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ROLE OF BCL-2 PROTEINS IN STRESS-INDUCED APOPTOSIS IN A NON-TRANSFORMED MAMMARY EPITHELIAL CELL LINE

By

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ABSTRACT OF THE THESIS

Role of Bcl-2 Proteins in Stress-induced Apoptosis in a Non-transformed Mammary Epithelial Cell Line

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Apoptosis is the process of programmed cell death characterized by morphological and physiological changes executed by cells in response to a stimulus. The apoptotic process contributes to the loss of milk secreting cells (i.e. mammary epithelial cells) that occurs in the bovine mammary gland once peak lactation has occurred. However, the specific mechanisms by which this occurs are unknown. The goal of this work was to examine the role of the Bcl-2 family of proteins in regulating apoptosis in the bovine mammary epithelial cell line, MAC-T.

The Bcl-2 family is divided into three categories based on protein function and combinations of four Bcl-2 homology (BH) domains. Anti-apoptotic multidomain proteins, such as Bcl-2 and Mcl-1, prevent apoptosis by binding to pro-apoptotic members of the family. Pro-apoptotic multidomain proteins, such as Bax, help to regulate apoptosis at the mitochondrial outer membrane. Pro-apoptotic BH3-only

proteins, such as Bad and Bim, act as messengers between the anti-apoptotic and pro-apoptotic proteins.

To investigate the roles of Bcl-2 proteins in apoptosis, MAC-T cells were treated with anisomycin (ANS), an activator of the intrinsic apoptotic pathway. ANS had little effect on *Bcl-2* or *Bax* mRNA levels while it induced a 40 to 60% decrease in levels of *Bad* mRNA. Protein expression of Bax did not change during apoptosis, while Bcl-2, Mcl-1, and Bad expression decreased to varying degrees. The greatest change in protein levels was observed for Mcl-1, whose expression was nearly non-detectable after 4 h of treatment with ANS. Bim was phosphorylated in response to ANS but this was not caused by JNK or p38 signaling. Knock-down of Bim using siRNA decreased the ability of ANS to induce apoptosis. Mcl-1 and Bim protein expression changed in a similar time frame, therefore, interactions between the two proteins were evaluated using co-immunoprecipitation experiments. Mcl-1 and Bim interacted both basally and after treatment with ANS. The significance of Bim phosphorylation, the kinase responsible for its phosphorylation, and the functional significance of the interaction between Bim and Mcl-1 in stress-induced apoptosis remain to be determined.

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ABBREVIATIONS

Akt	Protein kinase B
Apaf-1	Apoptotic protease activating factor 1
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
BCR	B cell antigen receptor
BH	Bcl-2 homology
Bim	Bcl-2 interacting mediator of cell death
CD95	Fas receptor
CD95L	Fas ligand
CREB	cAMP response element binding protein
Cyt c	Cytochrome c
DLC1	Dynein light chain 1
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
IGF-1	Insulin-like growth factor 1
IGFBP-3	Insulin-like growth factor binding protein 3
JNK	c-Jun N-terminal kinase
LBD	Light chain binding domain
Mcl-1	Myeloid cell leukemia sequence 1

MOMP	Mitochondrial outer membrane permeabilization
Mule	Mcl-1 ubiquitin ligase E3
PI3K	Phosphatidylinositol 3 kinase
SIMPs	Soluble intermembrane proteins
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

CHAPTER 1: REVIEW OF LITERATURE

Apoptosis

Apoptosis is the process of programmed cell death characterized by morphological and physiological changes executed by cells in response to a stimulus. Some of the major distinguishing features of apoptotic cell death include presentation of phosphatidylserine on the cell surface, caspase activation, mitochondrial outer membrane permeabilization (MOMP), loss of mitochondrial membrane potential, release of soluble intermembrane proteins (SIMPs) such as cytochrome c, DNA fragmentation, and cell membrane blebbing (Chipuk and Green, 2006; Debatin, 2004; Goldstein et al., 2000; Grimberg, 2000; Kroemer and Reed, 2000). There are several environmental insults that can induce apoptosis including tumor necrosis factor alpha (TNF-α), Fas ligand (CD95L), UV radiation, trophic factor deprivation, staurosporine, oxidative stress, ricin, and anisomycin (Bassik et al., 2004; Hori et al., 2008; Kulms and Schwarz, 2000; Putcha et al., 2003; Rajah et al., 2002; Zheng et al., 2008). The response to such insults is cell type-specific and stimulus-dependent.

Stressors can induce apoptosis using different pathways within the cell. There are two major pathways that culminate in apoptosis and converge at the mitochondria. These pathways are known as the extrinsic pathway and the intrinsic pathway (Figure 1). The extrinsic pathway of apoptosis requires ligand binding and the consequent activation of a death receptor on the cell surface. The intrinsic pathway of apoptosis

does not require binding to a ligand, but is often caused by damage to cellular components including ribosomes and DNA.

Extrinsic Pathway

The extrinsic pathway of apoptosis is activated by the binding of a ligand to a death receptor in the TNF family on the cell surface. Examples of death receptor ligands include TNF-α and CD95L (Kulms and Schwarz, 2000). When the Fas receptor (CD95) is activated by its ligand, the death domain in its cytoplasmic region recruits Fas-associating protein with death domain (FADD). FADD binds to caspase-8 and activates it by cleaving it (Kulms and Schwarz, 2000). Activated caspase-8 cleaves Bid to its truncated form, tBid (Luo et al., 1998). Bid is a member of the large family of Bcl-2 proteins that regulate apoptosis. This family of proteins will be discussed in further detail in the next section.

After Bid is cleaved, tBid translocates to and acts on the mitochondria by inactivating anti-apoptotic proteins and enhancing MOMP through the formation of pores (Kroemer and Reed, 2000; Kulms and Schwarz, 2000). Other TNF family ligands use this same signal transduction mechanism to induce apoptosis, but may require an adaptor protein to bind to the activated receptor before FADD recruitment. For example, TNF-α signaling requires that TRAF binds to the TNF-α receptor before FADD will bind.

Intrinsic Pathway

The intrinsic pathway of apoptosis is also known as the "stress pathway" and does not require a death receptor (Adams and Cory, 2007). The Bcl-2 proteins are heavily involved in the early events of the intrinsic pathway by controlling the mitochondrial membrane potential. The Bcl-2 proteins are categorized based on their functions and the presence of one or several Bcl-2 homology (BH) domains (Danial, 2007; Marzo and Naval, 2008). There are four BH domains in total and these are present in different combinations in the Bcl-2 proteins (Figure 2). The family of proteins is split into three categories: (1) pro-apoptotic BH3-only; (2) anti-apoptotic multi-domain; and (3) pro-apoptotic multi-domain proteins. The pro-apoptotic BH3-only proteins act as sensors or messengers that interact with the anti-apoptotic and pro-apoptotic multi-domain proteins to potentiate the apoptosis pathway.

The Bcl-2 proteins have been extensively studied; however, there are still specific details related to their mechanisms of action that are not fully understood. Factors that control cell survival and apoptosis can regulate Bcl-2 proteins by changing their abundance or by inducing post-translational modifications that affect protein-protein interactions and cellular localization. There are two major models describing Bcl-2 family interactions: the indirect activation model and the direct activation model (Adams and Cory, 2007; Danial, 2007). In the indirect activation model, an anti-apoptotic multi-domain Bcl-2 protein is bound to a pro-apoptotic multi-domain protein at the mitochondrial outer membrane and prevents its activation. Upon receiving an apoptotic stimulus, a BH3-only protein becomes activated and binds to the anti-

apoptotic multi-domain Bcl-2 protein and disrupts its interaction with the pro-apoptotic multi-domain protein. The "free" pro-apoptotic multi-domain protein is able to oligomerize with other pro-apoptotic multi-domain proteins at the mitochondrial outer membrane in order to form a pore and induce MOMP (Adams and Cory, 2007; Danial, 2007).

In the direct activation model, BH3-only proteins are referred to as either sensitizers or activators. Both sensitizer and activator BH3-only proteins are basally sequestered by anti-apoptotic multi-domain Bcl-2 proteins. This prevents them from interacting with the pro-apoptotic multi-domain Bcl-2 proteins. When an apoptotic stimulus is received, the amount of the sensitizer BH3-only protein increases and the anti-apoptotic multi-domain Bcl-2 proteins begin to release the activator BH3-only proteins due to differences in binding affinities between sensitizers and activators (Danial, 2007). The activator BH3-only proteins bind to the pro-apoptotic multi-domain proteins at the mitochondria and allow them to oligomerize and form pores.

Release of Soluble Intermembrane Proteins (SIMPs)

The intrinsic and extrinsic pathways of apoptosis converge at the mitochondria. The events that occur after MOMP are well-established in several systems (Danial, 2007; Kroemer and Reed, 2000; Kulms and Schwarz, 2000). Following the upstream events, pro-apoptotic members of the Bcl-2 family of proteins oligomerize in the mitochondrial outer membrane and form pores. SIMPs, such as cytochrome c, are released through these pores. Cytosolic cytochrome c binds to apoptotic protease-activating factor (Apaf-

1) and procaspase-9 to form a complex known as the apoptosome (Figure 3). ATP is required for procaspase-9 to bind to Apaf-1 and a different mechanism of cell death, necrosis, occurs if the amount of ATP in the cell is too low (Kroemer and Reed, 2000; Kulms and Schwarz, 2000). Procaspase-9 is activated in the apoptosome and triggers the later stages of apoptosis by cleaving caspase-3 (Debatin, 2004; Kroemer and Reed, 2000). Caspase-3 cleaves caspase-6 and caspase-7 to their active forms which inactivate vital proteins in the cell, such as DNA repair enzymes (e.g. PARP), causing DNA fragmentation and cell death (Hilmi et al., 2008).

The Bcl-2 Family of Proteins

Anti-apoptotic Multidomain Members

The anti-apoptotic members of the Bcl-2 family of proteins prevent apoptosis by sequestering pro-apoptotic BH3-only proteins and by binding to pro-apoptotic multidomain proteins to prevent MOMP. The proteins in this category contain different combinations of all four BH domains. Overexpression of the anti-apoptotic proteins contributes to cell survival and is often found in cancer cells (Debatin, 2004; Placzek et al., 2010; Taniai et al., 2004). There are many anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-w, Bcl-x_L, Bfl-1, Boo, and Mcl-1 (Marzo and Naval, 2008). This work focuses on Bcl-2 and Mcl-1, two proteins that are often involved in opposing stress-induced cell death.

Bcl-2

Bcl-2 was the first protein to be discovered in the Bcl-2 family. It was found to be upregulated in B-cell lymphoma 2 cells, thus earning the protein its name. Survival factors such as IGF-1 and IL-1β increase *Bcl-2* mRNA and protein levels by activating PI3K, Akt, CREB, and STAT3 (Chand et al., 2012; Hilmi et al., 2008; Pugazhenthi et al., 2000; van Golen et al., 2000). However, total protein levels of Bcl-2 are not altered during apoptosis in some cell types (Moulding et al., 2001; Rajah et al., 2002). Instead, the ability of Bcl-2 to interact with other Bcl-2 family members is regulated at the post-translational level by mechanisms such as phosphorylation.

Bcl-2 can be phosphorylated in the region between the BH3 and BH4 domains. Phosphorylation at this location inactivates the protein's survival function (Bassik et al., 2004; Danial, 2007). When apoptosis is caused by IGFBP-3 overexpression or by serum starvation, Bcl-2 is phosphorylated (Rajah et al., 2002; Wei et al., 2008) or cleaved by caspases (BH4 domain is removed) (Danial, 2007). Phosphorylation of Bcl-2 can affect its ability to bind other Bcl-2 family proteins. Bcl-2 binds to Bax, Bad, Bid, and Bim and phosphorylation disrupts its interaction with these proteins (Bassik et al., 2004). Under basal conditions, Bcl-2 is able to bind to Bim to sequester it at the mitochondria and prevent it from interacting with other pro-apoptotic proteins (Gomez-Bougie et al., 2005). Phosphorylated Bcl-2 is unable to protect cells from apoptosis because it cannot prevent MOMP. Bcl-2 can also bind to Bad during apoptosis. This interaction causes Bcl-2 to move away from its pro-apoptotic counterparts at the mitochondria (Ruffolo and Shore, 2003).

Mcl-1

Mcl-1(myeloid cell leukemia sequence 1) is another anti-apoptotic Bcl-2 protein. It is one of the only anti-apoptotic members that does not contain the BH4 domain and has a shorter half-life than the other proteins in this category (Michels et al., 2005). Levels of *Mcl-1* mRNA and protein increase in response to growth factors and differentiation factors and increases in mRNA levels typically correspond with increases in protein levels (Iglesias-Serret et al., 2003; Michels et al., 2005). Mcl-1 is required for survival in some cell types (Iglesias-Serret et al., 2003; Michels et al., 2005). Antisense depletion of *Mcl-1* mRNA decreases levels of the protein and increases sensitivity to apoptosis (Moulding et al., 2000; Song et al., 2013; van Delft et al., 2006). Apoptotic stimuli, such as staurosporine, infection with adenovirus, or treatment with TRAIL decrease transcription of Mcl-1 and levels of *Mcl-1* mRNA and protein (Cuconati et al., 2003; Iglesias-Serret et al., 2003; Michels et al., 2005; Moulding et al., 2001; Weng et al., 2005).

Mcl-1 is rapidly turned over in cells by ubiquitination and degradation in the proteasome. There are three steps involved in the ubiquitination of Mcl-1. A ubiquitin-activating enzyme (E1) uses ATP to activate the C-terminal end of ubiquitin. Then, the activated ubiquitin binds to a ubiquitin carrier protein (E2), Ubch7, and is ultimately attached to Mcl-1 by Mule (E3). Mule contains a region similar to the BH3 domain of the Bcl-2 proteins and this region allows it to bind to Mcl-1 (Zhong et al., 2005). The depleted Mcl-1 is rapidly regenerated under basal conditions, but a decrease in the amount of total Mcl-1 protein is observed early in apoptosis (Adams and Cory, 2007;

Cuconati et al., 2003; Danial, 2007; Du et al., 2010; Miller et al., 2013; Weng et al., 2005; Willis et al., 2005; Wuilleme-Toumi et al., 2007). In HeLa cells, the decrease in protein abundance observed during apoptosis results from a blockage of Mcl-1 synthesis, not an increase in the rate of protein degradation (Nijhawan et al., 2003). If degradation of Mcl-1 is blocked using a proteasome inhibitor, cells are more resistant to apoptosis (Nijhawan et al., 2003). Degradation of Mcl-1 can also be decreased by mutating Noxa so that it cannot bind to Mcl-1 (Willis et al., 2005). Noxa interacts with Mcl-1 during apoptosis and may play a role in its degradation (van Delft et al., 2006).

Mcl-1 is not just degraded during apoptosis, it can also be cleaved by caspases (Herrant et al., 2004; Michels et al., 2005; Weng et al., 2005). Mcl-1 is cleaved by caspases in response to activation of the extrinsic apoptosis pathway. Caspase cleavage of Mcl-1 generates fragments of two different sizes. The Mcl-1 fragments play anti-apoptotic roles in some cell types and are pro-apoptotic in others (Figure 4). In some cell types, the Mcl-1 fragments do not have pro-apoptotic functions, but instead have impaired anti-apoptotic functions and cannot counteract apoptosis induced by Bim_{EL} (Herrant et al., 2004). In other studies, it has been found that the C-terminal fragment of Mcl-1 is pro-apoptotic, interacts with tBid, and causes cell death when it is overexpressed in cells and may participate in a positive feedback loop during extrinsic apoptosis (Michels et al., 2005; Weng et al., 2005).

Mcl-1 interacts with other Bcl-2 family proteins by binding to their BH3 domains (Chen et al., 2005; Willis et al., 2005). Mcl-1 can bind to Bak. The interaction between Mcl-1 and Bak blocks MOMP (Adams and Cory, 2007; Danial, 2007). Mcl-1 can also bind

to the BH3-only proteins Bim and Noxa, but cannot bind to Bad. Mcl-1 is basally bound to the BH3 domain of Bak to prevent apoptosis and the interaction between Mcl-1 and Bim or Noxa disrupts the bond (Adams and Cory, 2007; Cuconati et al., 2003; Du et al., 2010; Miller et al., 2013; Puthalakath et al., 1999; Willis et al., 2005). When Mcl-1 is no longer bound to Bak, apoptosis can occur.

Pro-apoptotic Multidomain Members

The pro-apoptotic multidomain members of the Bcl-2 family help to control the permeability of the mitochondria during apoptosis and are required for intrinsic apoptosis to occur (Chu et al., 2009; Danial, 2007; Wei et al., 2001). The proteins in this category contain BH domains 1-3 and consist of three members: Bak, Bax, and Bok (Lalier et al., 2007). Bak and Bax will be discussed in further detail. These two proteins hetero- and homo-oligomerize at the mitochondrial outer membrane to form pores during apoptosis. In order to do this, both proteins undergo conformational changes. Bak and Bax have individual or redundant functions in response to cellular stressors; therefore elimination of one of the two does not always prevent apoptosis.

Bak

Bak is one of the "gatekeeper proteins" of the mitochondria. The protein is also known as Bcl-2 homologous antagonist/killer because it contains BH regions 1-3 and has a pro-apoptotic function. The BH3 domain is a critical region of the protein and without it, Bak cannot cause apoptosis (Dewson et al., 2008). Bak is less abundant in some

cancer cells and a lack of the protein may contribute to cancer cell survival (Partik et al., 1998; Wang et al., 2001). It has been found that Bak is required for cytochrome c release and apoptosis after treatment with tBid, cisplatin, or staurosporine (Wang et al., 2001). Transcription of Bak has been found to increase in response to several stressors, but its protein levels do not change (Moulding et al., 2001). Therefore it is thought that regulation of the amount of Bak is not one of the major mechanisms of its actions (Adams and Cory, 2007; Willis et al., 2005). Bak resides at the mitochondria both basally and during apoptosis and the interactions that Bak has with other proteins largely determine its function.

Bak undergoes conformational changes during apoptosis induced by UV radiation, actinomycin D, adenovirus infection, and staurosporine that allow it to induce MOMP (Cuconati et al., 2003; Llambi et al., 2011; Xiao et al., 2004). The N-terminal domain of Bak is exposed during apoptosis and has been proposed as an indicator of conformational changes (Dewson et al., 2008). Mcl-1 and Bcl-x_L interact with Bak under basal conditions (Cuconati et al., 2003; Du et al., 2010; Miller et al., 2013; Willis et al., 2005). Conformational changes in Bak have been shown to be blocked by treating cells with Mcl-1 and Bcl-x_L because they bind to the BH3 domain of Bak (Llambi et al., 2011; Willis et al., 2005). Bcl-2 is not able to have this same effect because it does not interact with Bak.

Early in apoptosis, the anti-apoptotic proteins are displaced from Bak by BH3-only proteins and MOMP occurs (Adams and Cory, 2007; Cuconati et al., 2003; Willis et al., 2005). While the disruption of the interaction between Bak and anti-apoptotic

proteins is important, it is not enough to induce apoptosis in all cell types (Danial, 2007; Willis et al., 2005). In order for apoptosis to occur, Bak must be fully activated by BH3-only proteins. The BH3-only proteins Bim, Bid, and Puma bind to Bak. The interaction between Bak and these proteins is transient and may only occur long enough for Bak to change its conformation (Danial, 2007). Overexpression of Bim has been shown to increase Bak oligomerization and tBid activates Bak and causes it to form oligomers (Cheng et al., 2001; Wei et al., 2001).

Bak is also capable of binding to itself and forming homo-oligomers (Adams and Cory, 2007; Dewson et al., 2008; Nijhawan et al., 2003). The homo-oligomers appear as clusters during apoptosis and colocalize with Bax (Nechushtan et al., 2001). When degradation of Mcl-1 is prevented using a proteasome inhibitor, Bak oligomerization is blocked and apoptosis does not occur (Nijhawan et al., 2003; Willis et al., 2005). This indicates that Mcl-1 prevents Bak oligomerization to protect cells. Bak oligomerizes using its BH3 domain. When Bcl-x_L is overexpressed, Bak does not form clusters during apoptosis because Bak binds to itself and binds to Bcl-x_L at the same site. The BH3 domain of Bak is only briefly exposed during apoptosis because it is hidden before oligomerization occurs and is not accessible after oligomerization occurs (Dewson et al., 2008).

Bax

Bax mRNA increases in response to several agents including cerulein, H_2O_2, and doxorubicin (Kaeser and Iggo, 2002; Moulding et al., 2001; Nakamura and Sakamoto,

2001; Yu et al., 2003). While *Bax* mRNA increases in response to the previously mentioned stressors, the amount of Bax protein does not always increase in response to apoptotic stimuli including ANS and serum starvation (Braun et al., 2011; Moulding et al., 2001; Tsuruta et al., 2004). This indicates that there are other mechanisms that regulate Bax.

Bax is regulated by its cellular localization. This protein typically resides in the cytosol as a soluble monomer and embeds in the mitochondria during apoptosis (Chu et al., 2009; Danial, 2007; Lalier et al., 2007; Tsuruta et al., 2004). Movement of Bax to the mitochondria has been found to be dependent on the activation of signaling molecules such as JNK (Chu et al., 2009). The changes in Bax localization are caused by conformational changes in the protein (Chu et al., 2009). Under basal conditions, Bax's hydrophobic helices are embedded within the amphipathic regions of the protein to create a stable and soluble structure (Lalier et al., 2007). During apoptosis, several features of Bax are exposed when its conformation changes. The hydrophobic helices of the protein are moved out of the core and increase the protein's affinity for the mitochondrial outer membrane. A mitochondrial targeting domain, a tail anchoring sequence, a hydrophobic groove, and the BH3 domain are also exposed (Chu et al., 2009; Lalier et al., 2007). The cooperation of all of these features allows Bax to embed itself in the mitochondrial outer membrane. Once Bax is embedded in the mitochondrial outer membrane, it must still undergo more conformational changes before it can oligomerize and form a pore. These changes are dependent on interactions with other proteins.

Other members of the Bcl-2 family are able to bind to Bax using its hydrophobic groove or its BH3 domain (Adams and Cory, 2007; Lalier et al., 2007). Bcl-2 and Bcl-x_L bind to the BH3 domain of Bax. The interaction between Bax and Bcl-2 earned the protein its full name, Bcl-2-associated X protein. The interaction between Bax and an anti-apoptotic protein is stabilized by a bond between the BH4 regions of the anti-apoptotic protein and the hydrophobic groove of Bax. When Bcl-x_L interacts with Bax, cells are more resistant to apoptosis (Chu et al., 2009). Some pro-apoptotic BH3-only proteins, including Bid, Bim, and Puma, are also able to bind to Bax and can displace pro-survival molecules (Braun et al., 2011; Chipuk et al., 2008; Gallenne et al., 2009; Gautier et al., 2011). When Puma binds to Bax, it promotes the protein's movement to mitochondria in order to cause apoptosis (Gallenne et al., 2009). In cell lines that constitutively overexpress Puma, cell survival depends on the upregulation of Bcl-x_L and subsequent inhibition of Bax (Gautier et al., 2011).

Bax is also capable of binding to itself and to Bak. Bax forms dimers in the mitochondrial outer membrane during apoptosis (Cheng et al., 2001; Chu et al., 2009). Homo- and hetero-oligomerization of Bax is a crucial step in mitochondrial pore formation during apoptosis. Pore formation results in the release of SIMPs from the mitochondria and activation of downstream effectors.

Pro-apoptotic BH3-only Members

The BH3-only members of the Bcl-2 family of proteins are pro-apoptotic and link the initial steps of the apoptosis pathway to the events at the mitochondria. These

proteins only contain one of the four BH domains, the BH3 domain. The BH3 domain is the region of the Bcl-2 proteins that allow them to bind to each other (Danial, 2007). BH3-only proteins have different binding affinities for the other Bcl-2 proteins and for this reason, may have different biological functions (Chen et al., 2005). The binding partners of the BH3-only proteins have been used to further classify them as sensitizers or activators. Sensitizers bind to anti-apoptotic proteins to block their actions and activators bind to pro-apoptotic proteins to activate them. There are several BH3-only proteins including Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa, and Puma (Adams and Cory, 2007; Chipuk et al., 2008). Other proteins have been proposed to be part of this category based on their structures and functions but these data have not been widely accepted (Marzo and Naval, 2008). In the present work, Bad and Bim were studied to determine if either plays a role in the ANS-induced intrinsic apoptosis pathway in bovine MECs.

Bad

Bad mRNA levels increase in response to apoptotic stimuli including high glucose treatment of human pancreatic islets of Langerhans and treatment with TNF-α (Federici et al., 2001; Moulding et al., 2001). Even though mRNA levels have been reported to increase, the abundance of Bad protein does not increase during apoptosis (Moulding et al., 2001; Sunayama et al., 2005). This indicates that there are other mechanisms aside from transcriptional regulation that are important in the control of Bad.

There are several studies that show Bad is regulated by post-translational modifications, namely phosphorylation. Depending on the location of Bad

phosphorylation, the protein's pro-apoptotic actions can be blocked or enhanced. Different kinases are responsible for blocking or enhancing Bad's functions. Pro-survival molecules such as Akt, PKA, PKC, and Raf1 phosphorylate Bad at Ser112, Ser136, or at both sites and block the effects of the protein (del Peso et al., 1997; Zha et al., 1996). JNK enhances Bad activity by phosphorylating it at Ser128 (Dhanasekaran and Reddy, 2008; Donovan et al., 2002). The phosphatase calcineurin dephosphorylates Bad to return it to its basal form (Kroemer and Reed, 2000).

Phosphorylation of Bad controls the protein's localization within the cell. Under conditions that favor cell survival, Bad is sequestered by 14-3-3 in the cytosol when it is phosphorylated at Ser112, Ser136, or both (Adams and Cory, 2007; Dhanasekaran and Reddy, 2008). Bad is found at the mitochondria when it is not phosphorylated at those sites (Zha et al., 1996). When Bad is phosphorylated at Ser128, it is no longer able to bind to 14-3-3 and can therefore move to the mitochondrial membrane and help promote cell death.

Bad has been reported to bind to other Bcl-2 family proteins. Bad is also known as Bcl-2-associated death promoter because it can interact with Bcl-2. Bad can also interact with Bcl-w and Bcl-x_L in order to prevent these anti-apoptotic proteins from binding to Bax and Bak (Adams and Cory, 2007; Ruffolo and Shore, 2003). Unlike some of the other pro-apoptotic BH3-only proteins, Bad cannot bind to Bax and Bak (Adams and Cory, 2007; Cheng et al., 2001; Chipuk et al., 2008). For these reasons, the protein is often referred to as a sensitizer. When Bad is phosphorylated in response to treatment with IL-3, it does not bind to Bcl-x_L but does bind to 14-3-3 (Zha et al., 1996).

When Bad is sequestered by 14-3-3, it is unable to bind to its anti-apoptotic counterparts, so this interaction promotes cell survival.

Bim

There are three different isoforms of Bim that are generated by alternative splicing: Bim_{EL}, Bim_{L}, and Bim_{S} (subscripts refer to extra-long, long, and short). Of the three, Bim_{EL} and Bim_{L} are the dominant forms in most cell types, but Bim_{S} is the most potent at inducing apoptosis (Day et al., 2004; Puthalakath et al., 1999). Bim is regulated at the transcriptional level by the forkhead transcription factor FOXO-3A in response to growth factor deprivation and ERK inhibition and contributes to apoptosis (Adams and Cory, 2007; Harada and Grant, 2012; Ley et al., 2005). Growth factor deprivation and ERK inhibition cause an increase in the levels of *Bim* mRNA and serum starvation increases the amount of Bim protein found in the cell (Braun et al., 2011; Ley et al., 2005). Bim transcription is not activated when growth factors are present because Akt phosphorylates FOXO-3A and the transcription factor becomes sequestered to 14-3-3 proteins (Harada and Grant, 2012).

Interactions between Bim and other proteins contribute to the regulation of its effects. Bim is known as an activator because it has been reported that Bim can bind directly to Bak and Bax in some cell types to mediate MOMP (Adams and Cory, 2007; Chipuk et al., 2008; Danial, 2007; Harada et al., 2004). Other studies have found that Bim cannot bind to Bak and Bax and have led to the proposal that Bim exerts its role in apoptosis by binding to and neutralizing the anti-apoptotic Bcl-2 proteins (Chen et al.,

2005; Gomez-Bougie et al., 2005). Bim is able to bind to several of the anti-apoptotic Bcl-2 proteins using its BH3 domain. The ability of Bim to bind to Bcl-2 has earned the protein its name, B cell lymphoma 2 interacting mediator of cell death. In some cell types, Bim is basally bound to Bcl-2 at the mitochondria and the amount of Bim bound to Bcl-2 increases during apoptosis (Gomez-Bougie et al., 2005; Zhu et al., 2004). Bim is also capable of binding to Bcl-x_L and Mcl-1 and interactions between Bim and Mcl-1 have been found to contribute to apoptosis in some cell types (Gomez-Bougie et al., 2005; Harada and Grant, 2012; Wuilleme-Toumi et al., 2007; Zhu et al., 2004).

In some cell types, Bim_{EL} and Bim_L can be sequestered to microtubules by binding to dynein light chain 1 (DLC1) using their light chain binding domains (LBD) (Adams and Cory, 2007; Day et al., 2004; Puthalakath et al., 1999). Bim_L binds to Bcl-2 and apoptosis occurs when the interaction between Bim and DLC1 is lost in response to an apoptotic stimulus such as B cell antigen receptor (BCR) ligation, staurosporine, doxorubicin, or taxol (Enders et al., 2003; Puthalakath et al., 1999). When Bim_{EL} and Bim_L are mutated to prevent them from binding to DLC1, their apoptotic effects increase and are comparable to Bim_S (Day et al., 2004; Puthalakath et al., 1999).

Bim is regulated at the post-translational level by phosphorylation. ERK is a kinase that is capable of phosphorylating Bim. When ERK phosphorylates Bim_{EL}, the pro-apoptotic protein is ubiquitinated and subsequently degraded in the proteasome (Adams and Cory, 2007; Harada and Grant, 2012; Hubner et al., 2008; Ley et al., 2005; Luciano et al., 2003). Bim_{EL} is phosphorylated at S69 in human cells by ERK and the two other isoforms Bim_L and Bim_S lack this site and therefore are not phosphorylated by ERK

(Luciano et al., 2003). Bim that has been mutated to prevent it from being phosphorylated by ERK has been found to have a greater effect on apoptosis (Hubner et al., 2008). It is thought that this is why Bim_S is more potent.

Phosphorylation also regulates the interactions of Bim with other proteins. Phosphorylation of Bim_{EL} prevents it from binding to Bax, but does not affect its ability to bind to Bcl-2 or Mcl-1 (Ley et al., 2005). When S69 of Bim is mutated in human cells so that it cannot be phosphorylated, Bim is able to bind to Bax and apoptosis is enhanced (Ley et al., 2005).

JNK is another kinase that can phosphorylate Bim. JNK phosphorylates Bim in response to some apoptotic stimuli including UV radiation and trophic factor deprivation (Harada and Grant, 2012; Hubner et al., 2008; Putcha et al., 2003). Phosphorylation by JNK may help potentiate the apoptotic effects of Bim by causing its release from DLC1 and allowing it to bind to Bcl-2 and Bcl-x_L to inactivate them (Adams and Cory, 2007; Dhanasekaran and Reddy, 2008; Lei and Davis, 2003; Ley et al., 2005).

CHAPTER 2: ROLE OF BCL-2 PROTEINS IN STRESS-INDUCED APOPTOSIS IN A NON-TRANSFORMED MAMMARY EPITHELIAL CELL LINE

Introduction

According to the Economic Research Service of the United States Department of Agriculture, milk production is one of the most valuable agriculture products in the United States livestock industry. Because dairy products are of economic importance in the United States, research has been performed to determine what factors affect milk production in cows. The mammary gland undergoes many changes throughout the life cycle of a cow. Cellular proliferation of different cell types occurs during puberty, pregnancy, and early lactation and apoptosis occurs during late lactation and involution (Colitti et al., 1999; Wilde et al., 1999). Milk yield decreases late in lactation as a result of apoptosis (Capuco et al., 2001) caused by hormone and growth factor withdrawal (Wilde et al., 1999). Growth factor withdrawal caused by serum starvation of various cell types has been shown to activate the intrinsic apoptosis pathway and for this reason, the intrinsic apoptosis pathway is of particular interest to those interested in milk yield (Braun et al., 2011; Moulding et al., 2001; Wei et al., 2008).

The intrinsic apoptosis pathway is controlled by the Bcl-2 family of proteins. The Bcl-2 family is divided into three categories based on their functions and presence of Bcl-2 homology (BH) domains (Danial, 2007; Marzo and Naval, 2008). The three main categories of Bcl-2 family proteins are (1) multidomain anti-apoptotic, (2) multidomain pro-apoptotic, and (3) BH3-only pro-apoptotic. Bcl-2 and Mcl-1 are two of the

multidomain anti-apoptotic proteins and are thought to prevent apoptosis by sequestering anti-apoptotic proteins or by preventing MOMP (Adams and Cory, 2007; Danial, 2007). Bax and Bak are multi-domain pro-apoptotic proteins and are responsible for causing MOMP by forming pores in the mitochondrial membrane during apoptosis (Chu et al., 2009; Danial, 2007; Wei et al., 2001). Bad and Bim are BH3-only pro-apoptotic proteins that are thought to contribute to apoptosis by either binding to anti-apoptotic proteins to prevent them from interacting with Bax and Bak or by directly activating Bax or Bak at the mitochondrial membrane (Adams and Cory, 2007).

Anisomycin (ANS) is an antibiotic that causes stress-induced apoptosis in MAC-T cells and activates JNK, p38, and ERK signaling pathways (Leibowitz and Cohick, 2009). As previously discussed, the early events in stress-induced apoptosis are not well established and are controlled by the Bcl-2 family of proteins. The goals of this study were to determine (1) if mRNA levels of *Bcl-2*, *Bax*, and *Bad* are regulated by ANS, (2) if protein levels and post-translational modifications of Bcl-2, Mcl-1, Bax, Bad, and Bim change during stress-induced apoptosis, and (3) if interactions between the proteins are affected by treatment with ANS.

Materials and Methods

Reagents

Phenol red-free (PRF) Dulbecco's Modified Eagle Medium with high glucose (DMEM-H), penicillin, and streptomycin were purchased from Life Technologies (Carlsbad, CA). DMEM with low glucose, gentamicin, bovine insulin, ANS, and fetal

bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IGF-1 (100% identical to bovine IGF-1) was obtained from Peprotech (Princeton, NJ). The JNK inhibitor SP600125 and the p38 inhibitor SB239063 were purchased from EMD Millipore (Bedford, MA). Antibodies were purchased as follows: Bad (ab90435) and HSP60 (ab31115) from Abcam (Cambridge, MA); Bax (sc-493) and Bcl-2 (sc-492) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Bim (2933), Mcl-1 (5453), phosphorylated C-Jun (9164), phosphorylated p38 (4511), and total p38 (9212) from Cell Signaling Technology, Inc. (Danvers, MA).

Cell Culture

The bovine mammary epithelial cell (MEC) line MAC-T (Huynh et al., 1991) was maintained at 37 °C with 95% humidity and 5% CO_2 in complete media consisting of DMEM-H containing 4.5 g/L D-glucose, 20 U/mL penicillin, 20 µg/mL streptomycin, 50 µg/mL gentamicin, 10% FBS, and 5 µg/mL bovine insulin. For experiments, cells were plated at a density of 1 x 10^4 cells/cm^2 and grown to confluence without insulin in PRF DMEM-H containing 10% FBS and antibiotics. Prior to exposure to treatments, cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free (SF) PRF DMEM-H with 0.2% BSA and 30 nM sodium selenite overnight. Cells were exposed to treatments in SF PRF DMEM-H with antibiotics, but no other additives.

Reverse Transcription Quantitative PCR (qRT-PCR)

For determination of mRNA expression, cells were lysed following treatment

in TRIzol Reagent (Life Technologies) and RNA was isolated using an RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. RNA concentration was measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) and integrity was assessed by visualization of the 28S and 18S ribosomal RNA subunits using agarose gel electrophoresis. RNA (2 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). For qRT-PCR, primer sets were generated using Primer Express 3.0 software (Applied Biosystems) and purchased from Sigma-Aldrich. qRT-PCR primers were as follows: *Bad* (forward = 5'-AATGAAGAGACGGAGGAGGAT-3', reverse = 5'-TTGAAGGAGACGTGAAACTCGTCG-3'), *Bax* (forward = 5'-CCAAGAAGCTGAGCGAGTGTCT-3', reverse = 5'-GCAGCTCCATGTTACTGTCCAA-3'), *Bcl-2* (forward = 5'-TGTGGATGACCGAGTACCTGAA-3', reverse = 5'-AGCCTCCGTTGTCCTGGAT-3'), and the housekeeping gene *Cyclophilin* (forward = 5'-GAGCACTGGAGAGAAAGGATTTGG-3', reverse = 5'-TGAAGTCACCACCCTGGCACATAA-3'). To validate each primer set, individual standard curves were established using serial dilutions (1:20 through 1:200,000) of pooled samples of RNA isolated from cells treated with 0.1 μM ANS or 100 ng/mL IGF-1 for 2 h. The slope and amplification efficiency of the standard curve for each primer set was determined. A standard curve slope of approximately -3.3 and amplification efficiencies between 90 and 110% were considered acceptable. Melt curves were evaluated to ensure that a single product was amplified. If the primers for the target and housekeeping genes amplify at the same rate, then the efficiency of these primers should be approximately equal. To determine this, ΔC_t ($C_{t\ target}$ - $C_{t\ reference}$) was plotted

for each dilution of the standard curve. As per the Applied Biosystems guidelines, the primers for the target and housekeeping genes amplify at the same rate if the absolute value of the slope for ΔC_t vs. log[RNA] is less than 0.1. Thus, the $2^{-\Delta\Delta Ct}$ method can be used to analyze the data. For qRT-PCR analysis, samples were diluted 1:400 and 5 µL of each sample was amplified in a 20 µL reaction containing 10 µL Power SYBR Green (Applied Biosystems) using the Applied Biosystems StepOne Plus™ Real-Time PCR System. Changes between treated and serum-free control samples were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western Immunoblotting

To examine protein expression in response to treatment, cells were washed twice with cold 1X PBS and collected by scraping in Complete Lysis Buffer (10 µg/mL aprotinin, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 50 mM HEPES, 10 µg/mL leupeptin, 1 mM phenylmethylsulfonylfluoride, 0.1% SDS, 10 mM sodium fluoride, 2 mM sodium orthovanadate, 1% Triton X-100, 10 µg/mL trypsin inhibitor). Cell lysates were incubated on ice for 30-40 min and then pelleted at 4 °C in a centrifuge for 15 min at 13,000 x g. The supernatants were aliquoted to prevent issues with freeze-thawing. Total protein content of lysates was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Samples were prepared by addition of Laemmli buffer containing β-mercaptoethanol and heating at 95 °C for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gels and transferred to 0.45 µm polyvinylidene difluoride (PVDF) membranes (EMD

Millipore). Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 (v/v) (TBS-T) and 5% non-fat dry milk (w/v) then incubated with primary antibodies at 4 °C overnight with gentle agitation. Membranes were then washed in TBS-T and incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated (HRP) secondary antibodies. Peroxidase activity was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ) and visualized using a FluorChem FC2 Imager (Cell Biosciences Inc, Santa Clara, CA). Following detection of proteins, membranes were stripped in Restore Plus Western Stripping Buffer (Thermo Scientific) for 10 min and then probed for other proteins and loading controls as described in Results.

Lambda Protein Phosphatase Experiments

After treatment, whole cell lysates were collected in Complete Lysis Buffer without phosphatase inhibitors (recipe same as above without EDTA, sodium fluoride, or sodium orthovanadate) and total protein content of lysates was determined as described above. Protein samples were treated with lambda protein phosphatase (LPP) (New England BioLabs, Ipswich, MA) and incubated at 30 °C for 30 minutes in order to remove the phosphate groups from the phosphorylated proteins. After treating the lysates with LPP, they were resolved using SDS-PAGE, transferred to a membrane, and blotted using appropriate antibodies.

siRNA Experiments

Cells were plated in complete media at 3 x 10^4 cells/cm^2. The following day, subconfluent cells (between 60-80% confluent) were transfected with 50 nM Bim oligo set 1 siRNA from Sigma-Aldrich (sense = CAGAGAAGGUGGACAAUUGdTdT, antisense = CAAUUGUCCACCUUCUCUGdTdT), 50 nM Bim oligo set 4 siRNA from Sigma-Aldrich (sense = GAUGUAAGUUCUGAGUGUGdTdT, antisense = CACACUCAGAACUUACAUCdTdT), 50 nM of a pool of siGENOME Human MAPK9 SMARTpool siRNA from Thermo Scientific, or 50 nM of siGENOME Non-targeting siRNA #3 from Thermo Scientific using TransIT-TKO Transfection Reagent (Mirus Bio LLC, Madison, WI) according to manufacturer's instructions. After 48 h, cells were washed twice with 1X PBS and incubated overnight in SF PRF DMEM-H with BSA and sodium selenite, then treated for analysis of gene knockdown or ANS-induced apoptosis. Gene knockdown of Bim was verified by Western immunoblotting as described above. Apoptosis was determined using the Sensolyte Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA). Caspase assay results were analyzed by a two-way ANOVA followed by a Bonferroni posttest using GraphPad Prism 5 (San Diego, CA).

Co-immunoprecipitation Experiments

To determine specific protein-protein interactions, co-immunoprecipitation assays were performed. After treatment, cells were collected in Modified RIPA buffer (1 µg/mL aprotinin, 1 mM EDTA, 1 µg/mL leupeptin, 1% NP-40, 1 µg/mL pepstatin, 1 mM phenylmethylsulfonylfluoride, 150 mM sodium chloride, 0.25% sodium deoxycholate, 1

mM sodium fluoride, 1 mM sodium orthovanadate, 50 mM Tris-HCl) and total protein content of whole cell lysates was determined as previously described. Protein G Plus/Protein A agarose bead suspension (EMD Millipore) was prepared by washing the beads once in 1X PBS. Primary antibody against Bim (0.22 µg/mg protein) or an equivalent concentration of rabbit IgG purified protein (Sigma-Aldrich) and the bead suspension were incubated under agitation at 4 °C for 1.5 h in order to bind the antibodies or IgG to the beads. Lysates were precleared by incubating them with the bead suspension alone for 3 h at 4 °C under agitation. The cleared lysates and antibody or IgG-coated beads were incubated at 4 °C under agitation for 3.5 h in order to pull down the respective protein. After pulling down protein, samples were washed three times in 1X PBS and then eluted from the beads by suspending them in Laemmli buffer containing β-mercaptoethanol and heating them for 5 min at 95 °C. Proteins were separated using SDS-PAGE and were immunoblotted for proteins as described above.

Results

Effect of ANS on mRNA or protein expression of Bcl-2, Bax, or Bad.

In order to determine if ANS regulates mRNA levels of *Bcl-2*, *Bax*, or *Bad*, MAC-T cells were treated with 0.1 or 1.0 µM ANS for 1, 2 and 6 h (Figure 5). Treatment with 0.1 or 1.0 µM ANS had no substantial effects on mRNA levels of *Bcl-2* or *Bax* at any time point, perhaps with the exception of 2 h with 1.0 µM ANS treatment, where *Bax* mRNA levels decreased about 40% (Figure 5A and B). However, *Bad* mRNA levels decreased

approximately 40 to 60% at 2 and 6 h after treatment with 0.1 and 1.0 μM ANS, respectively (Figure 5C).

To determine if similar patterns were reflected at the protein level, protein expression was evaluated in whole cell lysates. As shown in Figure 6, no major changes in protein levels were observed in response to 0.1 or 1.0 μM ANS treatment for Bax using Western Immunoblotting. The abundance of Bcl-2 decreased after treatment with 1.0 μM ANS at 2 h and with both 0.1 and 1.0 μM ANS at 6 h. In keeping with the small changes in *Bad* mRNA levels, there appeared to be a slight decrease in Bad protein levels at 1 and 2 h, while Bad protein levels appeared higher at 4 h with both concentrations of ANS. These data indicate that ANS does not induce apoptosis in MAC-T cells by regulating the mRNA or protein levels of *Bax*, though small changes may be induced for Bcl-2 and Bad. These changes could potentially affect the ratio between pro- and anti-apoptotic proteins.

Several groups have reported that Bcl-2 is phosphorylated during apoptosis (Bassik et al., 2004; Rajah et al., 2002; Wei et al., 2008). In order to determine if Bcl-2 is phosphorylated in response to ANS treatment, MAC-T cells were treated with 1.0 μM ANS for 1 h. Whole cell lysates were collected in the absence of phosphatase inhibitors and lysates were treated with either buffer alone or buffer and Lambda Protein Phosphatase (LPP) to remove phosphate groups from phosphorylated proteins. As shown in Figure 7, only one molecular weight form of Bcl-2 was observed by SDS-PAGE, suggesting that Bcl-2 is not phosphorylated basally or in response to ANS.

Treatment with ANS induces changes in Bim_{EL} and Mcl-1 expression.

Because protein levels of Bcl-2, Bax, and Bad did not change substantially in response to treatment with ANS, expression of other Bcl-2 family proteins was evaluated. Two molecular weight forms of Bim_{EL} were present under serum-free conditions: a higher molecular weight form of the protein at approximately 24 kDa and a lower molecular weight form of the protein at approximately 23 kDa (Figure 8, lane 1). After 1 h of ANS treatment, the lower molecular weight form of Bim_{EL} decreased with both doses of ANS (Figure 8, lanes 2 and 3). The higher molecular weight Bim_{EL} shifted slightly higher with both doses of ANS and also increased in intensity with the higher dose. With 0.1 µM ANS, this shift was lost at 2 and 4 h. With 1 µM ANS, the shift persisted at 2 h though the intensity of the band decreased to less than that observed with serum-free conditions, while at 4 h the shift was no longer observed and the intensity had significantly diminished below that observed with serum-free treatment. These data show that ANS treatment causes Bim_{EL} to be post-translationally modified.

Mcl-1 expression levels were also affected by treatment with ANS (Figure 8). After 2 h of treatment with either 0.1 or 1.0 µM ANS, the amount of Mcl-1 decreased. This effect was more pronounced with the higher dose of ANS and Mcl-1 was almost non-detectable after 4 h of treatment.

Phosphorylation of Bim_{EL} is increased by treatment with ANS.

In order to determine if the higher molecular weight band was a phosphorylated form of Bim_{EL}, cells were treated with 0.1 and 1.0 µM ANS for 30 m (Figure 9A) and 1 h

(Figure 9B). There are no commercially available antibodies that are reported to recognize phosphorylated bovine Bim. For this reason, whole cell lysates were collected in the absence of phosphatase inhibitors and lysates were treated with either buffer alone or buffer and Lambda Protein Phosphatase (LPP) in order to remove phosphate groups from phosphorylated proteins. When SF lysates were treated with LPP, the band corresponding to Bim_{EL} migrated faster and was more intense than the band that was present in the SF lysates without the enzyme, indicating that some phosphorylated Bim_{EL} was present under basal conditions. The higher molecular weight form of Bim_{EL} was evident in lanes containing lysates from cells treated with both 0.1 and 1.0 µM ANS at both 30 m and 1 h time points. When these lysates were treated with LPP, the band corresponding to Bim_{EL} was visible at a lower molecular weight. These results indicate that the change in the size of Bim_{EL} that occurs after treatment with ANS is the result of phosphorylation.

Bim_{EL} phosphorylation is not mediated by JNK or p38.

JNK and p38 are activated early in the intrinsic apoptosis pathway in MAC-T cells and JNK signaling is required for apoptosis to occur (Leibowitz and Cohick, 2009). JNK activation is involved in the transcriptional regulation and protein-protein interactions of the Bcl-2 family. In order to determine if activated JNK or p38 phosphorylate Bim_{EL} after treatment with ANS, MAC-T cells were pretreated with 50 µM SP600125 or 30 µM SB239063, respectively, for 45 m and then treated with 0.1 or 1.0 µM ANS for 1 h. When JNK or p38 was inhibited, Bim_{EL} was still phosphorylated in response to ANS

treatment at both concentrations (Figure 10). In order to further confirm that JNK does not phosphorylate Bim_{EL}, siRNA was used to knock-down the kinase. Bim_{EL} was still phosphorylated in response to treatment with ANS (Figure 11). These data indicate that neither JNK nor p38 activation are responsible for Bim_{EL} phosphorylation.

Knock-down of Bim attenuates ANS-induced apoptosis.

To determine if Bim plays a role in the ability of ANS to activate the intrinsic apoptotic pathway, siRNA oligos were designed to knock-down Bim. As shown in Figure 12A, Bim_{EL} protein expression was knocked down by both oligo 1 and 4, though Bim oligo 1 was slightly more effective (Figure 12A). In order to determine if Bim is required for ANS-induced apoptosis, caspase-3/7 cleavage was measured after 6 h of treatment with ANS (Figure 12B). Caspase cleavage increased in a dose-dependent manner when cells were treated with ANS and knock-down of Bim with Bim oligo 1 or Bim oligo 4 significantly blocked this effect ($p < 0.05$ or 0.01).

Knock-down of Bim does not prevent decreases in Mcl-1.

Bim_{EL} phosphorylation and Mcl-1 degradation occur in a similar timeframe in response to ANS treatment. In order to determine if Bim_{EL} phosphorylation mediates decreases in Mcl-1 expression, the levels of Mcl-1 were evaluated after knock-down of Bim_{EL} and treatment with ANS (Figure 13). Mcl-1 protein levels decreased when Bim was knocked down in the absence of ANS treatment. Decreases in Mcl-1 abundance also occurred in response to treatment with 0.1 or 1.0 μM ANS after 1 h of treatment

with ANS, irrespective of Bim_{EL} knock-down. These results indicate that the amount of Bim_{EL} present does not regulate Mcl-1 degradation.

Bcl-2 family proteins interact during apoptosis.

Members of the Bcl-2 family of proteins have been reported to regulate apoptosis by interacting with each other. In order to determine if ANS treatment affects interactions between Bim and Mcl-1, MAC-T cells were treated with 1.0 µM ANS for 30 m and whole cell lysates were collected in Modified RIPA Buffer. Protein samples were co-immunoprecipitated using Bim and Mcl-1 antibodies. Bim interacts with Mcl-1 under basal conditions and this interaction does not change in response to ANS treatment (Figure 14).

Discussion

The Bcl-2 family of proteins plays a major role in regulating apoptosis in response to cell stress. These proteins undergo changes in mRNA levels, protein abundance, and protein-protein interactions in response to apoptotic stimuli. Bcl-2, Mcl-1, Bax, Bad, and Bim were investigated in this work. It was found that *Bcl-2*, *Bax*, and *Bad* mRNA and protein abundance are not substantially regulated by ANS treatment (Figures 6 and 7). Minor changes in the levels of *Bad* mRNA and protein and Bcl-2 protein were detected and further studies are required to verify these results. Other groups have found that Bcl-2 is capable of binding Bax and Bad (Bassik et al., 2004; Gomez-Bougie et al., 2005) and the studies conducted herein do not allow us to rule out such interactions. It has

also been found that Bcl-2 is phosphorylated and "inactivated" during apoptosis (Bassik et al., 2004; Danial, 2007), but this was not seen in our experiments (Figure 7).

Because no major changes were detected in the mRNA and protein levels of *Bcl-2*, *Bax*, and *Bad*, Bim and Mcl-1 were studied. Bim_{EL} typically migrated as a doublet under basal conditions and was phosphorylated as a result of ANS treatment (Figures 9 and 10). Mcl-1 abundance decreased in response to ANS and this effect was more dramatic when 1.0 μM ANS was used (Figure 8).

Multiple signaling pathways are activated in response to ANS treatment in MAC-T cells, including JNK and p38 (Leibowitz and Cohick, 2009). These signaling pathways are activated in a timeframe similar to the changes seen in Bim_{EL} and Mcl-1 and there are studies that show that JNK phosphorylation of Bim during apoptosis may help to potentiate its pro-apoptotic functions (Harada and Grant, 2012; Hubner et al., 2008; Putcha et al., 2003). For these reasons, JNK and p38 were considered as potential upstream effectors. When JNK or p38 signaling was inhibited, Bim_{EL} was still phosphorylated in response to treatment with ANS and Mcl-1 still decreased (Figure 10). The inhibitor used to block JNK signaling (SP600125) is not 100% effective. SP600125 significantly decreases activation of downstream targets, such as C-Jun, but this may not be enough to completely prevent phosphorylation of Bim_{EL}. Unpublished data from our laboratory also suggest that SP600125 may have undesired effects by activating ERK. This effect is problematic because ERK has also been reported to phosphorylate Bim (Adams and Cory, 2007; Harada and Grant, 2012; Hubner et al., 2008; Ley et al., 2005; Luciano et al., 2003). For these reasons, Bim_{EL} phosphorylation and Mcl-1 degradation

were also evaluated when JNK was knocked down with siRNA (Figure 11). Bim_{EL} was still phosphorylated in response to ANS when JNK was knocked down with siRNA and Mcl-1 was still degraded. This suggests that JNK is not responsible for Bim_{EL} phosphorylation.

Since ERK is basally phosphorylated in MAC-T cells (Figure 9), it is possible that ERK is responsible for the phosphorylation of Bim_{EL}. ANS also activates ERK in MAC-T cells. Therefore future studies with ERK inhibitors or siRNA knock-down of ERK will be employed to determine if the ERK pathway is involved in ANS-induced phosphorylation of Bim_{EL}.

In order to determine if Bim is a critical protein in the apoptosis pathway, knock-down experiments were performed and caspase-3/7 activation was evaluated after treatment with ANS (Figure 12). It was found that Bim knock-down significantly decreased apoptosis caused by ANS treatment. Because Mcl-1 protein levels decrease in response to ANS treatment, future experiments will involve knock-down of Mcl-1 expression and measurement of caspase-3/7 activity when cells are treated with ANS to determine if changes in Mcl-1 are also biologically significant.

In order to determine if the decrease in Mcl-1 protein is the result of changes in Bim, Bim was knocked down with siRNA oligos and cells were treated with ANS (Figure 13). Mcl-1 levels decreased in response to Bim siRNA in the absence of treatment and were further decreased in response to ANS treatment. This may indicate that Bim and Mcl-1 are co-regulated.

Co-regulation of Bim and Mcl-1 has been reported in the literature. When RNAi is used to downregulate Bim, Mcl-1 protein abundance also decreases and vice versa

(Wuilleme-Toumi et al., 2007). mRNA regulation and qRT-PCR experiments to evaluate *Bim* and *Mcl-1* mRNA levels would help to determine if regulation is occurring at the mRNA level. Co-regulation of Bim and Mcl-1 may occur at the level of protein stability and interactions between Bim and Mcl-1 may act to stabilize the proteins. Bim and Mcl-1 interact with each other both basally and when cells are treated with ANS (Figure 14). In other work, Mcl-1 protein levels increased when cells were treated with a Bim BH3 peptide (Wuilleme-Toumi et al., 2007). This indicates that Bim stabilizes Mcl-1 by binding to the protein.

Further work may involve mutating the binding regions of Bim and Mcl-1 in order to prevent the proteins from interacting and protein abundance of the two proteins could be assessed via Western immunoblotting. Pulse chase experiments could also be used to determine if Bim knock-down affects expression of Mcl-1. New protein would be radiolabeled and differences in radiolabeled Mcl-1 could be assessed.

Interactions between the Bcl-2 proteins can help determine a potential mechanism of apoptosis. Bim and Mcl-1 interactions with Bak should be established to help determine a potential mechanism of commitment to MOMP. Two main models of Bcl-2 family member interactions have been proposed: the indirect activation model and the direct activation model (Adams and Cory, 2007; Danial, 2007). In the indirect model, anti-apoptotic Bcl-2 proteins bind to pro-apoptotic multi-domain proteins and prevent them from becoming activated and causing MOMP until a pro-apoptotic BH3-only protein binds the anti-apoptotic protein and disrupts its actions. In the direct model, BH3-only proteins are sequestered by anti-apoptotic proteins and are released

and bind to pro-apoptotic multi-domain proteins during apoptosis to activate them to cause MOMP.

Co-immunoprecipitation data could help distinguish between these two models of apoptosis. If the indirect activation model were supported, the association between Mcl-1 and Bim would increase with ANS treatment and if the direct activation model was correct, less Bim would be bound to Mcl-1 with ANS treatment. Preliminary data do not provide enough support in either direction, but further experiments with different time points would provide useful information.

In conclusion, this work begins to shed light on the role of the Bcl-2 family of proteins in stress-induced apoptosis in mammary epithelial cells. We found that mRNA and proteins levels of *Bcl-2*, *Bax*, and *Bad* were not substantially regulated during ANS-induced apoptosis, though small changes may be important in shifting the ratio of pro-apoptotic to pro-survival proteins. No evidence of post-translational modifications were observed for these three proteins. In contrast, ANS induced early post-translational modification of the pro-apoptotic protein Bim_{EL} while decreasing expression of the pro-survival protein Mcl-1. Knockdown of Bim_{EL} indicated that this protein is important for ANS-induced apoptosis. Preliminary co-immunoprecipitation experiments indicate that these two proteins may directly interact. Further work is required to determine how interactions between these two proteins contribute to stress-induced apoptosis in mammary epithelial cells.

FIGURES

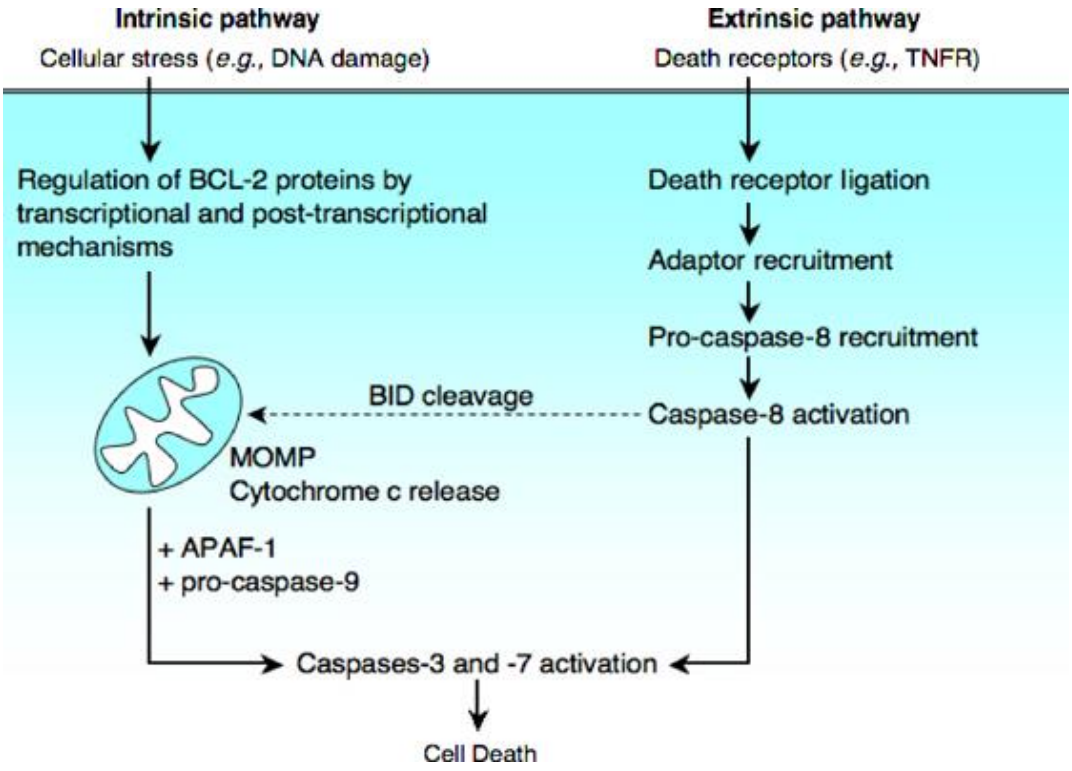

Figure 1. Apoptosis pathways in the cell. Programmed cell death occurs by two main pathways, the intrinsic and extrinsic apoptosis pathways. These two pathways occur independently and in response to different stressors, but both converge at the mitochondria. Reprinted by permission from Macmillan Publishers Ltd: [CELL DEATH AND DIFFERENTIATION] (Chipuk and Green, 2006), copyright (2006).

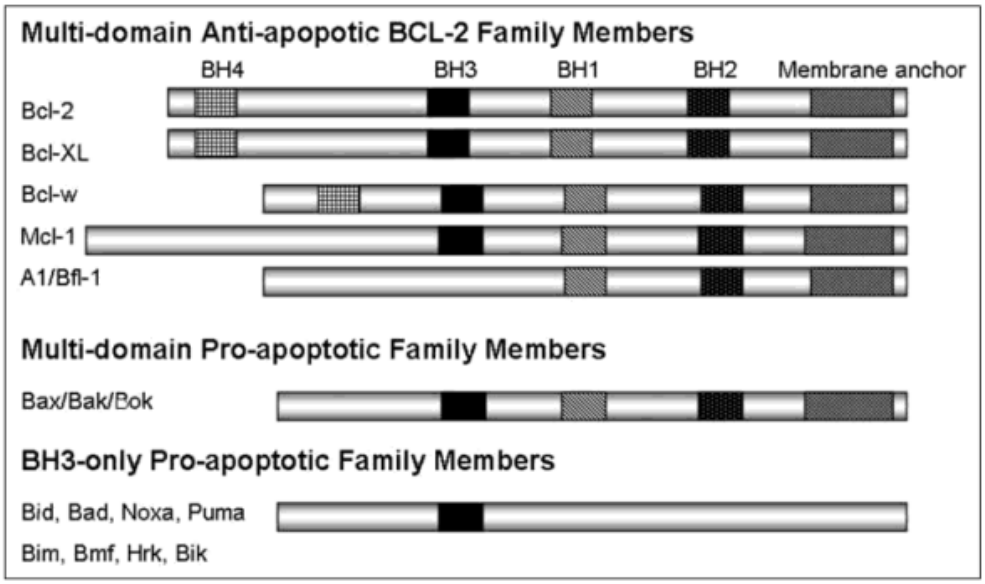

Figure 2. Characterization of the Bcl-2 family of proteins. The Bcl-2 family of proteins is split into three different categories based on the presence of four different BH domains (Hetz, 2010).

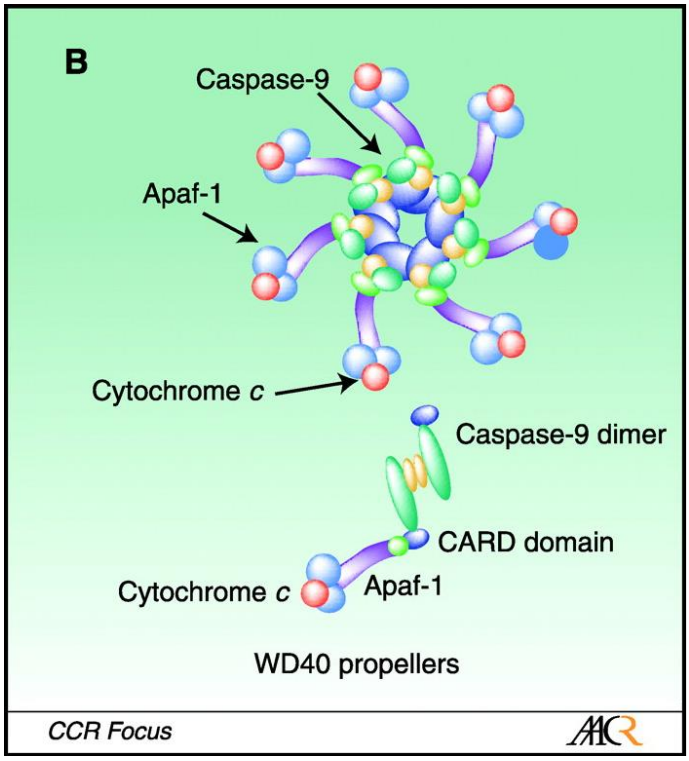

Figure 3. Apoptosome formation. The apoptosome forms when cyt c is released from the mitochondria during apoptosis. Cyt c bind to Apaf-1 and caspase-9 in a complex. Using ATP, caspase-9 is activated and can then move on to cleave other proteins in the apoptosis cascade (Danial, 2007). Reprinted from Clinical Cancer Research, Copyright 2007, 13/24, 7254-7263, Nika N. Danial, Bcl-2 Family Proteins: Critical Checkpoints of Apoptotic Cell Death, with permission from AACR.

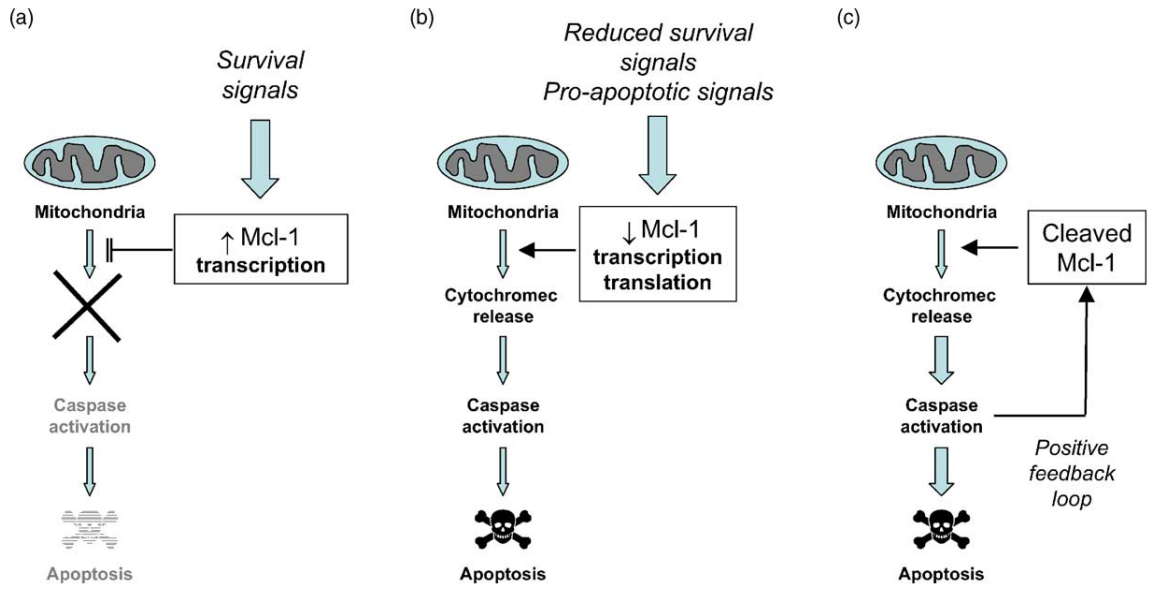

Figure 4. Apoptosis Regulation by Mcl-1. Apoptosis can be regulated in response to increased Mcl-1 transcription in response to survival signals (a), decreased Mcl-1 transcription as a result of pro-apoptotic signals (b), and cleavage of Mcl-1 by caspases (c). Mcl-1 has anti-apoptotic or pro-apoptotic effects depending on the pathway taken (Michels et al., 2005). Reprinted from The international journal of biochemistry and cell biology, 37/2, Jorg Michels, Peter W.M. Johnson, and Graham Packham, Mcl-1, 267-271, Copyright (2005), with permission from Elsevier.

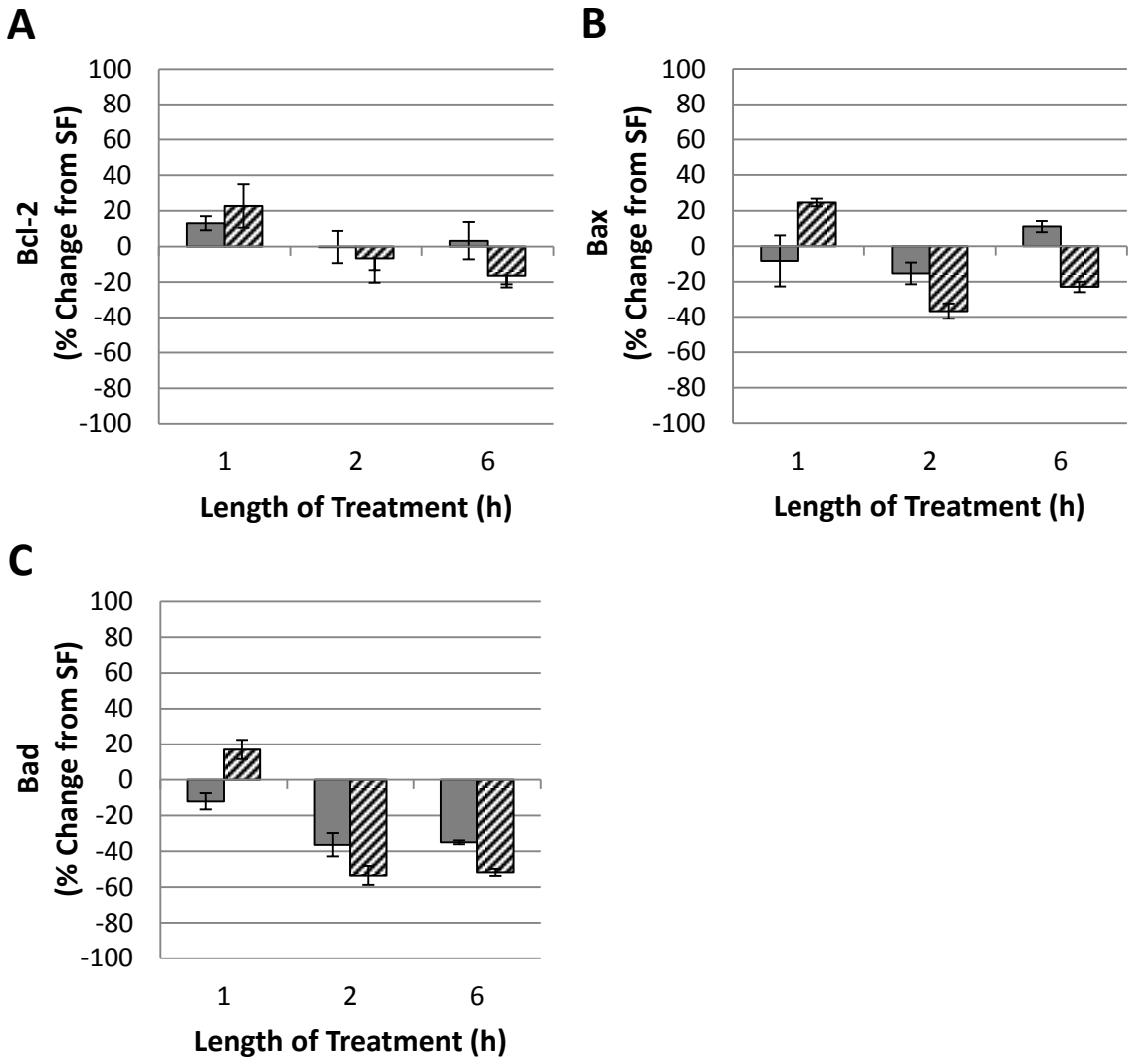

Figure 5. Effect of ANS on mRNA levels of *Bcl-2*, *Bax*, and *Bad*. MAC-T cells were treated with 0.1 (solid bars) or 1.0 (striped bars) µM ANS for the indicated times. RNA was isolated and mRNA levels were determined for *Bcl-2* (A), *Bax* (B), and *Bad* (C) and were corrected for *Cyclophilin*. Solid bars represent the mean ± SD of two experiments, striped bars represent the mean ± SD of three replicates from one experiment.

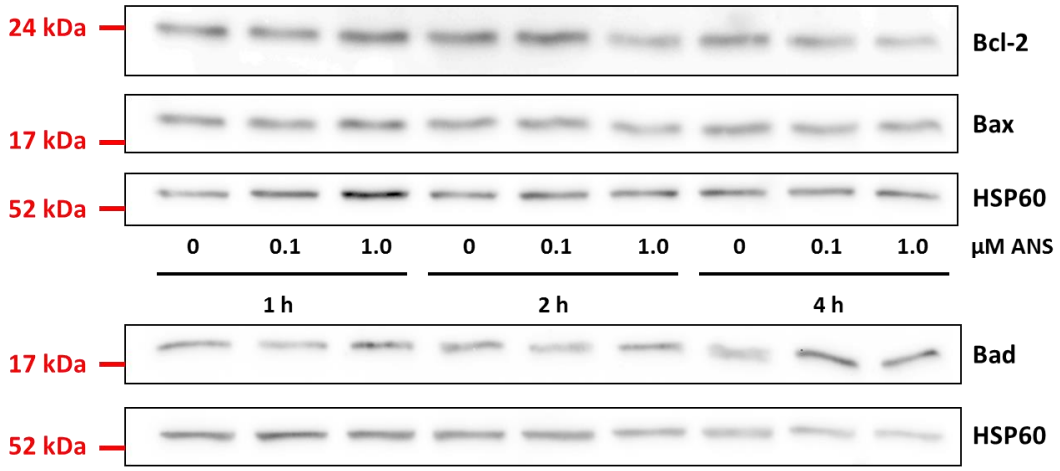

Figure 6. Treatment with ANS does not alter Bax protein expression but may induce small changes in Bcl-2 and Bad. MAC-T cells were treated with 0.1 or 1.0 µM ANS for the indicated times. Whole cell lysates (40 µg) were separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Bcl-2, Bax, Bad, and HSP60. Figure is representative of two independent experiments.

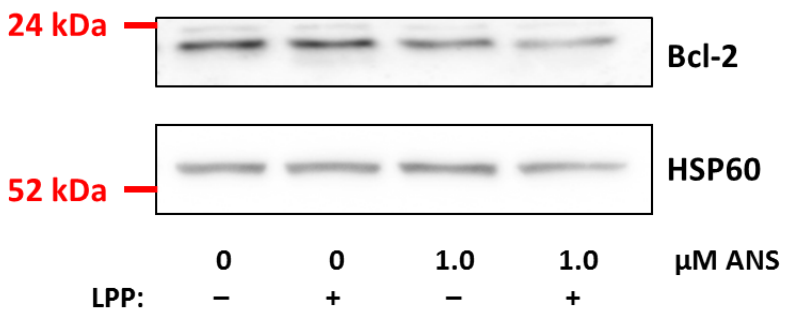

Figure 7. Bcl-2 is not phosphorylated in MAC-T cells. MAC-T cells were treated with 1.0 µM ANS for 1 h. Whole cell lysates (40 µg) were collected in Complete Lysis Buffer without phosphatase inhibitors and treated with Lambda Protein Phosphatase (LPP) and then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Bcl-2 and HSP60. Figure is representative of three independent experiments.

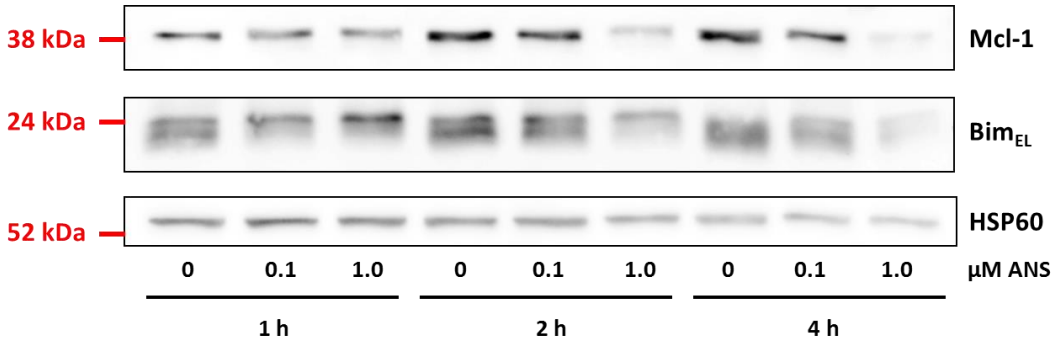

Figure 8. Treatment with ANS induces changes in Bim$_{EL}$ and Mcl-1 expression. MAC-T cells were treated with 0.1 or 1.0 µM ANS for the indicated times. Whole cell lysates (40 µg) were separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Mcl-1, Bim, and HSP60. Figure is representative of two experiments.

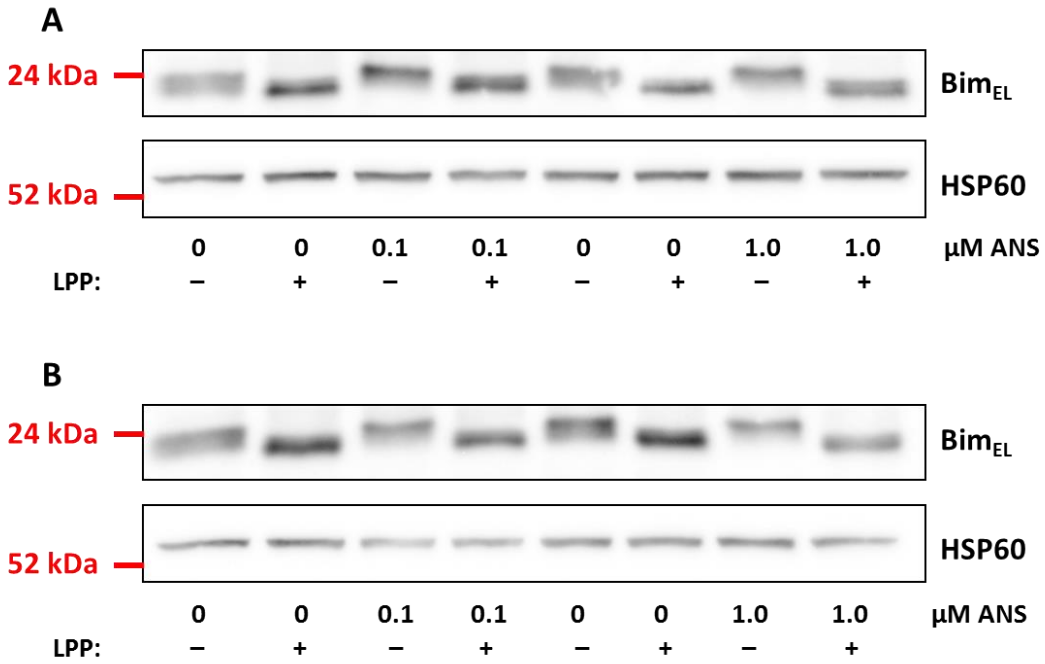

Figure 9. The upward shift in Bim$_{EL}$ in response to ANS is a result of phosphorylation. MAC-T cells were treated with 0.1 or 1.0 µM ANS for 30 m (A) or 1 h (B). Whole cell lysates (40 µg) were collected in Complete Lysis Buffer without phosphatase inhibitors and treated with buffer alone or with buffer and Lambda Protein Phosphatase (LPP) and then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Bim and HSP60. Figure is representative of three experiments.

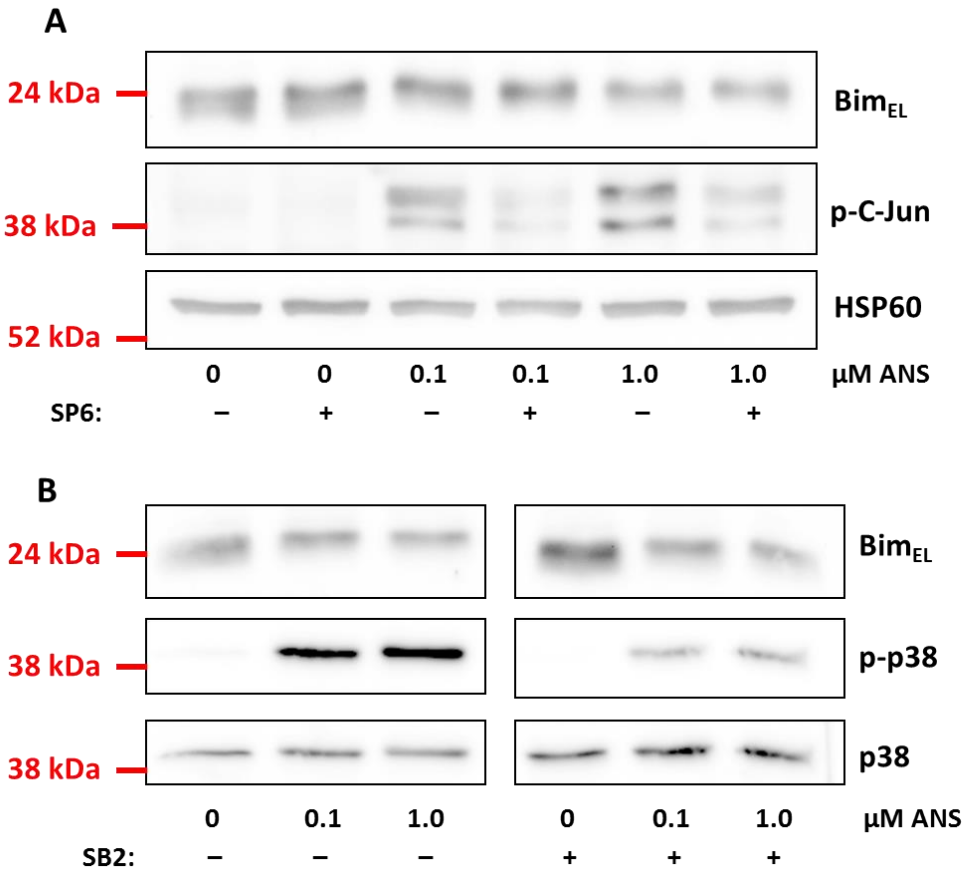

Figure 10. Bim$_{EL}$ phosphorylation is not mediated by JNK or p38. MAC-T cells were pretreated with 50 µM SP600125 (A) or 30 µM SB239063 (B) and then treated with 0.1 or 1.0 µM ANS for 1 h. Whole cell lysates (40 µg) were collected and then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Bim, p-C-Jun, p-p38, or HSP60. Figure is representative of three experiments.

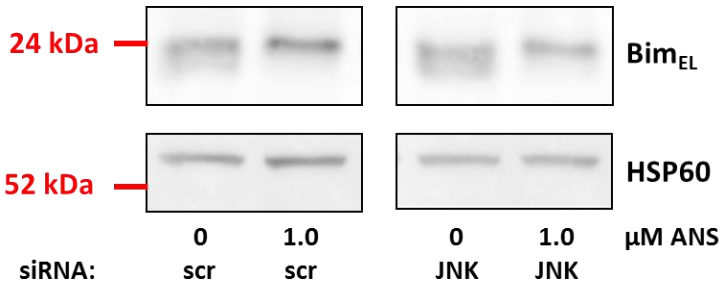

Figure 11. Bim$_{EL}$ phosphorylation is not mediated by JNK. MAC-T cells were transfected with 50 nM of non-targeting (scr) or JNK siRNA for 48 h and then treated with 1.0 µM ANS for 1 h. Whole cell lysates (40 µg) were collected and then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Bim or HSP60. Figure is representative of two experiments.

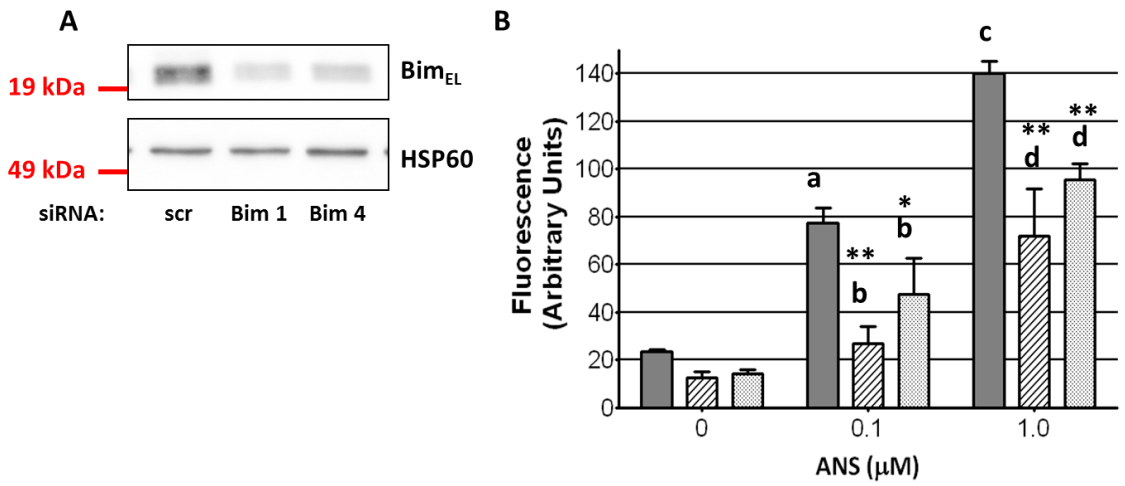

Figure 12. Knock-down of Bim attenuates ANS-induced apoptosis. (A) MAC-T cells were transfected with 50 nM of non-targeting (scr) or Bim siRNA oligo 1 or 4 for 48 h. Whole cell lysates (40 μg) were collected and then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Bim or HSP60. Figure is representative of two experiments. (B) MAC-T cells were transfected with 50 nM of non-targeting (dark bars), Bim oligo 1 (striped bars), or Bim oligo 4 (light bars) siRNA for 48 h and then treated with 0.1 or 1.0 μM ANS for 6 h. Caspase-3/7 activation was measured in arbitrary units. Bars represent the mean ± SD of three experiments. A two-way ANOVA was performed followed by a Bonferroni posttest. * indicates $p < 0.05$ and ** indicates $p < 0.01$.

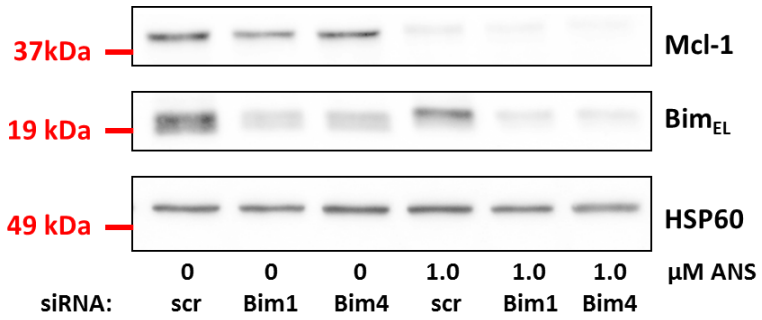

Figure 13. Knock-down of Bim does not prevent decreases in Mcl-1. MAC-T cells were transfected with 50 nM of non-targeting (scr) or Bim oligo set 1 or 4 siRNA for 48 h and then treated with 1.0 µM ANS for the indicated 1 h. Whole cell lysates (40 µg) were collected and then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Mcl-1, Bim, or HSP60. Figure is representative of two experiments.

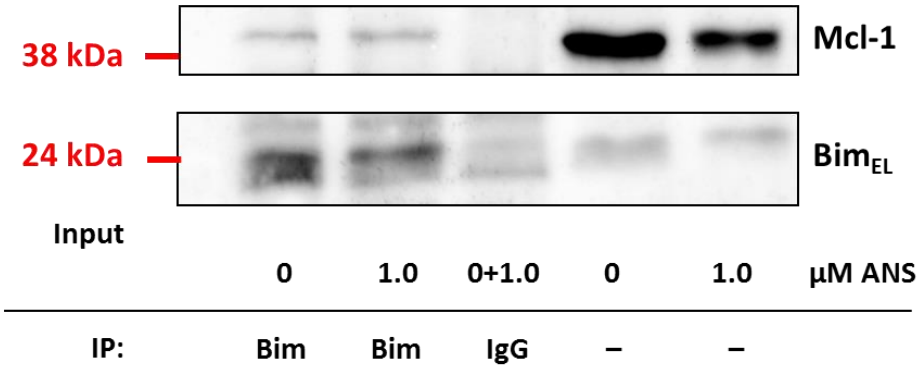

Figure 14. Bim interacts with Mcl-1 in MAC-T cells. MAC-T cells were treated with 1.0 µM ANS for 30 m. Whole cell lysates were collected in Modified RIPA buffer, immunoprecipitated with the indicated antibodies, then separated using SDS-PAGE (12.5% acrylamide) and immunoblotted with antibodies against Mcl-1 or Bim. Experiment performed once.

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