradish peroxidase to produce the fluorescent oxidation product, resorufin. A standard curve was prepared by diluting the provided CAT to 1000, 500, 250, 125, 62.5, and 0 mU/mL. Samples (500 ng) were diluted in 1X Reaction Buffer to 25 μl. Samples, standards, and controls were pipetted in triplicate into separate wells of a 96-well microplate. A solution of 40 μM H₂O₂ was added (25μl) to each well. After 30 min at 25°C, 50 μl of the Amplex Red/ HRP working solution was added to each well. The reaction was carried out for 30 min at 37°C and the plate read on a fluorescence microplate reader using excitation of 530 nm and emission detection at 590 nm. The change in fluorescence was reported by subtracting the sample value from that of a no-catalase control.
Assays for the activity of caspase-3, -8, and -9 of granulosa cells were carried out using the BD ApoAlert™ Caspase Assay Plate kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's recommendations. The included microplate contains fluorogenic substrates specific for the caspase-3, -8, or -9 immobilized in the wells. When cell lysate is applied to the wells, each caspase cleaves its substrate and a fluorescent product is released. To perform the assay, 50 μl of 2X Reaction Buffer/DTT Mix was added to each well and incubated at 37°C for 5 min. Granulosa cell lysate (50 μl) was added and the plate was incubated at 37°C for 2 h. The plate was analyzed in a fluorescent plate reader using an excitation of 380 nm and emission detection at 460 nm. All samples were analyzed in triplicate.

Statistical Analyses

One-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS [SAS User's Guide, 1987] was used to analyze differences among groups. Means were separated by least significant differences. When necessary, data were log-transformed to achieve normality and homogeneity of variances. Values of $P \leq 0.05$ were considered significant.
Results

Follicular characteristics

In all heifers, circulating concentrations of progesterone were undetectable on the day of estrus and increased to levels of $2.4 \pm 2.0$, $5.2 \pm 1.5$, and $7.7 \pm 2.0$ ng/ml on Days 4, 6, and 8 of the first follicular wave, respectively, indicating normal luteal function following ovulation. The first follicular wave was initiated $1.3 \pm 0.1$ (range = 1-2) days after ovulation, and in each heifer a single follicle deviated from the recruited cohort to become the dominant follicle. Follicular diameter following dissection remained constant among dominant follicles collected on Days 4, 6, or 8 while the number of granulosa cells recovered tended to decrease ($P = 0.07$) between Days 6 and 8 (Table 3.2). Concentrations of estradiol in follicular fluid were higher ($P < 0.01$) on Day 4 compared to Days 6 and 8 (Table 3.2).

Annexin V and PI staining

The percentage of granulosa cells in early stages of apoptosis (stained with annexin V alone) tended to increase ($P = 0.06$) between Days 4 and 6 (Fig. 3.1). The percentage of non-viable granulosa cells (stained for both annexin V and PI) in follicles collected on Day 4 were lower ($P = 0.05$) compared to those collected on Days 6 and 8.
**Bcl-2 family mRNA**

The ratios of relative levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to Bax were higher \( (P < 0.05) \) on Day 6 compared to Days 4 and 8 of the first follicular wave in granulosa cells (Fig. 3.2).

**Caspase-3, -8, and -9 activity**

The activity of caspases -3 and -9 did not change among granulosa cells collected on Days 4, 6, or 8 of the first follicular wave (Fig. 3.3). However, caspase-8 activity in granulosa cells decreased \( (P < 0.05) \) on Day 8 compared to Days 4 and 6.

**Levels of mRNA encoding oxidative stress response proteins**

In granulosa cells, relative levels of mRNA encoding GSHPx and MnSOD were higher \( (P < 0.01) \) in follicles collected on Day 8 compared to those collected on Days 4 or 6 (Fig. 3.4). Amounts of mRNA encoding Cu/ZnSOD were higher \( (P < 0.05) \) on Day 8 compared to Day 6. Levels of mRNA encoding CAT in granulosa cells were similar across all 3 days.

In theca interna, relative levels of mRNA encoding GSHPx and MnSOD were similar on Days 4, 6, and 8 (Fig. 3.5). Thecal expression of mRNA encoding Cu/ZnSOD decreased \( (P < 0.02) \) between Days 4 and 6 and remained low on Day 8. Levels of mRNA encoding CAT in theca interna were similar on Days 4, 6, and 8.
Levels of oxidative stress response proteins

In granulosa cells, relative amounts of GSHPx, MnSOD, Cu/ZnSOD, and CAT protein were variable among follicles collected on Day 4, 6, or 8 of the first follicular wave (Fig. 3.6). Similarly, relative expression of these proteins did not change in theca interna (Fig. 3.7).

Activity of oxidative stress response proteins

In granulosa cells, activity of GSHPx and CAT did not change among follicles collected on Day 4, 6, or 8 of the first follicular wave (Fig. 3.8A. and C.). Activity of SOD in granulosa cells was higher \( P = 0.05 \) on Day 6 compared to Day 4 of the first follicular wave (Fig. 3.8B.).
**Table 3.1** Quantitative RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>TTGCTGATGGAAGCTACAACTG</td>
<td>CCTTGGTGACAGGCCTTGAG</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>CACTGTGCCTGGGAAAGCGTA</td>
<td>AAAGTGTCCCAGGCCGTC</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>CGCATCGTGCCCTCTTTGAGTT</td>
<td>GCCGGGTCAACTGTCAGTCAT</td>
</tr>
<tr>
<td>18S</td>
<td>CCTTCCGCGAGGATCATTG</td>
<td>CGCTCCAAGATCCACTAC</td>
</tr>
</tbody>
</table>
Table 3.2 Characteristics of dominant follicles collected during the first follicular wave.\(^a\)

<table>
<thead>
<tr>
<th>Day of wave</th>
<th>Follicular diameter (mm)</th>
<th>Granulosa cells recovered (x 10^6)</th>
<th>Follicular fluid estradiol (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>13.0 ± 0.2(^b)</td>
<td>22.1 ± 2.5(^d)</td>
<td>299 ± 52(^b)</td>
</tr>
<tr>
<td>6</td>
<td>13.0 ± 0.7(^b)</td>
<td>30.2 ± 0.2(^e)</td>
<td>44 ± 7(^c)</td>
</tr>
<tr>
<td>8</td>
<td>13.6 ± 0.4(^b)</td>
<td>19.7 ± 4.2(^d)</td>
<td>48 ± 9(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Data are means ± SEM.

\(^b,e\) Values within each column without common superscripts differ (\(P < 0.05\)).

\(^d,e\) Values within each column with no common superscripts tend to differ (\(P = 0.07\)).
Figure 3.1 Percentage of granulosa cells in dominant follicles collected on Day 4, 6, or 8 of the first follicular wave stained with either annexin V alone (indicative of early apoptosis) or annexin V and PI (indicative of non-viability). Data are mean ± SEM. Letters A and B indicate a trend to differ among days within staining category ($P = 0.06$). Letters C and D indicate significant differences among days within staining category ($P = 0.05$).
Figure 3.2 Relative expression of Bcl-xL:Bax and Bcl-2:Bax mRNA in granulosa cells from dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. Letters indicate significant differences among days (P < 0.05).
Figure 3.3 Caspase activity of granulosa cells of dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. Letters indicate significant differences among days for each caspase ($P < 0.05$).
Figure 3.4 Amounts of GSHPx, MnSOD, Cu/ZnSOD, and CAT mRNA in granulosa cells of dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean cpm ± SEM. Letters indicate significant differences among days for each oxidative stress response gene ($P < 0.05$).
Figure 3.5 Amounts of GSHPx, MnSOD, Cu/ZnSOD, and CAT mRNA in theca interna of dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean cpm ± SEM. Letters indicate significant differences among days for each oxidative stress response gene ($P < 0.02$).
Figure 3.6 Amounts of GSHPx, MnSOD, Cu/ZnSOD, and CAT protein in granulosa cells of dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. There were no significant differences among days for any oxidative stress response proteins.
Figure 3.7 Amounts of GSHPx, MnSOD, Cu/ZnSOD, and CAT protein in theca interna of dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. There were no significant differences among days for any oxidative stress response proteins.
Figure 3.8 Activity of GSHPx (A.), SOD (B.), and CAT (C.) in granulosa cells of dominant follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. Letters indicate significant differences among days for activity of each oxidative stress response protein ($P = 0.05$).
Discussion

Each wave of follicular growth in cattle is characterized by recruitment of a cohort of follicles from which a single follicle is selected to become morphologically and steroidogenically dominant. If luteolysis does not occur during the growth phase of the dominant follicle, it undergoes atresia and a new follicular wave appears. Bao et al. [1998] suggested that functional atresia of the dominant follicle in cattle is initiated between Days 4 and 6 of the first follicular wave. This conclusion was based on a marked decrease in concentrations of estradiol in follicular fluid and reduced expression of mRNA encoding FSH receptor and several steroidogenic enzymes [Xu et al., 1995a, b]. We also observed a dramatic reduction in follicular fluid concentrations of estradiol between Days 4 and 6 of the wave. It is important to note that these alterations in steroid production and gene expression occurred before decreased follicular diameter or consistent loss of morphological integrity (e.g., degeneration of basement membrane) were evident [Xu et al., 1995a,b]. To our knowledge, this is the first study to characterize the temporal pattern of apoptosis in granulosa cells of the dominant follicle during the first follicular wave. Externalization of phosphatidylserine residues, a marker for early apoptosis, tended to increase between Days 4 and 6. The percentage of non-viable granulosa cells increased between Days 4 and 6, and remained > 20% on Day 8. The greater incidence of apoptosis and/or death in granulosa cells on Day 6 accompanied by dramatically reduced estradiol production leads us to conclude that atresia of the dominant follicle is likely initiated between Days 4 and 6 of the first follicular wave, as previously suggested by Bao et al. [1998]. However, it is important to note that in the
event of early luteal regression induced by PGF2α injection, some dominant follicles are
capable of ovulating 2 days after reaching maximal diameter [Fortune et al., 1991].
Therefore, it appears that in some cases, dominant follicles can be “rescued” from atresia.
The molecular and cellular characteristics that distinguish these follicles from those at
similar stage of development that fail to ovulate following luteolysis are not known.

The Bcl-2 family of proteins includes members which either promote (Bax) or
inhibit (Bcl-2, Bcl-xL) apoptosis. Overexpression of Bax accelerates apoptotic death
response to death signals [Oltvai et al., 1993]. In addition, Bax can heterodimerize with
Bcl-2 or Bcl-xL and prevent their effects on cellular survival [Oltvai et al., 1993]. In the
current study, the ratio of relative levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to
Bax increased on Day 6 compared to Day 4. This indicates that apoptosis initiated
between Days 4 and 6 is likely not regulated by the Bcl-2 proteins. The ratios of relative
levels of mRNA encoding Bcl-2 to Bax and Bcl-xL to Bax then decreased on Day 8
compared to Day 6 of the first follicular wave, indicating a shift in equilibrium towards
expression of the pro-apoptotic Bax gene. This supports the hypothesis that the Bcl-2
family of proteins are involved in the activation of apoptosis during atresia of dominant
bovine follicles. Recent evidence supports the concept that the translocation of Bax, Bcl-
2, and Bcl-xL between the mitochondria and the cytosol is as critical as changes in their
expression [Pawlowski et al., 2000; Hu et al., 2001]. A shift of Bcl-2 and Bcl-xL protein
to the mitochondria, where they can heterodimerize with mitochondria-associated Bax,
allows them to potentially block Bax-induced apoptotic events. Future studies that
address the intracellular translocation of these proteins are needed to further elucidate the role of the Bcl-2 family in bovine granulosa cell apoptosis.

The activation of execution caspases, such as caspase-3, -8, and -9, indicates the "point of no return" in the apoptotic pathway. These proteins either directly or indirectly cleave a broad array of proteins necessary for cell survival, such as those involved in DNA maintenance and repair and organization of intermediate filaments [Tewari et al., 1995; Caulin et al., 1997]. In the current study, an increase in caspase activity was not observed in granulosa cells of dominant follicles during the period encompassing Days 4, 6, and 8 of the first follicular wave. However, the activity of caspase 8 decreased on Day 8 compared to Days 4 and 6. This could possibly be explained by decreased NF-κB activity observed on Day 8 (Chapter 5, Fig 5.3). The transcription factor NF-κB is capable of up-regulating the pro-apoptotic genes, Fas and Fas ligand [Barkett et al., 1999; Aggarwal, 2000]. Activation of the Fas pathway leads to the cleavage and activation of caspase 8 [Ashkenazi et al., 1998]. Caspases activate endonucleases which cleave DNA at specific internucleosomal intervals, giving DNA a characteristic ladder appearance on agarose gels [Liu et al., 1997]. This internucleosomal DNA fragmentation increased in granulosa cells during atresia of bovine dominant follicles induced by progesterone treatment [Yang et al., 2000b]. Most likely, the activation of execution caspases occurs later (i.e. Day 9 or 10) during the wave than the time points examined in this study. The fact that some dominant follicles of the first wave are capable of ovulating early during the plateau phase of growth supports the idea that not all granulosa cells have not committed to the apoptotic pathway at this point [Fortune et al., 1991]. However, the
tendency for translocation of phosphatidylserine residues and the changes in mRNA encoding members of the Bcl-2 family of proteins indicates that the apoptotic pathway has been initiated during Days 4 and 6 of the first follicular wave. Therefore it appears that this period encompasses a critical turning point in the developmental pathway of the non-ovulatory dominant follicle when a signal (or series of signals) is delivered that attenuates production of estradiol, initiates apoptosis in granulosa cells, and directs the follicle down an atretic pathway.

A number of mechanisms have been proposed to induce apoptosis in granulosa cells. These include binding of ligands such as tumor necrosis factor-\(\alpha\) and Fas ligand to their respective receptors [Kaipia et al., 1996; Porter et al., 2000], inhibition of cell-cell contact [Trolice et al., 1996], presence or absence of specific growth factors [Quirk et al., 2000], and altered levels of hormones such as estrogens and androgens [Billig et al., 1993]. In addition to these mechanisms, follicular apoptosis may be induced by oxidative stress [Tilly et al., 1995]. To begin to address the role of oxidative stress in bovine follicular atresia, we examined the temporal relationship between apoptosis in granulosa cells and expression of proteins responsible for preventing oxidative stress. In contrast to our original hypothesis that decreased expression of genes encoding oxidative stress response proteins would lead to accumulation of ROS and thus trigger apoptosis of granulosa cells, we observed an increase in expression of genes encoding oxidative stress response proteins on Day 8 of the follicular wave. However, theses levels of mRNA did not translate into increased expression of the corresponding proteins or greater enzyme activity. Because this increase in gene expression occurred after elevated numbers of
apoptotic and non-viable of granulosa cells were observed on Day 6, our results do not support the hypothesis that apoptosis is initiated by oxidative stress in the bovine follicle. Instead, the unexpected increase in expression of 3 oxidative stress response genes in non-ovulatory follicles on Day 8 of the wave leads us to consider the possibility that these enzymes may play a physiological role in later stages of atresia. While our study focused on measurement of oxidative stress response proteins, measurement of additional endpoints such as intrafollicular levels of ROS or evidence of oxidative stress-induced cellular damage (such as lipid peroxidation) would further delineate the relationship between oxidative stress and apoptosis in bovine follicles.

In contrast to the consistent pattern of increased oxidative stress response gene expression observed in granulosa cells, the only corresponding change observed in theca interna was a decrease in amounts of Cu/ZnSOD mRNA on Day 6 of the follicular wave. Although apoptosis is rarely observed in theca interna cells [Palumbo et al., 1994], we cannot rule out the possibility that this disruption of the oxidative stress response system in the thecal compartment may influence the atretic process.

Our data demonstrate that changes in expression of oxidative stress response genes are associated with atresia of dominant follicles, but the factors regulating these changes remain to be elucidated. Previous studies have implicated estrogen as an inhibitor of apoptosis [Billig et al., 1993; Pelzer et al., 2000], and we observed an inverse relationship between concentrations of estradiol in follicular fluid and incidence of apoptotic and non-viable granulosa cells. The drop in estrogen production during follicular atresia may also play a role in regulating expression of oxidative stress response
proteins. In sheep and goats, follicular MnSOD activity and estradiol content were inversely related [Singh et al., 1998]. In our study, decreased concentrations of estradiol in follicular fluid on Day 6 of the follicular wave preceded increased levels of mRNA encoding GSHPx, MnSOD, and Cu/ZnSOD on Day 8. These results provide evidence that estradiol may inhibit expression of oxidative stress response genes in the bovine follicle. In cardiac myocytes, inhibition of apoptosis by estradiol is associated with reduced activity of nuclear factor κB (NF-κB), a known stimulator of MnSOD gene transcription [Pelzer et al., 2000]. Activation of NF-κB is associated with apoptotic cell death in a variety of cell types [May et al., 1998]. Therefore, reduced exposure of granulosa cells to estradiol during follicular atresia may stimulate NF-κB activity resulting in increased granulosa cell apoptosis and increased expression of oxidative stress response genes.

In summary, decreased production of estradiol is accompanied by increased numbers of non-viable granulosa cells in dominant follicles between Days 4 and 6 of the first follicular wave in cattle. Also associated with the dominant follicle’s descent into atresia are alterations in the relative expression of genes encoding the Bcl-2 family of proteins and an increase in expression of mRNA encoding oxidative stress response proteins in granulosa cells. Results of this study expand our knowledge of the mechanisms involved in atresia of bovine dominant follicles, provide the groundwork needed to identify molecular factors that regulate expression of genes involved in apoptosis, and determine the contribution of these genes to atresia of the dominant follicle.
CHAPTER 4

CHANGES IN STEROIDOGENIC CAPACITY OF DOMINANT BOVINE FOLLICLES DURING THE 1st FOLLICULAR WAVE*

*This chapter has been submitted for publication in Domestic Animal Endocrinology:

Abstract

Atresia of bovine dominant follicles is characterized by an abrupt decrease in concentrations of estradiol in follicular fluid. The purpose of this study was to elucidate the mechanisms responsible for this decrease in estrogen production. Dominant follicles were collected from Holstein heifers on Day 4, 6, or 8 of the first follicular wave (n = 5/Day/experiment). Amounts of 17α-hydroxylase mRNA in theca interna were 7-fold higher (P < 0.01) on Day 4 compared to Day 8. After 3 h in culture, secretion of androstenedione by theca interna collected on Day 4 (236 ± 44 pg/µg protein) tended to be lower (P = 0.055) compared to Day 6 (517 ± 162 pg/µg protein) and was lower (P < 0.05) compared to Day 8 (387 ± 51 pg/µg protein). In granulosa cells, amounts of aromatase mRNA decreased (P < 0.05) between Days 6 and 8. In vitro secretion of estradiol was higher in granulosa cells collected on Day 4 (3.5 ± 0.8 ng/10⁵ cells) compared to Day 6 (1.8 ± 0.6 ng/10⁵ cells; P< 0.05) and tended to be higher on Day 4.
than on Day 8 (2.2 ± 0.2 ng/10^5 cells; \( P = 0.058 \)). We conclude that the decline in estradiol production observed during atresia of the dominant follicle is not due to lack of androgen substrate for aromatization or down-regulated expression of the aromatase gene, but may be the direct result of decreased activity of the aromatase enzyme within granulosa cells.
Introduction

Follicular development occurs in a pattern of waves throughout the bovine estrous cycle [Savio et al., 1988; Sirlois et al., 1988; Ginther et al., 1989]. The beginning, or emergence, of a wave is defined as the first day 2 or more growing follicles ≥ 4 mm in diameter are detected using ultrasonography [Ginther et al., 1989]. From the group of recruited follicles, 2-6 are selected to continue growing until one follicle deviates, develops at a faster rate than others of the cohort, and is designated the dominant follicle of the wave. Most estrous cycles are characterized by either 2 or 3 waves of follicular development. In 2-wave cycles, the days of wave emergence are typically days 2 and 11. If an animal exhibits 3 waves during the estrous cycle, waves typically initiate on days 2, 9, and 16 [Sirlois et al., 1988]. Regardless of the number of waves per estrous cycle, the first wave of follicular development following ovulation displays a consistent pattern of growth compared to the other waves, making it well suited for studying many aspects of follicular growth and atresia.

Because only the dominant follicle of the final wave of each estrous cycle successfully ovulates, all other dominant follicle(s) undergo atresia. The hallmark of the healthy dominant follicle is a high capacity to produce estradiol [Ireland, 1987]. Cessation of estradiol production is one of the earliest events of atresia, preceding apoptotic death of granulosa cells and decreased follicular diameter [Austin et al., 2001]. Concentrations of estradiol in follicular fluid have been characterized for 1st wave dominant follicles on Days 4, 6, and 8 of the first follicular wave [Xu et al., 1995a,b]. Decreased concentrations of estradiol in follicular fluid and altered follicular expression
of genes involved in steroidogenesis are observed as early as day 6 of the wave [Xu et al., 1995; Bao et al., 1997]. The factors responsible for this apparent loss of steroidogenesis are not clear. Because concentrations of estradiol in follicular fluid represent the pooled accumulation of estradiol secreted by the follicle from the time of its formation, they may not accurately reflect the steroidogenic capacities of the theca interna and granulosa cells at specific time points during the lifespan of the dominant follicle. Estradiol biosynthesis is dependent on the activity of 17α-hydroxylase in theca interna for production of androstenedione, and aromatase in the granulosa cell which converts the androgen into estradiol [Rodgers, 1990]. It is possible that concentrations of estradiol in follicular fluid decrease because theca interna cells stop producing adequate amounts of androgen for aromatization. Another possibility is that androgen supplies are adequate but aromatase activity in granulosa cells decreases. To determine which of these mechanisms mediates the decline in estradiol biosynthesis during atresia of dominant follicles, theca interna and granulosa cells were characterized for their ability to produce androstenedione and estradiol at 3 times during the first follicular wave following ovulation. The dynamic capacity of follicular cells to produce estradiol is critical to gaining a better understanding of how and when atresia is initiated in the non-ovulatory dominant follicle.
Materials and methods

Animals

All animal procedures were approved by the University of Arizona Care and Use Committee and were within guidelines established by the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Sexually mature Holstein heifers were housed in dry lots, fed high quality alfalfa hay and flaked corn with mineral supplement, and provided with constant access to water and shade. Experiments were conducted from October through August.

Ultrasonography and collection of follicles

Estrous cycles of heifers were synchronized by injection of 25 mg PGF$_{2\alpha}$ (Lutalyse, Pharmacia). Beginning on the day of estrus, blood samples were collected by coccygeal venipuncture and ultrasonographic examination of ovaries was performed daily as previously described [Turzillo et al., 1990] using a real-time B-mode linear array ultrasound scanner equipped with a 7.5 MHz intrarectal probe (Aloka SSD-550V Zug, Switzerland). Examinations were recorded on videotape (digital Handycam, Hi8™ Recording Tape, Sony Electronics Inc., Park Ridge, NJ). Ovulation was identified by the disappearance of a large follicle preceded by estrus. Following ovulation, diameters and positions of all follicles $\geq 4$ mm in diameter were analyzed. Day 1 of the follicular wave was defined as the day 2 or more follicles $\geq 4$ mm in diameter were first observed. These follicles continued to grow until 1 follicle deviated from the cohort to become dominant while other follicles in the wave regressed.
The ovary bearing the dominant follicle was surgically removed via flank incision on Day 4, 6, or 8 of the first follicular wave (n=5 per day). Ovaries were placed in dissection medium (1X MEM with Earle's salts and 25 mM HEPES, without L-glutamine; Life Technologies, Rockville, MD) and transported on ice 4 miles to the laboratory. The dominant follicle was dissected from the ovarian stroma. Follicular diameter was measured using calipers and follicular fluid was aspirated and stored at -20°C. The collapsed follicle was cut into 4 pieces. Theca interna, along with the basement membrane and granulosa cells, was separated from the theca externa and remaining stroma using fine forceps. Using an angled, finely pulled Pasteur pipette, granulosa cells were scraped from the basement membrane and theca interna. Granulosa cells were collected in dissection medium, centrifuged 15 min at 800 x g, resuspended in 1 ml fresh dissection medium, and counted with a hemocytometer. Granulosa cells were either repelleted and stored at -80°C until preparation of RNA or cultured as described below.

Template generation and cRNA probe synthesis

Complementary DNA (cDNA) encoding bovine aromatase and bovine 17α-hydroxylase was kindly provided by Dr. H.A. Garverick at the University of Missouri [Xu et al., 1995]. The 579-basepair (bp) cDNA encoding aromatase (corresponding to bp 166-746 of GenBank accession no. U18447) and a 407-bp BamHI fragment of the 17 α-hydroxylase cDNA (corresponding to bp 573-980 of GenBank accession no. M12547) were ligated into the pBluescript® SK plasmid (Stratagene, La Jolla, CA). A linear
antisense template encoding 18S ribosomal RNA (pTRI RNA 18S) was purchased from Ambion, Inc. (Austin, TX). The identity and orientation of all cDNAs were verified by dideoxy sequencing [Sanger et al., 1977].

To provide a shorter transcription product that could be used simultaneously with other cRNA probes for ribonuclease protection assay, the template for aromatase was linearized at sites within the cDNA insert using TaqI. The cDNA encoding 17α-hydroxylase was linearized at an XbaI site within the vector. Antisense [32P] UTP-labeled cRNA probes were transcribed from linearized cDNA templates using T7 polymerase and the MAXiscript™ In Vitro Transcription Kit (Ambion, Inc.) according to the manufacturer’s recommendations. Because of the high abundance of 18S ribosomal RNA, the 18S antisense riboprobe was generated at 1/10 the specific activity of the other riboprobes. Antisense cRNA probes were purified on a denaturing acrylamide gel and used for hybridization within 1 day.

Ribonuclease protection assays

Total RNA was extracted from granulosa cells and theca interna by the guanidinium isothiocyanate-phenol-chloroform extraction procedure [Chomczynski et al., 1987] using TRIZol® Reagent (Life Technologies). Optical densities at 260 and 280 nm were measured and used to determine the quantity and purity of RNA samples. Ribonuclease protection assays were carried out using the RPA III™ Ribonuclease Protection Assay Kit (Ambion, Inc.) according to the manufacturer’s recommendations. Assays for aromatase mRNA, 17α-hydroxylase mRNA, and 18S ribosomal RNA were
done simultaneously using 3 μg of RNA isolated from granulosa cells or theca interna. All hybridizations were carried out at 50°C for 15 h, followed by incubation with a 1:50 dilution of RNase A/TI cocktail (Ambion, Inc.) and resolution of protected fragments on a 5% acrylamide/8M urea gel. Following electrophoresis at 260 V for 2 h, gels were transferred to filter paper, covered with plastic wrap, and placed in an InstantImager Electronic Autoradiography System (Parkard Instrument Company, Meriden, CT) for 15 min to quantify size and relative abundance of all protected fragments. Gels were also exposed with 1 intensifying screen overnight to Hyperfilm MP autoradiography film (Amersham Pharmacia Biotech Inc., Piscataway, NJ) at -80°C.

**Theca cell culture**

All materials for culture of theca interna and granulosa cells were obtained from Life Technologies (Rockville, MD) unless otherwise noted. Theca interna from each follicle was cut into 63–85 pieces (based on the calculated surface area of the follicles, which ranged in diameter from 13.5 to 16 mm). Tissue was placed in 24-well plates (3 pieces per well) and cultured in 0.5 ml medium consisting of 1X MEM with Earle’s salts (without L-glutamine) supplemented with 50 μg/ml penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 5 μg/ml transferrin (Collaborative Biomedical Products, Bedford, MA), 1 μg/ml insulin, and 40 ng/ml cortisol (Steraloids, Inc., Newport, RI) at 37°C with 5% CO₂. Media were collected at 3, 6, 12, and 24 h of culture. Total protein was isolated from theca interna following the final collection of
media by homogenation in lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.35% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF) with a hand held Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). The lysate was centrifuged twice at 6610 g for 10 min at 4°C. Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Inc., Rockford, IL) according to the manufacturer’s recommendations.

*Granulosa cell culture*

Granulosa cells were cultured in 6-well plates (1 x 10^6 per well) in 3 ml medium per well. The medium for granulosa cell cultures was identical to that used for theca interna cultures, except that 1% fetal bovine serum was added in the presence or absence of 10^-7 M testosterone (Steraloids) as substrate for aromatase and estradiol production. Cells were incubated at 37°C with 5% CO₂. Media were collected at 3, 6, 12, and 24 h of culture. Estradiol secretion was shown as the difference in estradiol concentrations between samples incubated with and without testosterone and expressed as ng estradiol produced per 10^5 cells per 3 h.

*Hormone assays*

Concentrations of progesterone in serum were measured using the Coat-A-Count Progesterone radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) according to the manufacturer’s recommendations. This assay was previously validated
in our laboratory [Sanders et al., 2002]. Serum was not extracted prior to assay. Sensitivity of the assay, calculated as 2 standard deviations below the mean cpm at maximum binding, was 0.02 ng/ml. The intra-assay CV was 15.2%.

Concentrations of estradiol were measured in follicular fluid and media samples using the Double Antibody Estradiol radioimmunoassay (Diagnostic Products Corp.) according to the manufacturer's recommendations as previously validated for use in our laboratory [Sanders et al., 2002]. Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, was 1.5 pg/ml. The intra- and inter-assay CV were 5.91 and 9.5%, respectively. Production of estradiol is expressed as the difference in estradiol concentrations between samples incubated with and without testosterone in ng estradiol produced per $10^5$ granulosa cells of dominant follicles per 3 h.

Concentrations of androstenedione in media samples were measured using the Coat-A-Count Androstenedione radioimmunoassay (Diagnostic Products Corp.), according to the manufacturer's recommendations and previously validated for use in our laboratory [Sanders et al., 2002]. Sensitivity of the assay method was 0.05 ng/ml. The intra- and inter-assay CV were 3.51 and 5.55%, respectively. Androstenedione production is expressed as pg androstenedione produced per μg protein per 3h.
Statistical analyses

One-way and two-way analysis of variance (ANOVA) was used to analyze differences among groups. The Shapiro-Wilk Statistic and $F_{\text{max}}$ Test were utilized to assess normality and heterogeneity of variances, respectively. Means were separated by Duncan's Multiple Range Test; $P$-values $< 0.05$ were considered significant. All data are presented as mean $\pm$ SEM.
Results

Follicular characteristics and mRNA expression

Mean circulating concentrations of progesterone were undetectable on Day 0 and increased to 2.4 ± 2.0, 5.2 ± 1.5, and 7.7 ± 2.0 ng/ml on Days 4, 6, and 8 of the first follicular wave, respectively, indicating normal luteal function following ovulation. The first follicular wave was initiated 1.25 ± 0.11 (range = 1-2) days following ovulation, and in each heifer a single dominant follicle was selected from the recruited cohort. Follicular diameter and number of granulosa cells following dissection were similar among follicles collected on Days 4, 6, or 8 (Table 4.1).

Compared to follicles collected on Day 4 of the wave, concentrations of estradiol in follicular fluid decreased ($P < 0.01$) in follicles on Day 6, and remained low on Day 8 (Table 4.1). In theca interna, amounts of mRNA encoding 17α-hydroxylase were lower ($P < 0.05$) in Day 8 follicles compared to those collected on Day 4 (Table 4.1). Aromatase mRNA was not detectable in theca interna of any follicles (data not shown).

In granulosa cells, amounts of mRNA encoding aromatase decreased ($P < 0.05$) in follicles collected on Day 8 compared to those collected on Day 6. Detectable amounts of mRNA encoding 17α-hydroxylase were not observed in granulosa cells of any follicles (data not shown).

Androstenedione production

After 3 h of culture, androstenedione production tended to be lower ($P = 0.055$) in theca interna collected on Day 4 compared to Day 6, and was lower on Day 4 compared
to Day 8 ($P < 0.05$; Fig. 4.1). At 6, 12 and 24 h of culture, androstenedione production was lower ($P < 0.05$) in theca interna collected on Day 4 compared to Days 6 and 8.

In theca interna collected on Day 4, androstenedione production decreased ($P < 0.05$) between 3 and 6 h of culture and again between 6 and 12 h (Fig. 4.1). Amounts of androstenedione produced by theca interna collected on Day 4 were similar between the 12 and 24 h time points. In theca interna collected on Day 6, androstenedione production decreased between 3 and 6 h, 6 and 12 h, and 12 and 24 h ($P < 0.05$). Androstenedione secretion by theca interna collected on Day 8 was similar between 3 and 6 h, but decreased ($P < 0.05$) between 6 and 12 h and 12 and 24 h of culture.

**Estradiol production**

After 3 h in culture, estradiol secretion was higher in granulosa cells collected on Day 4 (3.5 ± 0.8 ng/10$^5$ cells) compared to Day 6 (1.8 ± 0.6 ng/10$^5$ cells; $P < 0.05$) and tended to be higher on Day 4 than on Day 8 (2.2 ± 0.2 ng/10$^5$ cells; $P = 0.058$; Fig. 4.2). At 6, 12, and 24 h of culture, estradiol secretion did not differ among follicles collected on Day 4, 6, and 8.

Secretion of estradiol by granulosa cells collected on Day 4 of the first follicular wave decreased ($P < 0.05$) between 3 and 12 h of culture, and between 6 and 24 h ($P < 0.05$). In granulosa cells from follicles collected on Day 6, estradiol secretion was similar at 3, 6, and 12 h, and did not fall until 24 h of culture ($P < 0.05$). Secretion of estradiol by granulosa cells collected from Day 8 follicles demonstrated a pattern similar to that of
granulosa cells collected on Day 4, decreasing ($P < 0.05$) between 3 and 12 h, and between 6 and 24 h (Fig. 4.2).
Table 4.1 Characteristics of dominant follicles collected during the first follicular wave.

<table>
<thead>
<tr>
<th>Day of wave</th>
<th>Follicular diameter (mm) (^a)</th>
<th>Granulosa cells recovered (x (10^6)) (^a)</th>
<th>Follicular Fluid estradiol-17β (ng/ml) (^a)</th>
<th>17α-OH mRNA in theca interna (^b)</th>
<th>Aromatase mRNA in granulosa cells (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>13.8 (0.4')</td>
<td>15.6 ± 2.5(^c)</td>
<td>320 ± 42(^c)</td>
<td>97 ± 37(^c)</td>
<td>194 ± 37(^c,d)</td>
</tr>
<tr>
<td>6</td>
<td>14.2 (0.7')</td>
<td>19.0 ± 4.0(^c)</td>
<td>98 ± 33(^d)</td>
<td>45 ± 25(^c,d)</td>
<td>264 ± 29(^c)</td>
</tr>
<tr>
<td>8</td>
<td>15.1 (0.6')</td>
<td>17.7 ± 3.6(^c)</td>
<td>83 ± 17(^d)</td>
<td>13 ± 4(^d)</td>
<td>138 ± 44(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Data are mean ± SEM.
\(^b\) Data are mean CPM ± SEM.
\(^c,d\) Values within each column with no common superscripts differ \((P < 0.05)\).
Figure 4.1 Secretion of androstenedione by theca interna of dominant follicles collected on Day 4, 6, or 8 following initiation of the 1st follicular wave expressed as pg androstenedione produced/μg protein/3 h. Data are mean ± SEM. At each culture time point, differences among Days 4, 6, and 8 are indicated by letters (P < 0.05). Differences across culture time points are indicated by numbers (P < 0.05).
Figure 4.2 Secretion of estradiol, defined as the difference in estradiol concentrations between samples incubated with and without testosterone, in granulosa cells of dominant follicles collected on Day 4, 6, or 8 following initiation of the 1st follicular wave. Means (± SEM) are expressed as ng estradiol produced/10^5 cells/3 h. At each culture time point, differences among Days 4, 6, and 8 are indicated by letters (P < 0.05). Differences across culture time points are indicated by numbers (P < 0.05).
Discussion

In dominant bovine follicles, cessation of estradiol production is one of the earliest events of follicular atresia, preceding apoptotic death of granulosa cells and reduced follicular diameter [Austin et al., 2001]. The purpose of this study was to characterize of the steroidogenic capacities of the individual follicular cell populations (i.e. granulosa and theca interna) in dominant follicles during the first follicular wave following ovulation to gain insight into the cellular events leading to a decline in estradiol production during atresia. In the present study steroidogenesis by both theca interna and granulosa cells decreased over the 24 h culture period. This is most likely due to de-differentiation of the cells as they remain in culture [Skinner et al., 1988; Roberts et al., 1990]. It is likely that the in vivo environment is most closely reflected by the earliest time point in vitro. Therefore, for the purposes of this discussion we will focus primarily on differences in steroidogenesis noted after 3 h of culture.

During steroidogenesis in bovine follicles, cholesterol is converted to pregnenolone by cytochrome P450 side-chain cleavage in theca interna. Pregnenolone is then converted to dehydroepiandrosterone by P450 17α-hydroxylase. Dehydroepiandrosterone is converted to androstenedione, which diffuses into granulosa cells and is used as substrate for estradiol synthesis by cytochrome P450 aromatase [Rodgers, 1990]. We found that in theca interna, mRNA encoding 17α-hydroxylase decreased by 50% between Days 4 and 6 of the first follicular wave. However, this decrease was variable, and was not significant until Day 8. Xu et al. [1995a] also reported a decrease in 17α-hydroxylase mRNA expression during the first wave using in...
situ hybridization, but noted a significant decrease earlier in the wave, between Days 4 and 6. Because concentrations of estradiol in follicular fluid, expression of P450 side-chain cleavage mRNA, and amounts of P450 17α-hydroxylase mRNA decreased between Days 4 and 6 in their studies, Bao et al. [1998] suggested that reduction in the ability of dominant follicles to produce estradiol on Day 6 is due to insufficient androgen production by thecal cells. However, despite the 50% decrease in 17α-hydroxylase mRNA on Day 6 in the present study, we found that production of androstenedione after 3 h in vitro was actually higher by theca interna of follicles collected on Day 6 compared to Day 4. Therefore the decline in follicular fluid estradiol between Days 4 and 6 of the wave observed in the present study and by Xu et al. [1995] does not appear to be caused by insufficient supply of androstenedione from theca interna.

Cytochrome P450 aromatase is the enzyme responsible for converting androstenedione to estradiol in granulosa cells of the bovine follicle. We found that amounts of mRNA encoding aromatase in granulosa cells of dominant follicles were similar on Days 4 and 6 of the first follicular wave, but decreased between Days 6 and 8. These results are consistent with Xu et al. [1995a] who reported similar changes in aromatase mRNA during the first wave using in situ hybridization. Because the fall in aromatase mRNA occurred after the drop in concentrations of follicular fluid estradiol on Day 6 in both studies, it appears that decreased expression of the aromatase gene is not the underlying cause of decreased estradiol production between Days 4 and 6. After 3 hours in culture, secretion of estradiol was lower in granulosa cells collected on Day 6 compared to those collected on Day 4. Therefore, despite high thecal production of
androstenedione and maintenance of aromatase mRNA, it appears that the activity of the aromatase enzyme declines between Days 4 and 6, leading us to conclude that this is the cause of the fall in follicular fluid estradiol observed by Day 6 of the follicular wave. Rhodes et al. [2001] observed a > 2-fold decline in concentrations of estradiol follicular fluid of dominant follicles between Days 3 and 5 after ovulation without a concomitant change in aromatase activity. However, it is important to note that dominant follicles in their study were still growing (4.7 mm larger diameter on Day 5 vs. Day 3) while the dominant follicles in the present study had already reached maximal diameter. This discrepancy is indicative of fundamental differences in follicular dynamics between the beef heifers used by Rhodes et al. [2001] and the dairy heifers in the present study. Badinga et al. [1992] reported that aromatase activity, defined in their studies as conversion of [1,2-\textsuperscript{3}H] testosterone into H\textsubscript{2}O and estrogens in follicle wall of first wave dominant follicles, did not differ between Days 5 and 8 of the estrous cycle, corresponding roughly to Days 4 and 7 of the wave respectively. However, the presence of theca interna in follicle wall complicates comparison of these results with those derived from isolated granulosa cells. Because thecal supply of androgen does not appear to be limited on Day 6 of the wave, we cannot rule out the possibility that in vivo, theca interna contributes some factor other than androgen that stimulates aromatase activity.

Loss of steroidogenic capacity precedes other signs of atresia, such as granulosa cell apoptosis or breakdown of the follicle wall, which lead to regression of the dominant follicle. Here, we identified the changes in granulosa cells that lead to the disruption of the steroidogenic pathway during atresia. Our findings indicate that decreased ability of
granulosa cells to produce estradiol precedes a loss in aromatase mRNA and is independent of thecal contribution of androstenedione. Therefore reduced secretion of estradiol into follicular fluid observed on Day 6 of the follicular wave is likely the direct result of decreased activity of the aromatase enzyme within the granulosa cells. Future studies are needed to elucidate the cause of the loss of aromatase activity in granulosa cells and thus provide insight into mechanisms that initiate the early stages of follicular atresia.
CHAPTER 5

REGULATION OF NUCLEAR FACTOR-κB (NF-κB) ACTIVITY AND APOPTOSIS BY ESTRADIOL IN BOVINE GRANULOSA CELLS*

This chapter has been submitted for publication in Molecular and Cellular Endocrinology:


Abstract

Although atresia of bovine follicles is associated with apoptosis of granulosa cells, the signals initiating this cell death have not been resolved. The ubiquitous transcription factor NF-κB has been implicated as an important regulator of genes controlling apoptosis, and previous studies indicate that estradiol may modulate NF-κB activation. Since production of estradiol is the hallmark of healthy follicles, we hypothesized that estradiol activates NF-κB and thus inhibits apoptosis in granulosa cells of dominant follicles. Dominant follicles were collected on Day 4, 6, and 8 of the first follicular wave following ovulation and assayed for NF-κB activity. Concentrations of estradiol in follicular fluid decreased \( (P < 0.05) \) on Day 6 compared to Day 4 of the wave. Amounts of active NF-κB found in the nucleus of granulosa cells were lower \( (P < 0.05) \) on Day 8 compared to Days 4 and 6. In vitro, NF-κB activity and minimal incidence of apoptosis (< 4%), as measured by annexin V and TUNEL assays, were
associated with production of estradiol. However, pharmacological inhibition of NF-κB 

in vitro led to a suppression of apoptosis. These results indicate that follicular NF-κB 

activation is associated with estradiol production. However, whether NF-κB is playing a 

pro- or anti-apoptotic role in granulosa cells of the dominant bovine follicle remains to be 

elucidated.
Introduction

Atresia is the physiological fate of greater than 99% of all ovarian follicles. In the bovine ovary, apoptosis of granulosa cells is an early event in follicular atresia [Van Wezel et al., 1999; Yang et al., 2000a]. Apoptosis may be initiated in granulosa cells by atretogenic factors [i.e. TNFα and Fas ligand; Kaipia et al., 1996; Porter et al., 2000], and/or withdrawal of trophic stimuli [i.e. steroids and growth factors; Billig et al., 1993; Quirk et al, 2000]. These extracellular signals are presumed to affect the relative expression of pro- vs. anti-apoptotic genes and thus determine granulosa cell fate. The ubiquitous transcription factor NF-κB has also been implicated as an important regulator of genes involved in apoptosis. In the unactivated state, NF-κB is sequestered as heterodimers or homodimers in the cytosol bound to inhibitory IκB proteins [Karin et al., 2000; Bauerle et al., 1996]. The interaction between NF-κB and IκB masks the nuclear localization sequence of NF-κB and interferes with sequences important for DNA binding [Chen et al., 1998; Cramer et al., 1997; Ghosh et al., 1995]. In response to one of over 150 extracellular signals, IκB kinase (IKK) is activated, leading to phosphorylation of specific serine residues on IκB proteins [Karin et al., 2000; Ghosh et al., 1998]. A cascade of phosphorylation, ubiquitination, and degradation of IκB un_masks the nuclear localization signal of NF-κB, leading to its translocation from the cytosol to the nucleus where it binds to the promoters and enhancers of numerous target genes [Karin et al., 2000; Read et al., 2000].
While NF-κB was initially considered a pro-apoptotic factor, due to its involvement in the expression of some apoptotic genes such as Fas and Fas ligand, more recent work supports the role of NF-κB as an anti-apoptotic factor [Chen et al., 2001]. Studies of NF-κB in the ovary have been limited. However, there is evidence that NF-κB could be involved in the attenuation of apoptosis in the follicle. Through increased expression of the anti-apoptotic proteins X-linked inhibitor of apoptosis protein (Xiap) and Flice-like inhibitory protein (FLIP), NF-κB prevented tumor necrosis factor α-induced apoptosis in rat granulosa cells [Xiao et al., 2001; Xiao et al., 2002]. The same researchers demonstrated that treatment of rat granulosa cells with FSH activated NF-κB, stimulated XIAP expression, and inhibited apoptosis; and that FSH-stimulated growth of follicles in vitro was suppressed by inhibition of NF-κB activation [Wang et al., 2002]. To our knowledge, however, the role of NF-κB in apoptosis in the bovine follicle has not been examined.

Studies have indicated that estradiol-17β may modulate NF-κB activation [Caulin-Glaser et al., 1996; Harnish et al., 2000; Pelzer et al., 2000; Pelzer et al., 2001]. The hallmark of the healthy bovine dominant follicle is a high capacity to produce estradiol [Ireland, 1987]. Cessation of estradiol production is one of the earliest events of atresia, preceding apoptotic death of granulosa cells [Austin et al., 2001]. We hypothesize that estradiol-17β stimulates follicular activation of NF-κB, thus inhibiting apoptosis in granulosa cells of healthy dominant follicles. To address this hypothesis, we used bovine follicles collected during the first follicular wave following ovulation. This wave of follicular development displays a consistent pattern of growth compared to the
other waves, making it well suited for studying many aspects of follicular growth and atresia.
Materials and Methods

Animals

All animal protocols were approved by the University of Arizona Use and Care Committee and were within guidelines established by the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching [1999]. Sexually mature Holstein heifers were housed in dry lots, fed high quality alfalfa hay and flaked corn with mineral supplement, and provided with constant access to water and shade. Experiments were conducted from October to April.

Experiment 1: Apoptosis, gene expression, and NF-κB activity in vivo

Ultrasonography and Collection of Follicles

Follicular development was monitored daily via ultrasonography as previously described [Turzillo et al., 1990] using a real-time B-mode linear array ultrasound scanner equipped with a 7.5 MHz intrarectal probe (Aloka SSD-550V Zug, Switzerland). Examinations were recorded on videotape (digital Handycam, Hi8™ Recording Tape, Sony Electronics Inc., Park Ridge, NJ). The presence or absence of a corpus luteum was determined upon initial ultrasonographic examination. Animals with a CL received (i.m.) 25 mg prostaglandin F2α (PGF2α; Lutalyse, Pharmacia & Upjohn, Peapack, NJ) to induce luteolysis and a new follicular wave. Ovulation was confirmed by the
disappearance of a large follicle followed by development of a new follicular wave. Following ovulation, diameters and positions of all follicles ≥ 4 mm in diameter were analyzed. Day 1 of the follicular wave was defined as the day 2 or more follicles ≥ 4 mm in diameter were first observed. These follicles continued to grow until 1 follicle deviated from the cohort to become dominant while other follicles in the wave regressed.

The ovary bearing the dominant follicle was surgically removed via flank incision on Day 4, 6, or 8 of the first follicular wave (n=5 per day). Ovaries were placed in dissection medium (1X MEM with Earle’s salts and 25 mM HEPES, without L-glutamine; Life Technologies, Rockville, MD), and transported immediately on ice to the laboratory. The dominant follicle was dissected from the ovarian stroma. Follicular diameter was measured using calipers and follicular fluid was aspirated and stored at −20°C. The collapsed follicle was cut into 4 pieces. Theca interna, along with the basement membrane and granulosa cells, was separated from the theca externa and remaining stroma using fine forceps. Using an angled, finely pulled Pasteur pipette, granulosa cells were scraped from the basement membrane and theca interna. Granulosa cells were collected in dissection medium, centrifuged 15 min at 800 x g, resuspended in 1 ml fresh dissection medium, and counted with a hemocytometer. A small portion of granulosa cells from each follicle (100,000) was set aside for annexin and propidium iodide (PI) staining while the remaining cells were repelleted for isolation of nuclear extract.
Annexin V and Propidium Iodide (PI) Staining of Fresh Cells

Early-stage apoptosis of granulosa cells was assessed by staining with annexin V using the Vybrant™ Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR) according to the manufacturer’s recommendations with modifications. Annexin V binds phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane early in apoptosis. To distinguish intact, apoptotic cells from cells lacking intact plasma membranes (non-viable), PI is used to stain double-stranded nucleic acids. Granulosa cells (approximately 100,000) were washed in cold PBS, centrifuged at 800 × g for 15 min, and resuspended in 100 μl of 1X Annexin-Binding Buffer. Alexa Fluor 488 annexin V (5 μl) and PI (1 μl of a 100 μg/ml working solution) were added and cells were incubated at room temperature for 15 min. Cells were then spread on a glass slide, coverslipped, and viewed with an Olympus BX50WI fluorescence microscope using a FITC filter. At least 800 granulosa cells per follicle were observed. Numbers of cells that stained for annexin V exclusively or annexin V and PI were recorded.

Preparation of Nuclear Extract

Granulosa cells were washed in 1 ml TBS. Cells were centrifuged again (15 min; 800 × g) and resuspended in freshly prepared cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1.0 mM DTT, 0.5 mM PMSF; 400 μl per 1 million cells). Cells were allowed to swell on ice for 15 min. After addition of 10%
Nonidet NP-40 (Fluka Biochemika, Buchs, Switzerland; 25 µl per 1 million cells), cells were vigorously vortexed for 10 sec and centrifuged 30 sec at 10,000 x g. Supernatant containing cytoplasm and RNA was removed. The pellet containing nuclear extract was resuspended in freshly prepared ice-cold buffer C (20 mM HEPES pH 7.9, 0.4 NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF; 50 µl per 1 million cells). Samples were placed on a shaking platform for 15 min at 4°C, and then centrifuged (5 min, 10,000 x g, 4°C). Supernatant was divided into aliquots and stored at –80°C.

Electrophoretic mobility shift assay (EMSA)

Double-stranded DNA oligonucleotides (3.5 pmol) containing consensus sequences for the NF-κB binding site were labeled with 2.0 µl [γ-32P]-ATP (3000 Ci/mmol at 5 mCi/ml) and 1.0 µl T4 polynucleotide kinase at 37°C for 10 min, followed by the addition of 1.0 µl 0.5 M EDTA and 39 µl TE buffer. Labeled probes were purified on G-25 quickspin columns (Roche Pharmaceuticals, Nutley, New Jersey). Nuclear extract (5 µg) was incubated 10 min at room temperature in binding buffer (5 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 2.5mM DTT, 10% glycerol, 0.5% NP-40) before addition of 2 µl labeled consensus sequence and 30 min incubation at room temperature. Nuclear acid-protein complexes were resolved on a 4% acrylamide gel in 0.5X TBE. Gels were dried 2 hrs at 80°C before detection of bands by autoradiography.
Hormone Assays

Concentrations of progesterone were measured in serum using the Coat-A-Count Progesterone radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA) according to the manufacturer’s recommendations following validation in our laboratory [Sanders et al., 2002]. The progesterone antiserum exhibits extremely low cross-reactivity with related steroids (androstenedione and estradiol, not detectable; pregnenolone and testosterone, 0.1%). Serum was not extracted prior to assay. Sensitivity of the assay, calculated as 2 standard deviations below the mean cpm at maximum binding, is 0.02 ng/ml. The intra-assay CV was 15.2%.

Concentrations of estradiol-17β were measured in follicular fluid using the Double Antibody Estradiol radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) according to the manufacturer's recommendations and validated for use in our laboratory [Sanders et al., 2002]. The estradiol antiserum exhibits extremely low cross-reactivity with related steroids (androstenedione, 0.004%; estriol, 0.235%; progesterone and testosterone, not detectable). Sensitivity of the assay, calculated as two standard deviations below the mean CPM at maximum binding, is 1.5 pg/ml. The intra- and inter-assay CV were 5.91 and 9.5%, respectively.
Experiment 2: Apoptosis in vitro

Granulosa cell culture

Dominant follicles were collected from Holstein heifers on Day 4, 6, or 8 of the first follicular wave \((n=5/\text{Day})\) as described above. Follicular fluid was aspirated and stored at \(-20^\circ\text{C}\). Granulosa cells were cultured in 6-well plates \((1 \times 10^6 \text{ per well})\) in 3 ml base medium consisting of 1X MEM with Earle’s salts (without L-glutamine) supplemented with 50 \(\mu\text{g/ml}\) penicillin/streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 5 \(\mu\text{g/ml}\) transferrin (Collaborative Biomedical Products, Bedford, MA), 1 \(\mu\text{g/ml}\) insulin, 40 ng/ml cortisol (Steraloids, Inc., Newport, RI), and 1\% fetal bovine serum (Invitrogen Corporation) at \(37^\circ\text{C}\) with 5\% CO\(_2\). Cells were cultured in the presence or absence of \(10^{-7} \text{ M}\) testosterone as substrate for aromatase and estradiol production. Granulosa cells were also cultured in 8-well chamber slides \((400,000 \text{ per well})\) in the presence or absence of testosterone. Media were collected after 3 h of culture and assayed for estradiol as described previously by our laboratory [Sanders et al., 2002]. After 24 h, cells cultured in 6-well plates were collected, nuclear extract was prepared, and EMSA was carried out as described above. Cells cultured on chamber slides were used for annexin V and TUNEL assays.
Annexin V and propidium iodide (PI) staining of cultured cells

After 24 h, granulosa cells cultured in duplicate in chamber slides were assessed for early-stage apoptosis by staining with annexin V using the Vybrant™ Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR), according to the manufacturer’s recommendations, and fixed with acetone. Cells were viewed with an Olympus BX50WI fluorescence microscope using a FITC filter. At least 400 granulosa cells per follicle were observed. Numbers of cells that stained for annexin V exclusively or annexin V and PI were recorded.

In Situ 3' End Labeling (TUNEL)

After 24 h, granulosa cells cultured in duplicate in 8-well chamber slides were fixed with paraformadehyde, rinsed twice with PBS, and late-stage apoptosis was assessed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). All slides were incubated with TUNEL reaction mixture for 60 min at 37°C in a humidified chamber. Slides were viewed with a Olympus BX50WI fluorescence microscope using a FITC filter. At least 400 granulosa cells per follicle were observed.

Experiment 3: NF-κB activity in vitro
To further elucidate the role of NF-κB on apoptosis of granulosa cells in vitro, an inhibitor of NF-κB activity was added to culture media. The peptide SN50 (BIOMOL International, Plymouth Meeting, PA) contains the nuclear localization sequence of NF-κB p50 (residues 360-369) linked to a cell-permeabilization sequence (the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor) which inhibits translocation of the NF-κB active complex into the nucleus. Granulosa cells were also cultured with the inactive control peptide SM50, which also contains the nuclear localization sequence of the transcription factor NF-κB p50 linked to the hydrophobic of the signal peptide of Kaposi fibroblast growth factor, but has amino acid substitutions (Lys-363 to Asn and Arg-364 to Gly), and thus has no measurable effect on NF-κB activity. In a preliminary experiment, the dose of SN50 which effectively inhibited NF-κB activation was determined by incubating granulosa cells from follicles collected on Day 4 of the first follicular wave in 6-well plates as described above with 50, 100, 200, 300, or 400 μg/ml SN50. Nuclear extract was prepared and NF-κB activity was assessed by EMSA as described above. Maximal inhibition of NF-κB activity (81%) was obtained at concentrations of SN50 ≥ 200 μg/ml (Fig 5.9A).

For experiment 3, dominant follicles were collected on Day 4 of the first follicular wave (n=5) as described above. Follicular fluid was aspirated and stored at -20°C. Granulosa cells were cultured in 6-well plates and chamber slides as described above in base medium without testosterone, base medium with 10^{-7} M testosterone, base medium with 10^{-7} M testosterone and 200 μg/ml SN50, or base medium with 10^{-7} M testosterone
and 200 μg/ml SM50. Media were collected after 3 h of culture and assayed for estradiol. Nuclear protein was prepared after 24 h from cells cultured in 6-well plates. NF-κB activation was analyzed by EMSA as described above. Apoptosis was assessed in cells cultured in chamber slides using assays for Annexin V and TUNEL as described above.

**Statistical analyses**

One-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS (SAS) was used to analyze differences among groups. Means were separated by Duncan's Multiple Range Test; P-values <0.05 were considered significant. The Shapiro-Wilk Statistic and $F_{\text{max}}$ Test were utilized to assess normality and heterogeneity of variances, respectively. Dixon's test was used to identify outliers. All data are presented as mean ± SEM.
Results

Experiment 1: Apoptosis and NF-κB activity in vivo

Follicular characteristics

In all heifers, circulating concentrations of progesterone were undetectable on the day of estrus and increased to levels of 1.8 ± 0.1, 3.0 ± 0.4, and 4.2 ± 0.9 ng/ml on Days 4, 6, and 8 of the first follicular wave, respectively, indicating normal luteal function following ovulation. The first follicular wave was initiated 1.1 ± 0.3 (range = 1-2) days after ovulation, and in each heifer a single follicle deviated from the recruited cohort to become the dominant follicle. Follicular diameter following dissection tended to increase (P = 0.06) in dominant follicles collected on Day 8 compared to Day 4, while the number of granulosa cells recovered remained constant among Days 4, 6, and 8 (Table 5.1). Concentrations of estradiol in follicular fluid were higher (P < 0.01) on Day 4 compared to Days 6 and 8 (Table 5.1).

Annexin V staining

The percentage of granulosa cells in early stages of apoptosis (stained with annexin V alone) did not differ among follicles collected on Days 4, 6, or 8 (Fig. 5.1). The percentage of non-viable granulosa cells (stained for both annexin V and PI) was also similar among follicles collected on Day 4, 6, or 8 (Fig. 5.1).
NF-κB activity

Amounts of active NF-κB found in the nucleus of granulosa cells of dominant follicles collected on Day 8 of the first follicular wave were lower ($P < 0.05$) compared to Days 4 and 6 (Fig. 5.2).

Experiment 2: Apoptosis in vitro

Estradiol production

Compared to cells cultured without testosterone, granulosa cells produced higher amounts of estradiol ($P < 0.05$) when cultured with testosterone (Fig. 5.3A). After 3 h of culture, granulosa cells collected from follicles on Day 4 of the first follicular wave produced higher ($P < 0.05$) amounts of estradiol compared to granulosa cells from Day 6 and 8 follicles when cultured in the presence of testosterone (Fig. 5.3A). When cultured without testosterone, granulosa cells collected on Days 4, 6, and 8 follicles produced similar amounts of estradiol (Fig. 5.3A).

NF-κB activity

In the presence of testosterone in vitro, NF-κB activity was higher ($P < 0.05$) in granulosa cells collected from follicles on Day 4 of the first follicular wave compared to granulosa cells collected on Days 6 or 8 (Fig. 5.3B). When cultured without testosterone, granulosa cells from follicles collected on Days 4, 6, and 8 demonstrated similar amounts of activated NF-κB (Fig. 5.3B). Granulosa cells from follicles collected on Day 4 had higher ($P < 0.05$) amounts of activated NF-κB when cultured with testosterone (Fig.
5.3B). In contrast, the presence of testosterone did not affect activation of NF-κB in granulosa cells collected on Day 6 or 8 of the first follicular wave (Fig. 5.3B).

**Annexin V staining**

One follicle collected on Day 6 and cultured with testosterone was excluded after identification as an outlier. In the presence of testosterone, the percentage of granulosa cells in early stages of apoptosis (stained with annexin V) was higher \( (P \leq 0.05) \) in cells from follicles collected on Day 6 compared to Days 4 and 8 of the first follicular wave (Fig. 5.4A). When cultured without testosterone, granulosa cells obtained from follicles on Day 4 demonstrated less \( (P < 0.05) \) early-stage apoptosis than those from follicles collected on Day 6 of the first follicular wave, while the percentage of early-stage apoptotic granulosa cells from follicles collected on Day 8 was intermediate (Fig. 5.4A). Within each day of follicle collection, the presence of testosterone did not influence the percentage of early-stage apoptotic granulosa cells (Fig. 5.4A).

**TUNEL**

In the presence of testosterone, the percentage of granulosa cells in late stages of apoptosis (TUNEL positive) did not differ among cells from follicles collected on Day 4, 6, or 8 of the first follicular wave (Fig. 5.4B). When cultured without testosterone, the incidence of late-stage apoptosis in granulosa cells from follicles collected on Days 4 and 8 was lower \( (P < 0.05) \) than in cells from follicles collected on Day 6 of the first follicular wave (Fig. 5.4B). Within Day, the presence of testosterone decreased \( (P < 0.05) \) the
incidence of late apoptosis in granulosa cells from follicles collected on Days 4 and 6, while testosterone did not influence the percentage of granulosa cells in early apoptosis from follicles collected on Day 8 of the first follicular wave (Fig. 5.4B).

Experiment 3: NF-κB activity in vitro

NF-κB activity

The addition of SM50 (200 μg/ml) to culture media containing testosterone did not affect NF-κB activity compared to culture with testosterone alone (data not shown). However, the addition of SN50 (200 μg/ml) to culture media containing testosterone inhibited NF-κB activity by 81% compared to SM50 (Fig. 5.5B) or testosterone alone (data not shown).

Estradiol production

After 3 h of culture, granulosa cells from follicles collected on Day 4 produced higher ($P < 0.05$) amounts of estradiol when cultured with testosterone than when cultured in its absence (Fig. 5.5). The addition of 200 μg/ml SM50 or SN50 to granulosa cells cultured with testosterone did not influence the production of estradiol (Fig. 5.5).

Annexin V staining

After 24 h of culture, the percentage of granulosa cells in early stages of apoptosis (stained with annexin V) was higher ($P < 0.05$) in cells cultured without testosterone compared to those cultured with testosterone alone, testosterone and SM50, or
testosterone and SN50 (Fig. 5.6A). Addition of SN50 to culture media caused the percentage of early apoptotic granulosa cells to decrease ($P < 0.05$) compared to granulosa cells cultured with testosterone and SM50 or testosterone alone (Fig. 5.6A).

**TUNEL**

After 24 h of culture, the percentage of granulosa cells in late stages of apoptosis (stained with TUNEL) was higher ($P < 0.05$) in cells cultured without testosterone compared to those cultured with testosterone alone, testosterone and SM50, or testosterone and SN50 (Fig. 5.6B). The addition of SN50 to culture media caused the percentage of late apoptotic granulosa cells to decrease ($P < 0.05$) compared to granulosa cells cultured with either testosterone and SM50 or testosterone alone (Fig. 5.6B).
Table 5.1 Characteristics of dominant follicles collected during the first follicular wave.\(^a\)

<table>
<thead>
<tr>
<th>Day of wave</th>
<th>Follicular diameter (mm)</th>
<th>Granulosa cells recovered (x 10^6)</th>
<th>Follicular fluid estradiol (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>14.7 ± 1.7(^b)</td>
<td>15.5 ± 2.2(^d)</td>
<td>261 ± 50(^d)</td>
</tr>
<tr>
<td>6</td>
<td>16.0 ± 0.7(^b,c)</td>
<td>16.0 ± 2.0(^d)</td>
<td>58.9 ± 18(^e)</td>
</tr>
<tr>
<td>8</td>
<td>18.7 ± 0.9(^c)</td>
<td>19.5 ± 2.8(^d)</td>
<td>16.9 ± 5(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Data are means ± SEM.

\(^{b,c}\) Values within each column without common superscripts tend to differ (\(P = 0.06\)).

\(^{d,e}\) Values within each column without common superscripts differ (\(P < 0.05\)).
Figure 5.1 Percentage of granulosa cells in dominant follicles collected on Day 4, 6, or 8 of the first follicular wave stained with either annexin V alone (indicative of early apoptosis) or annexin V and PI (indicative of non-viability). Data are mean ± SEM. There were no significant differences among days within staining category ($P > 0.05$).
Figure 5.2 Relative amounts of activated NF-κB in the nucleus of granulosa cells collected from dominant follicles of the first follicular wave following ovulation. Data are mean ± SEM. Letters indicate significant differences among days ($P < 0.05$).
Figure 5.3 Concentrations of estradiol in media of granulosa cells after 3 h (A.) and NF-κB activity as measured by EMSA in nuclear protein from granulosa cells after 24 h (B.) of culture with or without substrate for aromatase (testosterone; T). Cells were obtained from follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. Letters indicate significant differences among days within treatment ($P < 0.05$). Numbers indicate significant differences between treatments for each Day ($P < 0.05$).
Figure 5.4 Percentage of granulosa cells in early-stage (stained with Annexin V; A.) and late-stage (TUNEL positive; B.) apoptosis following 24 h of culture in the presence or absence of testosterone (T). Cells were from follicles collected on Day 4, 6, or 8 of the first follicular wave. Data are mean ± SEM. Letters A and B indicate significant differences among days within treatment ($P < 0.05$). There were no significant differences in Annexin V staining between treatments for any Day ($P > 0.05$). Numbers indicate significant differences in the percentage of TUNEL positive cells between treatments for each Day ($P < 0.05$).
Figure 5.5  

A. EMSA showing shifted bands of activated NF-κB in the nucleus of granulosa cells cultured with 50, 100, 200, 300, or 400 μg/ml SM50 or SN50.  

B. Quantification of the inhibitory effect of doses of SN50 on NF-κB activation compared to culture with SM50 at similar concentrations. Maximal inhibition (81%) was obtained at doses ≥ 200 μg/ml SN50.
Figure 5.6 Concentrations of estradiol in media after culture of granulosa cells for 3 h without or with testosterone or with testosterone (T) and SM50 or SN50 (200 µg/ml). Cells were from follicles collected on Day 4 of the first follicular wave. Data are mean ± SEM. Letters indicate significant differences among treatments ($P < 0.05$).
Figure 5.7 Percentage of granulosa cells in early-stage (stained with Annexin V; A.) or late-stage (TUNEL positive; B.) apoptosis following 24 h of culture without testosterone (T), with T alone, or with T and SM50 or SN50 (200 μg/ml). Cells were from follicles collected on Day 4 of the first follicular wave. Data are mean ± SEM. Letters indicate significant differences among treatments ($P < 0.05$).
Discussion

In dominant bovine follicles, apoptotic death of granulosa cells is an early event in follicular atresia, but is preceded by cessation of estradiol production [Van Wezel et al., 1999; Yang et al., 2000a; Austin et al., 2001]. The mechanistic relationship between this loss of steroidogenesis and the initiation of apoptosis is unknown. Previous work has indicated that the apoptotic pathway may be initiated between Days 4 and 6 of the first follicular wave [Xu et al., 1995a,b]. Therefore it appears that this period encompasses a critical turning point in the developmental pathway of the non-ovulatory dominant follicle when a signal (or series of signals) is delivered that attenuates production of estradiol, initiates apoptosis in granulosa cells, and directs the follicle down an atretic pathway. Because the ubiquitous transcription factor NF-κB is a key mediator of pro- and anti-apoptotic gene expression, the purpose of this study was to characterize the role of NF-κB in the initiation of apoptosis of granulosa cells of dominant follicles during the first follicular wave following ovulation.

As reported previously [Xu et al., 1995a,b], concentrations of estradiol in follicular fluid of dominant follicles declined on Day 6 of the first follicular wave and remained low on Day 8. As observed in our previous studies (Chapter 3, Fig 3.1) the change in the percentage of granulosa cells in early stages of apoptosis (stained with annexin V alone) demonstrated a trend to increase on Day 6 of the first follicular wave. These data indicate that apoptosis has been initiated over the period encompassing Day 4 and 6 of the first follicular wave. Amounts of active NF-κB found in the nucleus of
granulosa cells of dominant follicles declined on Day 8 of the first follicular wave compared to Days 4 and 6. This supports the hypothesis that NF-κB plays an anti-apoptotic role in granulosa cells of dominant bovine follicles.

As dominant follicles of the first follicular wave undergo atresia in vivo, activation of NF-κB by granulosa cells is inhibited. This inhibition is preceded by a decline in follicular fluid levels of estradiol. Recent studies have indicated that estradiol-17β may modulate NF-κB activation [Caulin-Glaser et al., 1996; Harnish et al., 2000; Pelzer et al., 2000; Pelzer et al., 2001]. To directly address the role of estradiol in NF-κB activation, granulosa cells from Day 4, 6, and 8 dominant follicles were cultured in vitro in the presence or absence of testosterone as a substrate for estradiol production. Production of high amounts of estradiol was associated with higher amounts of NF-κB activity and fewer cells in early apoptosis. These data support the hypothesis that estradiol influences NF-κB activity and prevents apoptosis in granulosa cells of dominant bovine follicles.

To directly determine the effect of NF-κB on granulosa cell apoptosis, an inhibitor of NF-κB activation was added to culture media in Experiment 3. The addition of this inhibitor did not affect estradiol production when testosterone was provided. However, the addition of the NF-κB inhibitor significantly decreased the incidence of apoptosis after 24 h of culture compared to controls. This would suggest that, in vitro, NF-κB activation is necessary for induction of apoptosis of granulosa cells. Although this contradicts our hypothesis, it is an intriguing finding. NF-κB is capable of affecting
the transcription of both pro- and anti-apoptotic genes. The pro-apoptotic genes, Fas and Fas ligand (FasL) are up-regulated by NF-κB activation [Barkett et al., 1999; Aggarwal, 2000]. There is evidence that the Fas pathway is involved in the initiation and progression of atresia in dominant bovine follicles. Porter et al. [2001] demonstrated that FasL mRNA is higher in dominant bovine follicles collected on Day 11 compared to Day 5 of the estrous cycle (these days compare roughly to Day 10 and Day 4 of the wave). It has also been shown that apoptosis of bovine granulosa cells induced by serum withdrawal in vitro is mediated partially by the Fas pathway [Quirk et al., 2000]. Therefore, it is possible that inhibition of NF-κB activity in vitro blocked apoptosis induced via the Fas pathway. Measurement of the relative changes in Fas and FasL mRNA expression following addition of the NF-κB inhibitor would address this question.

In rat granulosa cells, expression of the gene encoding anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP) has been shown to be up-regulated as a result of TNFα-induced NF-κB activation in vitro. In fact, addition of the NF-κB inhibitor SN50 resulted in suppression of FSH stimulated NF-κB-DNA binding, XIAP expression, and follicular growth [Wang et al., 2002]. The current experiments were conducted without the addition of FSH to culture media. Whether NF-κB is affecting transcription of XIAP in the current experiments is unknown and should be examined in the future.

In conclusion, our findings provide evidence that 1) estradiol influences NF-κB activity and 2) NF-κB is involved in apoptosis of granulosa cells. However, whether this role of NF-κB is pro- or anti-apoptotic remains to be established. Future experiments
should be conducted to identify genes that are regulated by NF-κB in granulosa cells of the bovine dominant follicle.
CHAPTER 6

CONCLUSIONS

Atresia is by far the most common physiological fate of ovarian follicles, and efforts to elucidate the cellular and molecular regulation of this phenomenon are of critical importance to ovarian physiology. Reproductive failure is the leading cause of financial loss to livestock producers, costing U.S. beef and dairy producers $386 million annually [Bellows et al., 2002]. Control and manipulation of the estrous cycle is crucial to successfully impregnation in a time-effective manner. Identification of factors that initiate atresia could lead to improved methods to either prevent atresia or induce regression of persistent, non-ovulatory follicles that have escaped atresia (i.e. cysts). A better understanding of the events controlling follicular atresia can lead to improved pregnancy rates in dairy and beef cattle, and increased profits.

Experiments conducted using the bovine ovary also are relevant to human reproduction, where ovarian disorders account for 40% of infertility cases in women 15-44 years of age [Abma et al., 1997]. Women and cattle have similar reproductive cycle lengths (28 and 21 day, respectively). In addition, like the human, the cow is a monovulatory species [Gore et al., 1995]. Finally, both cattle and women exhibit wave-like patterns of follicular development and atresia during the reproductive cycle. Information obtained from studies using the cow could bring insight to treatments for disorders caused by abnormal atresia in women such as cystic conditions or premature ovarian failure.
Atresia is accompanied by apoptosis of granulosa cells [Jolly et al., 1994; Van Wezel et al., 1999; Yang et al., 2000a]. However, the factors controlling the initiation of apoptosis in dominant bovine follicles have not been elucidated. My overall hypotheses were that oxidative stress is involved in the atresia of non-ovulatory dominant bovine follicles, and NF-κB is involved in preventing apoptosis in the healthy dominant follicle. Previous studies have demonstrated that oxidative stress may have a role in initiating apoptosis [Tilly et al., 1995]. Therefore, my first specific aim was to determine if expression of oxidative stress response proteins in dominant bovine follicles is related to follicular health. There were no differences in levels of oxidative stress response genes in either theca interna or granulosa cells of non-ovulatory compared to preovulatory dominant follicles. This would suggest that these enzymes which function to prevent oxidative stress are not differentially regulated to prevent atresia of the preovulatory follicle. However, in this study I also compared levels of oxidative stress response genes between healthy and atretic follicles. Amounts of mRNA encoding the oxidative stress response proteins GSHPx and MnSOD were more highly expressed in granulosa cells of healthy (highly estrogenic) compared to atretic dominant bovine follicles, suggesting that higher expression of oxidative stress response proteins in granulosa cells of healthy bovine follicles may allow these follicles to avoid apoptosis caused by oxidative stress. This preliminary experiment provided evidence to support the hypothesis that oxidative stress contributes to the apoptosis that occurs as follicles undergo atresia, and indicates increased expression of oxidative stress response proteins may protect follicular cells against apoptosis induced by oxidative stress. This provided the groundwork for my next
set of experiments which examined the temporal relationship between apoptosis in granulosa cells and expression of proteins responsible for preventing oxidative stress.

My second specific aim was to characterize the initiation of apoptosis and expression of oxidative stress response genes, proteins, and activities in dominant bovine follicles at 3 times during the first wave of follicular development. The well characterized pattern of follicular development and atresia during the 1st wave following ovulation provides an excellent model to determine when apoptosis is initiated during atresia of non-ovulatory dominant follicles, and if prevention of oxidative stress is involved in attenuation of apoptosis. I found that decreased production of estradiol is accompanied by increased numbers of granulosa cells in early apoptosis in dominant follicles on Day 6 of the first follicular wave. Studies suggest that estradiol plays an anti-apoptotic role in the granulosa cell [Billig et al., 1993]. Therefore, the increase in early-apoptotic granulosa cells may be the result of the decrease in estradiol production noted on Day 6. Also associated with the dominant follicle’s descent into atresia were alterations in the relative expression of genes encoding the Bcl-2 family of proteins and an increase in expression of mRNA encoding oxidative stress response proteins in granulosa cells. The changes in expression of the Bcl-2 family of proteins, which function to regulate the execution phase of apoptosis, should be studied further. Specifically, examination of translocation from the cytosol to the mitochondria would further clarify the roles of the Bcl-2 family during the initiation of atresia. The increase in mRNA encoding oxidative stress response proteins in granulosa cells on Day 8 of the 1st follicular wave is perplexing. However, it is not accompanied by increases in protein
or enzyme activity, and therefore, should not confer any immediate physiological
significance.

The transcription factor NF-κB is capable of regulating genes involved in
preventing oxidative stress and has been characterized as a mediator of cell survival.
Studies have suggested that NF-κB activity may be regulated by estradiol-17β, a major
determinant of follicular health. It is well established that follicular fluid estradiol
decreases by Day 6 of the 1st follicular wave. However, amounts of estradiol in follicular
fluid represent the pooled accumulation of estradiol secreted by the follicle from the time
of its formation, and do not accurately reflect the steroidogenic capacities of the theca
interna and granulosa cells at specific time points during the lifespan of the dominant
follicle. Therefore, my third specific aim was to characterize the follicular steroidogenic
capacities of theca interna and granulosa cells collected from dominant follicles on Days
4, 6, and 8 of the first follicular wave. I found that the reduced secretion of estradiol into
follicular fluid observed on Day 6 of the follicular wave is likely the direct result of
decreased activity of the aromatase enzyme within the granulosa cells, and is independent
of thecal contribution of androstenedione. Given that expression of the aromatase gene
does not decrease during the time, we must speculate that either translation of the mRNA
encoding aromatase into protein decreases or the activity of the enzyme itself lessens
between Days 4 and 6 of the first follicular wave following ovulation.

With this information, my final experiments were designed to characterize the
role of activated NF-κB in apoptosis of dominant follicles and NF-κB regulation by
estradiol. The trend of differences noted in the percentage of granulosa cells, isolated
from follicles at Day 4, 6, or 8 of the first follicular wave, that were early-stage apoptotic after 24 h in vitro (Chapter 5, Fig. 5.4) mimicked the trend in differences noted in follicles collected on Days 4, 6, and 8 of the first follicular wave. This suggests that our in vitro system is a valid model for studying granulosa cell apoptosis. Furthermore, I found that production of high amounts of estradiol was associated with higher amounts of NF-κB activity and fewer cells in early apoptosis. These data support the hypothesis that estradiol influences NF-κB activity and prevents apoptosis in granulosa cells of dominant bovine follicles. However, when NF-κB activity was directly inhibited, apoptosis was suppressed in cultured granulosa cells, independent of estradiol production. My findings support the hypothesis that estradiol influences NF-κB activity and that NF-κB is involved in the apoptosis of granulosa cells. However, whether this role is pro- or anti-apoptotic remains to be established. It is possible that NF-κB is involved in both stimulating and suppressing apoptosis, depending on the stage of follicular development and the intracellular milieu. NF-κB may stimulate expression of such anti-apoptotic proteins as XIAP in the healthy dominant follicle, and late inducing transcription of FasL to initiate apoptosis in the atretic follicle. Future experiments should be conducted to address which genes NF-κB is controlling in granulosa cells of the bovine dominant follicle.

The experiments described here were designed to begin to elucidate the signals that trigger the sequence of events leading to apoptosis and atresia of bovine follicles. They provide the framework for the direction of future experiments designed to further clarify the complex interplay of signals that determine a follicle’s fate.
Appendix A

Supplemental Results

Appendix A.1 Supplemental results to Chapter 3

Fluorescent real-time quantitative RT-PCR was used to determine differences in mRNA expression of eNOS and iNOS in theca interna as described in Chapter 3 (Table A.1). Expression of mRNA encoding eNOS tended to decrease ($P = 0.065$) in theca interna in follicles collected on Day 8 compared to Day 4 of the 1st follicular wave (Figure A.1). Amounts of mRNA encoding iNOS in theca interna in follicles collected on Days 4, 6, and 8 of the 1st follicular wave did not change (Figure A.1).
Table A.1 Quantitative RT-PCR primers

<table>
<thead>
<tr>
<th></th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>CCTTCCGCTACCAGCCAGA</td>
<td>CAGAGATCTCACCGGTTGGCCA</td>
</tr>
<tr>
<td>iNOS</td>
<td>GGATCTCTCTTTGCAAGTCCAAGTC</td>
<td>GATAGCTTGGAGTGAGACTCGTC</td>
</tr>
<tr>
<td>18S</td>
<td>CCTTCCGGAGGATCCATTG</td>
<td>CGCTCCCAAGATCAAACCTAC</td>
</tr>
</tbody>
</table>

Figure A.1 Amounts of mRNA encoding eNOS and iNOS in theca interna of follicles collected on Days 4, 6, and 8 of the 1st follicular wave. Letters indicate a trend to differ ($P=0.065$).
Appendix B

Methods

Appendix B.1 Radioimmunoassays

Stock Solutions

A. Estradiol (E2) Standards

Methods and calculations for preparation:

<table>
<thead>
<tr>
<th>Table B.1.1 Estradiol standards.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration in assay (200μl)</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>0.625pg</td>
</tr>
<tr>
<td>1.25pg</td>
</tr>
<tr>
<td>2.5pg</td>
</tr>
<tr>
<td>5pg</td>
</tr>
<tr>
<td>10pg</td>
</tr>
<tr>
<td>20pg</td>
</tr>
<tr>
<td>40pg</td>
</tr>
<tr>
<td>80pg</td>
</tr>
</tbody>
</table>

1. Weigh out 1.6mg of Estradiol, dilute in 10ml 100% ethanol (160μg/ml; stock solution A).
2. Dilute stock solution A 1:100 in ethanol (160ng/ml; stock solution B)
3. Dilute stock solution B 1:1000 in MEM. (1600pg/ml; stock solution C)
4. Perform serial 1:1 dilutions in MEM to obtain the other 9 concentrations.
   1600pg/ml → 800pg/ml → 400pg/ml …→ 3.125pg/ml (final conc)

B. E2 Pools

Methods and calculations for preparation:

<table>
<thead>
<tr>
<th>Table B.1.2 Estradiol pools.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration in assay (200μl)</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>1pg</td>
</tr>
<tr>
<td>5pg</td>
</tr>
<tr>
<td>25pg</td>
</tr>
</tbody>
</table>
1) Start with stock solution C from E2 standards, and dilute 1:3.2 with MEM.  
1600pg/ml → 500pg/ml

2) With this solution, perform a 1:4 serial dilution in MEM to obtain 125pg/ml.  
500pg/ml → 125pg/ml

3) Perform a 1:5 dilution with the 125pg/ml dilution in MEM to obtain pools.  
125pg/ml → 25pg/ml → 5pg/ml

C. Progesterone Standards
Methods and calculations for preparation:

Table B.1.3 Progesterone standards.

<table>
<thead>
<tr>
<th>Concentration in assay (100μl)</th>
<th>Concentration per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25pg</td>
<td>62.5pg/ml</td>
</tr>
<tr>
<td>12.5pg</td>
<td>125pg/ml</td>
</tr>
<tr>
<td>25pg</td>
<td>250pg/ml</td>
</tr>
<tr>
<td>50pg</td>
<td>500pg/ml</td>
</tr>
<tr>
<td>100pg</td>
<td>1000pg/ml</td>
</tr>
<tr>
<td>200pg</td>
<td>2000pg/ml</td>
</tr>
<tr>
<td>400pg</td>
<td>4000pg/ml</td>
</tr>
<tr>
<td>800pg</td>
<td>8000pg/ml</td>
</tr>
</tbody>
</table>

1) Weigh out 8mg of progesterone and dilute with 10ml 100% ethanol.  
8mg/10ml = 0.8mg/ml = 800μg/ml (Stock A)

2) Perform a 1:100 dilution in 100% ethanol with Stock A to make Stock B.  
800μg/ml → 8μg/ml = 8000ng/ml (Stock B)

3) Perform a 1:1000 dilution in MEM.  
8000ng/ml → 8ng/ml = 8000pg/ml (Stock C)

4) Perform 1:1 serial dilutions of Stock C in MEM.  
8000pg/ml → 4000pg/ml → ... 62.5pg/ml (final concentration)

D. Progesterone Pools
Methods and calculations for preparation:

Table B.1.4 Progesterone pools

<table>
<thead>
<tr>
<th>Concentration in assay (100μl)</th>
<th>Concentration per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>15pg</td>
<td>150pg/ml</td>
</tr>
<tr>
<td>60pg</td>
<td>600pg/ml</td>
</tr>
<tr>
<td>240pg</td>
<td>2400pg/ml</td>
</tr>
</tbody>
</table>

1) Start with Stock solution C from the progesterone standard (8000pg/ml) and  
dilute it 1:3.33 in MEM.  
8000pg/ml → 2400pg/ml

2) Use this solution to perform a 1:4 serial dilution in MEM.
2400pg/ml → 600pg/ml → 150pg/ml (final concentrations)

E. Androstenedione Standards
Methods and calculations for preparation:

**Table B.1.5 Androstendione standards**

<table>
<thead>
<tr>
<th>Concentration in assay (100µl)</th>
<th>Concentration per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5pg</td>
<td>125pg/ml</td>
</tr>
<tr>
<td>25pg</td>
<td>250pg/ml</td>
</tr>
<tr>
<td>50pg</td>
<td>500pg/ml</td>
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<tr>
<td>100pg</td>
<td>1000pg/ml</td>
</tr>
<tr>
<td>200pg</td>
<td>2000pg/ml</td>
</tr>
<tr>
<td>400pg</td>
<td>4000pg/ml</td>
</tr>
<tr>
<td>800pg</td>
<td>8000pg/ml</td>
</tr>
</tbody>
</table>

1) Weigh out 8mg of androstendione and dilute with 10ml 100% ethanol.
   8mg/10ml = 0.8mg/ml = 800µg/ml (Stock A)
2) Perform a 1:100 dilution in 100% ethanol with Stock A to make Stock B.
   800µg/ml → 8µg/ml = 8000ng/ml (Stock B)
3) Perform a 1:1000 dilution in MEM.
   8000ng/ml → 8ng/ml = 8000pg/ml (Stock C)
2) Perform 1:1 serial dilutions of Stock C in MEM.
   8000pg/ml → 4000pg/ml → ... 125pg/ml (final concentration)

F. Androstenedione Pools
Methods and calculations for preparation:

**Table B.1.6 Androstenedione pools**

<table>
<thead>
<tr>
<th>Concentration in assay (100µl)</th>
<th>Concentration per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20pg</td>
<td>200pg/ml</td>
</tr>
<tr>
<td>100pg</td>
<td>1000pg/ml</td>
</tr>
<tr>
<td>500pg</td>
<td>5000pg/ml</td>
</tr>
</tbody>
</table>

1) Start with Stock solution C from the androstendione standard (8000pg/ml) and dilute it 1:1.6 in MEM.
   8000pg/ml → 5000pg/ml
2) Use this solution to perform a 1:5 serial dilution in MEM.
   5000pg/ml → 1000pg/ml → 200pg/ml (final concentrations)
Estradiol Double Antibody Radioimmunoassay (DPC)

All components except the Precipitating Solution (P.S.) must be at room temperature before use.

1. Label 38 uncoated tubes, plus additional tubes for samples. T (total counts), NSB (nonspecific binding), BO (maximum binding), and standards are performed in triplicate. Pools and samples are run in duplicate.
2. Pipet 200μl MEM into the NSB and BO tubes, and 200μl of the standards, pools, or samples into their corresponding tube sets. Pipet tips may be re-used for various standards and pools as long as pipetting is always done from a lower to higher concentration.
3. Add 100μl of Estradiol Antiserum (Blue) to all tubes except NBS and T tubes. Vortex.
4. Incubate at room temperature for 2 hours.
5. Add 100μl of 125I Estradiol to all tubes. Vortex. (Remove the T tube at this point; it is ready to be counted.)
6. Incubate for 1 hour at room temperature.
7. Add 1 ml of cold Precipitating Solution (P.S.) to all tubes. Vortex.
8. Incubate for 10 minutes at room temperature.
9. Centrifuge for 15 minutes at 3000xg.
10. Carefully invert tubes on a piece of absorbent paper towel making sure to remove all residual liquid.
11. Count each tube for 1 minute on a gamma counter. Analyze data.

Progesterone Coat-A-Count Radioimmunoassay (DPC)

All components must be at room temperature before use.

1. Label 6 uncoated tubes for the T tubes (total counts), and the NSB (nonspecific binding) tubes. Label 31 Ab-coated tubes (tubes 7-39) for the BO tubes (maximum binding), standards, and pools. Label additional tubes for samples. The T tubes, NSB tubes, BO tubes, and the standards are performed in triplicate while the pools and samples are performed in duplicate.
2. Pipet 100μl of MEM into the NSB and the BO tubes, and 100μl of each standard, pool, or sample into their corresponding tube set. Pipet tips may be reused for standards and pools as long as pipetting is done from a low to high concentration only.
3. Add 1 ml of 125I Direct Progesterone to every tube. Vortex. (Set aside the T tube; it is ready to be counted.)
4. Incubate for 3 hours at room temperature.
5. Invert tubes on an absorbent sheet of paper towel and tap gently making sure to remove any residual liquid.
6. Count each tube for 1 minute on a gamma counter. Analyze data.

**Androstenedione Coat-A-Count Radioimmunoassay (DPC)**

All components must be at room temperature before use.

1. Label 6 uncoated tubes for the T tubes (total counts), and the NSB (nonspecific binding) tubes. Label 30 Ab-coated tubes (tubes 7-36) for the BO tubes (maximum binding), standards, and pools. Label additional tubes for samples. The T tubes, NSB tubes, BO tubes, and the standards are performed in triplicate while the pools and samples are performed in duplicate.

2. Pipet 100μl of MEM into the NSB and the BO tubes, and 100μl of each standard, pool, or sample into their corresponding tube set. Pipet tips may be reused for standards and pools as long as pipetting is done from a low to high concentration only.

3. Add 1 ml of $^{125}$I Direct Androstenedione to every tube. Vortex. (Set aside the T tube; it is ready to be counted.)

4. Incubate for 2 hours at room temperature.

5. Invert tubes on an absorbent sheet of paper towel and tap gently making sure to remove any residual liquid.

6. Count each tube for 1 minute on a gamma counter. Analyze data.
Appendix B.2 RNA isolation using TRIzol® (Invitrogen)

1. HOMOGENIZATION
   a. Tissue: Homogenize tissue samples in 1 ml of TRIzol reagent per 100 mg of tissue using power homogenizer. The sample volume should not exceed 10% of the volume of TRIzol used.
   b. Cells: Lyse cells in TRIzol by repetitive pipetting. Use 1 ml of reagent per 10x10^6 cells.

2. PHASE SEPARATION
   Incubate the homogenized samples for 5 min at 26°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml chloroform per 1 ml TRIzol. Shake tubes vigorously by hand for 15 s and incubate at 26°C for 2 min. Centrifuge at 12,000 x g for 15 min at 4°C.

3. RNA PRECIPITATION
   Transfer the aqueous phase to a new tube, and save the organic phase if isolation of protein is desired. Precipitate the RNA from the aqueous phase by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIzol. Incubate samples at 26°C for 10 min and centrifuge at 12,000 x g for 10 min at 4°C.

4. RNA WASH
   Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding 1 ml per 1 ml of TRIzol. Mix the sample by vortexing and centrifuge at 7500 x g for 5 min at 4°C.

5. REDISSOLVING THE RNA
   Briefly dry the RNA pellet. Dissolve RNA in Rnase-free water.
Appendix B.3 Riboprobe synthesis using MAXIscript kit (Ambion Inc.)

Template DNA
Template DNA should be linearized by complete restriction digestion that cleaves distally of the promoter to allow transcription of an antisense strand of DNA. Final concentration of linearized template should be 0.5-1.0 µg/µl.

Transcription Reaction
1. Thaw reagents on ice, except 10X transcription buffer at 26°C.
2. Vortex nucleotides and 10X transcription buffer and centrifuge all reagents.
3. Add indicated reagents in order to 1.5 ml tube at room temp for each probe.

<table>
<thead>
<tr>
<th></th>
<th>DNA template (0.5 µg)</th>
<th>10X transcription buffer</th>
<th>10mm ATP</th>
<th>10mm CTP</th>
<th>10mm GTP</th>
<th>10mm UTP COLD</th>
<th>α-32P UTP</th>
<th>T7 RNA polymerase</th>
<th>DEPC H2O</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td>All other probes</td>
<td>variable</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>2.5</td>
<td>1.0</td>
<td>variable</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*1.0µl of a 1:10 dilution of α-32P UTP

4. Mix contents by flicking tube with finger then microfuge briefly.
5. Incubate reaction 1 hour at 37°C.
6. Add 1 µl Dnase I to remove DNA template.
7. Incubate 37°C 15 minutes.
8. Add 1 µl 0.5 M EDTA to stop the reaction.
9. Remove 1µl and add to 4 ml of scintillation fluid to count (label before purifying)
10. Gel purify probes to remove free nucleotides.

Gel Purification of Probes
1. Prepare denaturing polyacrylamide gel.
   - For 30 ml:
     14.4 g Urea
     3.0 ml 10X TBE
     3.8 ml 40% Acrylamide (acryl: bis-acryl = 19:1)
     11.4 ml ddH2O
   - Stir at room temp till dissolved
   - Just before pouring add:
360 µl 10% APS
48 µl TEMED
  • Pipette quickly into plates.

2. Add 11 µl of gel loading buffer (add equal volume of loading buffer as reaction volume).
3. Incubate 3 min at 90°C.
4. Running buffer: 1400 ml 1X TBE. Blow out bubbles and rinse wells to eliminate urea. Load samples.
5. Run until leading dye is near bottom of gel at ~ 250V.
6. Remove gel and wrap in saran wrap. Tape to cassette and expose for ~10 sec.
   • Mark gel location on film.
   • Cut out full length probe (the slowest migrating, most intense band on gel)
   • Put gel back on film to develop ladder.
7. Place gel chunk in microfuge tube with 350 µl of elution buffer.
8. Incubate overnight at 37°C.
9. Centrifuge at maximal speed 15 min at 4°C and remove supernatant to new tube.
10. Remove 1 µl of purified probe and add to 4 ml of scintillation fluid and check counts along with unpurified probe (user 7 on beta counter).
11. Store probes at -20°C in shielded radioactive box.
Appendix B.4 Ribonuclease protection assay using RPA III Kit (Ambion, Inc.)

**Day 1**
1. On ice thaw riboprobes, sample RNA, yeast RNA, ammonium acetate, hybridization buffer, 100% ethanol.
2. Mix sample RNA (5-20 μg), probe (80,000 CPM), ddH₂O.
3. Set up 2 controls: each with 10.0 μg yeast RNA (1 will receive RNase).
4. Co-precipitate the probes and sample RNA: Add 1:10 dilution of ammonium acetate and 2.5X volume of 100% ethanol. Incubate at -80°C for 4-10 h.
5. Centrifuge 15 min, 4°C, 12,000 x g.
6. Remove supernatant, rinse with 50 μl of 70% ethanol.
7. Centrifuge 5 min, 4°C, 12,000 x g.
8. Air dry pellet and resuspend in 10 μl hybridization buffer.
9. Incubate 4 min at 90°C.
10. Incubate 42°C overnight.

**Day 2**
1. Prepare 1:50 dilution of T1 Rnase in RNase Digestion III Buffer.
2. Add 150 μl diluted RNase solution to each sample (except 1 yeast control). Add 150 μl of RNase Digestion buffer without RNase to other yeast control.
3. Incubate 30 min at 37°C.
4. Add 225 μl RNase Inactivation/Precipitation III solution.
5. Incubate -80°C for 2 h.
6. Prepare 5% acrylamide/8M urea gel as for riboprobe synthesis.
7. Centrifuge samples 15 min, 4°C, 12,000 x g.
8. Wash pellets with 50 μl of 75% ethanol.
9. Air dry pellets and resuspend in 10 μl gel loading buffer.
10. Incubate 4 min at 90°C. Place on ice until loading on gel.
11. Load sample on gel, run at 260 V until leading dye is near bottom of gel.
12. Transfer gel to filter paper, cover with plastic, and expose to film overnight.
Appendix B.5 Protein isolation following RNA isolation with TRIzol

1. Remove the remaining aqueous phase overlying the interphase, and precipitate the DNA from the interphase and organic phase with 0.3 ml of 100% ethanol per 1ml of TRIzol. Mix samples by inversion. Store the samples at 26°C for 3 min and centrifuge at 2000 x g for 5 min at 4°C.

2. Transfer the phenol-ethanol supernatant to a new tube and precipitate the proteins with 1.5 ml per 1.0 ml TRIzol of isopropyl alcohol.

3. Incubate samples for 10 min at 26°C.

4. Centrifuge 12,000 x g for 10 min, 4°C.

5. Remove the supernatant and wash the protein pellet 3 times in 0.3M guanidine hydrochloride, 95% ethanol. During each wash, incubate the pellet in the wash solution for 20 min at 26°C and centrifuge at 7500 x g for 5 min at 4°C.

6. Dry the protein pellet and dissolve in 1% SDS by pipetting. Store at ~20°C.
Appendix B.6 Western Analysis

A. Solutions for Running SDS-PAGE

4X Tris/SDS pH 8.8 (500 mL)
91.0 g Tris-base
2.0 g SDS
300.0 mL ddH₂O
- pH to 8.8 with HCl
- Bring to 500 mL with ddH₂O
- Filter thru 0.45 micron filter
- Store at 4°C.

4X Tris/SDS pH 6.8 (100mL)
6.05 g Tris-base
0.4 g SDS
40.0 mL ddH₂O
- pH to 6.8 with HCl
- Bring to 100 mL with ddH₂O
- Filter thru 0.45 micron filter
- Store at 4°C

5X SDS Electrophoresis Buffer (1L)
15.1 g Tris-base
72.0 g Glycine
5.0 g SDS
Bring to 1 L with ddH₂O.

2X Sample Loading Buffer (10 mL)
0.152 g Tris-base
2.0 ml Glycerol
0.2 g SDS
0.2 mL β-mercaptoethanol
4.0 mL ddH₂O
- pH to 6.8 with HCl
- Add 0.1 mg bromophenol blue
- Bring to 10 mL with ddH₂O.
- Store at 4°C or frozen in aliquots

B. Separating Gel
In a 25 mL flask, mix Separating Gel components based on the % polyacrylamide desired (see separating gel chart). Swirl gently to mix. Pour immediately to about 0.5 cm below where the combs will be. Overlay with a layer of water or isobutanol.

Table B.6.1 Stock Solutions for 2 1.5 mm gels (ml)

<table>
<thead>
<tr>
<th>Final acrylamide concentrations in the separating gel (%)</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide/ 0.8% bisacrylamide</td>
<td>2.50</td>
<td>3.00</td>
<td>3.50</td>
<td>3.75</td>
<td>4.00</td>
<td>4.50</td>
<td>5.00</td>
<td>7.50</td>
</tr>
<tr>
<td>4x Tris-CI/SDS, pH 8.8</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>H₂O</td>
<td>8.75</td>
<td>8.25</td>
<td>7.75</td>
<td>7.50</td>
<td>7.50</td>
<td>6.75</td>
<td>6.75</td>
<td>3.75</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
C. Stacking Gel
1. Pour off the layer of isobutyl alcohol completely. In a 25ml sidearm flask, mix:
   - 0.65 ml acrylamide/0.8% bisacrylamide
   - 1.25 ml 4X Tris/SDS, pH 6.8
   - 3.05 ml ddH₂O
   - 25 ul 10% Ammonium Persulfate
   - 5 ul TEMED
2. Swirl gently to mix. Pour immediately.
3. Using a Pasteur pipette, slowly add the stacking gel solution along one edge of one of the spacers until the solution reaches the top of the lower glass plate. Insert the Teflon comb into the stacking gel solution. Add additional stacking gel to completely fill the spaces in the comb, if necessary.
4. Allow the stacking gel solution to polymerize 30 to 45 minutes, then gently remove comb and rinse wells with running buffer.

D. Sample Preparation and Loading
1. Put protein samples into microfuge tubes.
2. Add 2X loading buffer; mix at least 1:1 with sample.
4. Cool on the bench top 2 minutes. Quick spin in the microfuge to get all the liquid into the bottom of the tube.
5. Put gels into the apparatus as directed by the instructions. Fill both lower and upper chambers with appropriate amounts of 1X SDS/electrophoresis buffer into the lower buffer chamber. Rinse out wells with pipette full of running buffer. Load each sample onto the gel, recording the order. Samples should sink to the bottom of each well.

E. Running the Gel
- The gel should be run at approximately 120 V (about 25 mA) for 1 hour 15 min. Depending on the size of the gel and the protein of interest (run heavier proteins longer), the dye front should be about 1 cm from the bottom of the gel.
- When done, remove the spacers from the sides of the gel, then use one to pry one glass plate away from the gel. Mark one corner so you can later determine the orientation. Next, either stain the gel with Coomassie stain or begin protein transfer to nitrocellulose.
F. Coomassie Staining

<table>
<thead>
<tr>
<th>Coomassie Gel Stain</th>
<th>Destain 1</th>
<th>Destain 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% MeOH</td>
<td>50% MeOH</td>
<td>5% MeOH</td>
</tr>
<tr>
<td>0.05% Coomassie</td>
<td>10% acetic acid</td>
<td>7.5% Acetic Acid</td>
</tr>
<tr>
<td>10% acetic acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Put gel into stain for at least one hour or overnight for best results. Recycle stain, then destain gel with Destain 1, about 3 changes 15 min apart. Gel can then be destained further with Destain 2, or stored in Destain 2. Make sure gel is completely immersed at all times so it doesn’t dry out.

G. Protein transfer to nitrocellulose

NTB – Nitrocellulose Transfer Buffer (3L)

- 9.09g Tris base
- 43.2g Glycine
- 600ml Methanol
- Bring to 3L with ddH2O

1. While gel is running, equilibrate 2 scotch-brite pads and 2 pieces of filter paper per gel in NTB (nitrocellulose transfer buffer).
2. With gloves on, equilibrate one piece of nitrocellulose, cut to the same size as gel, per gel in NTB.
3. When gel is finished running, place one pad onto the sandwich cassette, then one piece of filter paper. Open glass plates and remove gel, then lay it onto the filter paper. Put one piece of membrane on the gel so that there are no bubbles in between. Put filter paper then pad over this. Put sandwich in so that gel is on the negative side of apparatus.
4. Run transfer at approximately 200 mA for at least one hour.

H. Protein Detection

TTBS (2L)

- 58.44 g NaCl
- 4.85 g Tris-HCl
- 3 ml Tween-20
- Bring to 2 L with ddH2O

After nitrocellulose transfer, place nitrocellulose in small dish (like tip box) and check for protein transfer by using Ponceau S stain. Pour into dish and let sit for 5 minutes on rocker. Pour stain back into jar. Rinse nitrocellulose in ddH2O. Mark ladder with ballpoint pen.
1. Wash 3 x 5 minutes in TTBS on rocker.
2. Block nitrocellulose with 5% Milk in TTBS: 5 g non-fat evaporated dry milk per 100 ml TTBS for 30 minutes to overnight (in cold room if O.N.).
3. Use preferred dilution of primary antibody in 3% milk. Incubate on rocker 1 hour.
4. Wash 3 x 5 min in TTBS
5. Use secondary antibody of choice (see below for HRP) and block in 3% milk for one hour.
6. Wash 2 x 5 min in TTBS. Wash 1 x 5 min in TBS.
7. Detect Western.

I. “Mosal” Western Detection

**Luminol (Sigma A-8511, 3-aminophthalhydrazide)**
0.44 g in 10 ml DMSO
Aliquot into 200 μl volumes and store at –80°C

**p-Coumaric Acid (Sigma C-9008)**
0.15 g in 10 ml DMSO
Aliquot into 88 μl volumes and store at –80°C

**1 M Tris-HCl pH 8.5 (500 mL)**
78.8 g Tris-HCl in 500 ml H₂O
pH to 8.5

Thaw aliquots of Luminol and p-coumaric acid.

<table>
<thead>
<tr>
<th>Solution 1</th>
<th>40 mL</th>
<th>20 mL</th>
<th>10 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminol</td>
<td>200 μL</td>
<td>100 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>88 μL</td>
<td>44 μL</td>
<td>22 μL</td>
</tr>
<tr>
<td>1 M Tris-HCl pH 8.5</td>
<td>2 mL</td>
<td>2 mL</td>
<td>500 μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>17.71 mL</td>
<td>17.86 mL</td>
<td>4.43 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂ (30%)</td>
<td>12 μL</td>
<td>6 μL</td>
<td>3 μL</td>
</tr>
<tr>
<td>1 M Tris-HCl pH 8.5</td>
<td>2 mL</td>
<td>0</td>
<td>500 μL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>18 ml</td>
<td>0</td>
<td>4.5 μl</td>
</tr>
</tbody>
</table>

- Mix solutions 1:1 and incubate blots in this for one minute.
- Remove excess liquid from blot, and then place into ziploc bag in film cassette.
- Expose film for 30 sec-30 min. Develop film.
Appendix B.7 Annexin Staining of fresh cells using Vybrant Apoptosis Assay Kit #2 (Molecular Probes)

1. Re-suspend cells (~100,000) in cold PBS.
2. Prepare 1X Annexin-Binding Buffer.
3. Prepare 100μg/ml solution of propidium iodide (PI) in 1X Annexin-Binding Buffer.
4. Re-centrifuge the washed cells (800 x g for 15 min), discard the supernatant and re-suspend the cells in 1X Annexin-Binding Buffer, diluting cell density to ~10⁶ cells/ml.
5. Add 5 μl of Alexa Fluor 488 annexin V (Component A) and 1 μl of the 100 μg/ml PI working solution to each 100 μl of cell suspension.
6. Incubate the cells at room temperature for 15 min.
7. Spread suspension on microscope slide, coverslip, and view with Olympus BX50WI fluorescence microscope using FITC filter.
8. Count at least 800 cells, noting if they were unstained, stained with only annexin V, or stained with both annexin V and PI.
Appendix B.8 Reverse transcription of RNA using SuperScript ™ III RNase Reverse Transcriptase (Invitrogen)

1. Add the following components to a nuclease-free microcentrifuge tube to 14 µl:
   1.0 µl Random primers (50-250 ng)
   2.0 µg RNA
   1.0 µl dNTP mix (10 mM)
   Sterile, distilled water
2. Heat mixture to 65°C for 5 min, and incubate on ice 1 min.
3. Add:
   4.0 µl 5X First-strand buffer
   1.0 µl 0.1 M DTT
   1.0 µl SuperScript III RT (200 units/ml)
4. Mix by pipetting gently up and down. Incubate at 25°C for 5 min.
5. Incubate at 50°C for 60 min.
6. Inactivate the reaction by heating at 70°C for 15 min.
7. Store at −20°C.

Appendix B.9 Real-time PCR using Rotor-Gene (Corbett Research Mortlake, Australia)

1. Add the following to a 0.2 ml tube:
   2.0 µl cDNA (0.008 µg/ml)
   0.4 µl MgCl2 (25 mM)- makes reaction 3.5 mM
   0.6 µl sterile, distilled water
   1.0 µl Forward primer ((5 mM)
   1.0 µl Reverse primer (5 mM)
   5.0 µl SYBR Green Master Mix (Qiagen)
   10.0 µl Total reaction volume

2. Running conditions:
   • 95°C 15 min (Activates Hotstart Taq)
   • 45 cycles of:
     95°C 15 sec
     58°C 15 sec
     72°C 20 sec
   • Ramp 72°C - 99°C over 15 min
Appendix B.10 Glutathione Peroxidase Assay Kit (Cayman Chemical)

Preparation of reagents:
1. Assay buffer:
   Dilute 2 ml of Assay Buffer concentrate with 18 ml of HPLC-grade water. Store at 4°C up to 2 months.
2. Sample Buffer:
   Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water. Store at 4°C up to 1 months.
3. Control Glutathione Peroxidase:
   Aliquot and store at -20°C. Prior to use, transfer 10 μl of the enzyme and dilute with 490 μl of diluted Sample buffer. The diluted enzyme is stable on ice for 4 h.
4. Co-substrate Mixture:
   Reconstitute by adding 2 ml of HPLC-grade water to each vial and vortex well. Keep at 25°C while assaying. Stored at 4°C up to 2 days.
5. Cumene Hydroperoxide
   The reagent is ready to use as supplied.

Performing the assay:
1. Background or Non-enzymatic Wells: Add 120 μl of Assay Buffer and 50 μl co-substrate mixture to 3 wells of 96-well microplate.
2. Positive Control Well (bovine erythrocyte GSHPx): Add 100 μl Assay Buffer, 50 μl co-substrate mixture, and 20 μl diluted GSHPx (control) to 3 wells.
3. Sample Wells: Add 100 μl Assay Buffer, 50 μl co-substrate mixture, and 20 μl sample diluted in Sample Buffer to 3 wells. To obtain reproducible results, the amount of GSHPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min.
4. Initiate the reactions by adding 20 μl cumene hydroperoxide to all the wells being used. Note the precise time of initiation and add the cumene hydroperoxide as quickly as possible.
5. Carefully shake the plate for a few seconds to mix.
6. Read the absorbance once every min at 340 nm using a microplate reader to obtain at least 5 time points. The initial absorbance of the sample wells should be between 1.2 and 0.5.

Determination of the Reaction Rate:
1. Plot the absorbance values as a function of time to obtain the rate of the the linear portion of the curve.
2. Determine the rate of ΔA340/min for the background wells and subtract this rate from the rate of each sample well.
   Use the following formula to calculate the GSHPx activity, given one unit of GSHPx activity is defined as the amount of enzyme that will cause oxidation of 1.0 nmol of NADPH to NADP⁺ per minute at 25 °C, with the NADPH extinction coefficient being 0.00373 μM⁻¹:  
GSHPx activity = \frac{\Delta A_{340/\text{min}}}{0.00373 \mu \text{M}^{-1}} \times \frac{0.19 \text{ ml}}{0.02 \text{ ml}} \times \text{Sample dilution} = \text{nmol/min/ml}

Example Samples (10 µg protein)
- #18662: \( y = -0.0582x + 0.5268 \)
- #951-2: \( y = -0.0713x + 0.5377 \)
- #18849: \( y = -0.048x + 0.5699 \)

Controls
- neg contr: \( y = -0.0078x + 0.5735 \)
- pos contr: \( y = -0.0444x + 0.5239 \)

Figure B.10.1 GSHPx activity plots
Appendix B.11 SOD Assay Kit-WST (Dojindo Molecular Technologies)

Preparation of solutions:
1. WST Working Solution: Dilute 1 ml WST Solution with 19 ml Buffer Solution.
2. Enzyme Working Solution: Mix the Enzyme Solution by pipetting, and dilute 15 μl Enzyme Solution with 2.5 ml Dilution Buffer.
3. SOD Solution (for assay monitoring): Dilute SOD with Dilution Buffer to prepare SOD Standard Solutions of 200, 100, 50, 10, 5, 1, 0.1, 0.05, 0.01, and 0.001 U/ml.

Performing the assay:
1. In a 96-well microplate, add 20 μl sample solution (sample) or sterile water (blank1 and blank 3) to each well.
2. Add 200 μl WST Working solution to each well and mix.
3. Add 20 μl Dilution buffer to the wells of blank 2 and blank 3.
4. Add 20 μl Enzyme Working Solution to each sample well and the well of blank 1, then mix thoroughly.
5. Incubate the plate for 20 min at 37°C.
6. Read the absorbance at 450 nm using a microplate reader.

Calculation of SOD activity:

\[
\text{SOD activity (inhibition rate %)} = \left( \frac{(A_{B1} - A_{B3}) - (A_{\text{sample}} - A_{B2})}{A_{B1} - A_{B3}} \right) \times 100
\]

Example Samples (5 μg protein)

<table>
<thead>
<tr>
<th>Heifer #</th>
<th>Absorbance (450 nM)</th>
<th>Inhibition Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>18662</td>
<td>0.557</td>
<td>7.76</td>
</tr>
<tr>
<td>951-2</td>
<td>0.456</td>
<td>8.48</td>
</tr>
<tr>
<td>18849</td>
<td>0.536</td>
<td>7.91</td>
</tr>
</tbody>
</table>

Figure B.11.1 SOD Inhibition curve.
Appendix B.12 Amplex Red Catalase Assay Kit (Molecular Probes)

Preparation of solutions:
1. Prepare a 10 mM stock solution of Amplex Red reagent: Allow one vial of Amplex red reagent and DMSO to warm to room temperature. Just prior to use, dissolve the contents of the vial of Ampex red reagent in 100 μl DMSO.
2. Prepare a 1X working solution of Reaction Buffer by adding 4 ml 5X Reaction Buffer stock solution to 16 ml deionized water.
3. Prepare a 100U/ml solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP in 200 ml 1X Reaction Buffer.
4. Prepare a 20 mM H₂O₂ working solution by diluting the ~3% H₂O₂ stock solution into the appropriate volume of water.
5. Prepare a 1000 U/ml solution of catalase by dissolving the contents of the vial of catalase in 100 μl water.
6. Prepare a catalase standard curve by diluting the 1000 U/ml catalase in 1X Reaction Buffer to 1000, 500, 250, 125, 62.5, and 0 U/ml.

Performing the assay:
1. Dilute the catalase-containing samples in 1X Reaction Buffer to a total volume of 25 μl.
2. Pipet 25 μl of the diluted experimental samples, standard curve samples and controls into separate wells of a 96-well microplate.
3. Prepare a 40 mM H₂O₂ solution by adding 10 μl 20 mM H₂O₂ solution to 4.99 ml 1X Reaction Buffer.
4. Pipet 25 μl 40 mM H₂O₂ solution to each microplate well containing the samples and controls.
5. Incubate the reaction for 30 min at room temperature.
6. Prepare a working solution of 100 μM Amplex Red reagent containing 0.4 U/ml HRP by adding 50 μL Amplex Red reagent stock solution and 20 μl HRP stock solution to 4.93 ml 1X Reaction Buffer.
7. Add 50 μl Amplex Red/HRP working solution to each well containing samples and controls.
8. Incubate the reaction for 30 min at 37°C protected from light.
9. Measure the fluorescence in a microplate reader using excitation in the range of 530-560 nm and emission at 590 nm.

Calculation of catalase activity:
Report the change in fluorescence by subtracting the sample value from that of the no-catalase control.
Example Samples (0.5 μg protein)

<table>
<thead>
<tr>
<th>Heifer #</th>
<th>Change in fluorescence</th>
<th>Catalase activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18662</td>
<td>3732</td>
<td>11.3</td>
</tr>
<tr>
<td>951-2</td>
<td>4782</td>
<td>16.4</td>
</tr>
<tr>
<td>18849</td>
<td>5636</td>
<td>21.1</td>
</tr>
</tbody>
</table>

Figure B.12.1 Catalase activity plot
Appendix B.13 Caspase Assays using BD ApoAlert™ Capase Assay Plate (BD Biosciences)

Preparation of solutions:
1. Aliquot a sufficient volume of 2X Reaction Buffer for the number of assays to be performed and add DTT to the 2X Reaction Buffer: add 10 ml 1 M DTT per 1 ml 2X Reaction Buffer.

Performing the assay:
1. Add 50 µl 2X Reaction Buffer/DTT mix to each well of the 96-well microplate that will be used.
2. Transfer 50 µl of the appropriate cell lysates to the wells. Seal the plate with parafilm and incubate at 37°C for 2 h in an incubator.
3. Analyze the plate in a fluorescent plate reader (excitation: 380 nm; emission; 460 nm).

Calculation of relative caspase activity:
1. Subtract the fluorescence of a no-lysate control from the fluorescence of each sample.
Appendix B.14 Preparation of nuclear extract

1. Wash 0.5 – 1X10^6 cells with TBS, then centrifuge 15 min, 800 x g.
2. Resuspend pellet in 400 μl (per each 10^6 cells) cold Buffer A by gentle pipetting.
   Buffer A: 10 mM HEPES pH 7.9
   10 mM KCl
   0.1 mM EDTA
   0.1 mM EGTA
   1.0 mM DTT (4°C) *NEED TO ADD FRESH
   0.5 mM PMSF (-20°C) *NEED TO ADD FRESH

   **For 2 ml Cold Buffer A: Add 4 μl 0.5 M DTT
   Add 1 μl 1M PMSF

   *******KEEP EVERYTHING ON ICE!!!! **************

3. Allow cells to swell on ice 15 minutes.
4. Add 25 μl (per 400 μl Cold Buffer A) 10% Nonidet NP-40.
5. Vigorously vortex 10 seconds.
6. Centrifuge 30 sec at 10,000 x g.
7. Transfer supernatant (cytoplasm & RNA) to a fresh tube.
   To RNA tube: Add 400 μl (per original 400) Buffer B (Room Temp).
   Add 600 μl phenol/chloroform (1:1).
   Mix immediately & store at -80°C until ready to prepare RNA.
   Buffer B: 10 mM Tris pH 7.5
   7 M urea
   1 % SDS
   0.3 M NaAc
   20 mM EDTA

8. Re-suspend nuclear pellet from step 6 in 50 μl ice-cold Buffer C (4°C).
   Buffer C: 20 mM HEPES pH 7.9
   0.4 M NaCl
   1 mM EDTA
   1 mM EGTA
   1 mM DTT **NEED TO ADD FRESH
   1 mM PMSF **NEED TO ADD FRESH

   **For every 2 ml Buffer C: Add 4 μl 0.5 M DTT
   Add 2 μl 1M PMSF

9. Vigorously rock 4°C 15 minutes on shaking platform.
10. Centrifuge 5 min, 10,000 x g, 4°C (~55 μl).
11. Freeze in aliquots (~ 10 μl) at −80°C.
Appendix B.15 Electrophoretic mobility shift assay (EMSA) using Gel Shift Assay System (Promega)

Preparation of probe:
1. Assemble the following reaction in a microcentrifuge tube:
   - 2.0 µl NF-κB Consensus Oligonucleotide (1.75 pmol/µl)
   - 1.0 µl T4 Polymerase Kinase 10X Buffer
   - 1.0 µl [γ-32P]ATP (3000Ci/mmol at 10mCi/ml)
   - 5.0 µl Nuclease-Free Water
   - 1.0 µl T4 Polynucleotide Kinase (5-10 u/µl)
2. Incubate at 37°C for 10 min.
3. Stop the reaction by adding 1.0 µl 0.5 M EDTA.
5. Purify the probes using G-25 quickspin columns (Roche Pharmaceuticals)
   - Invert the column several times.
   - Remove top and bottom caps and allow to drain.
   - Centrifuge with collection tube 1100 x g, 2 min.
   - Apply labeled probe to center of column bed.
   - Place column in new collection tube.
   - Centrifuge 1100 x g, 4 min.
   - Add 50 µl TE to center of column bed and centrifuge 1100 x g, 4 min.
   - Store purified probe in collection tube at -20°C.

Preparation of gel (done day before assay):
For 4% acrylamide gel:
1.0 ml 10X TBE
1.25 ml acrylamide/bis/acrylamide 37.5:1 (40% w/v)
0.75 ml 40% acrylamide
625 µl 80% glycerol
16.2 ml water
   - Stir at room temp until dissolved.
   - Just before pouring add:
     150 µl 10% APS
     10 µl TEMED
**Performing the assay:**

1. Assemble the following reactions in sterile microcentrifuge tubes in the order shown:

   Reaction #1 (negative control):
   - 7.0 μl Nuclease-Free Water
   - 2.0 μl Gel shift Binding 5X Buffer
   - 0 μl HeLa Nuclear Extract
   - 9.0 μl total volume

   Reaction #2 (positive control):
   - 5.0 μl Nuclease-Free Water
   - 2.0 μl Gel shift Binding 5X Buffer
   - 2.0 μl HeLa Nuclear Extract
   - 9.0 μl total volume

   Reaction #3 (specific competitor):
   - 4.0 μl Nuclease-Free Water
   - 2.0 μl Gel shift Binding 5X Buffer
   - 2.0 μl HeLa Nuclear Extract
   - 1.0 μl unlabeled competitor oligo (1.75 pmol NF-κB Consensus Oligo)
   - 9.0 μl total volume

   Reaction #4 (nonspecific competitor):
   - 4.0 μl Nuclease-Free Water
   - 2.0 μl Gel shift Binding 5X Buffer
   - 2.0 μl HeLa Nuclear Extract
   - 1.0 μl unlabeled noncompetitor oligo (1.75 pmol AP2 Consensus Oligo)
   - 9.0 μl total volume

   Sample Reactions:
   - ? μl Nuclease-Free Water
   - 2.0 μl Gel shift Binding 5X Buffer
   - 5.0 μg Sample Nuclear Extract
   - 9.0 μl total volume

2. Incubate the reactions at room temperature for 10 min.
3. Add 2 μl labeled consensus sequence and incubate 30 min at room temperature.
4. Resolve nuclear acid-protein complexes on a 4% acrylamide gel in 0.5X TBE at 300V.
5. Dry gels 2 h at 80°C and detect bands by autoradiography.
Appendix B.16 Preparation of RNA after nuclear protein isolation

1. Centrifuge cytosolic fractions 1 min at 15,000 x g.

2. Transfer the aqueous phase to a new tube and precipitate with 1 ml 95% ethanol for 2 hr at –80°C.

3. Centrifuge samples 12,000 x g, 15 min, 4°C. Wash each RNA pellet once with 70% ethanol and resuspend in 60 µl DEPC-treated water.
Appendix B.17 Annexin Staining of cells cultured in chamber slides using Vybrant Apoptosis Assay Kit #2 (Molecular Probes)

1. After the culture period, wash the cells in cold PBS.
2. Prepare 1X Annexin-Binding Buffer.
3. Prepare 100μg/ml solution of propidium iodide (PI) in 1X Annexin-Binding Buffer.
4. Add 5 μl Alexa Fluor 488 annexin V (Component A) and 1 μl 100 μg/ml PI working solution to each 100 μl of 1X Annexin-Binding Buffer.
5. Add 100 μl annexin V/PI/Annexin-Binding Buffer to each well.
6. Incubate the cells at room temperature for 15 min protected from light.
7. Remove the annexin V/PI/Annexin-Binding Buffer and wash the cells in cold PBS.
8. Add 100 μl acetone to each well and incubate 5 min at room temperature protected from light.
9. Wash the cells in cold PBS 3 times.
10. Dissemble the chamber slides; cover the samples with fluorescent mounting medium and coverslip.
11. View with Olympus BX50WI fluorescence microscope using FITC filter.
12. Count at least 800 cells, noting if they were unstained, stained with only annexin V, or stained with both annexin V and PI.
Appendix B.18 TUNEL Staining of cells cultured in chamber slides using In Situ Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals)

**Preparation of TUNEL reaction mixture (for 5 wells):**
1. Remove 100 ml Label solution from bottle 2 for two negative controls.
2. Add total volume of bottle 1 to the remaining 450 ml Label Solution in bottle 2 to obtain 500 ml TUNEL reaction mixture.
3. Mix well.

**Labeling of cells:**
1. Fix air-dried cell samples with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 1 h at 25°C.
2. Rinse slides with PBS and incubate in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice.
3. Rinse slides twice with PBS.
4. Add 50 μl TUNEL reaction mixture to sample.
5. Incubate slide in a humidified chamber for 60 min at 37°C in the dark.
6. Rinse slide 3 times with PBS.
7. Disassemble the chamber slides; cover the samples with fluorescent mounting medium and coverslip.
8. View with Olympus BX50WI fluorescence microscope using FITC filter.
9. Count at least 800 cells, noting if they were unstained or TUNEL positive.
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