ROLE OF NFκB AND BCL-2 IN IGFBP-3 MEDIATED INTRINSIC APOPTOSIS

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ABSTRACT OF THE THESIS

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The overall goal of this work was to investigate the mechanisms by which IGFBP-3 mediates ribotoxin-induced apoptosis in the non-transformed bovine mammary epithelial cell line MAC-T. For this work, the ribotoxins anisomycin (ANS) and deoxynivalenol (DON), which both activate the intrinsic apoptotic pathway, were investigated. Similar to previous results with ANS, DON increased mRNA and protein levels of IGFBP-3 and knockdown of IGFBP-3 with small interfering (si)RNA attenuated DON-induced apoptosis. These results confirm a role for IGFBP-3 as a key player of intrinsic apoptosis associated with the ribotoxic stress response. However, the specific mechanism by which this occurs is largely unknown.

The early stages of intrinsic apoptosis are carefully controlled by members of the Bcl-2 family of proteins which is comprised of both pro- and anti-apoptotic members. The overall ratio between these pro- and anti-apoptotic proteins ultimately dictates the sensitivity or resistance of the cell to apoptotic stimuli. To investigate a role for Bcl-2 proteins, MAC-T cells transfected with IGFBP-3 or control siRNA were treated with ANS or DON and cell lysates were analyzed for changes in expression of Bcl-2 family proteins. IGFBP-3 knockdown significantly increased protein and mRNA levels of the pro-survival protein Bcl-2. Since knockdown of IGFBP-3 did not affect the levels of pro-

apoptotic proteins Bax and Bak, the increase in Bcl-2 increased the ratio towards one that favors survival. Given the relationship between Bcl-2 and the NFκB pathway, IGFBP-3 knockdown cells were treated with or without the NFκB inhibitor phenethyl caffeate (PC). Inhibition of NFκB with PC decreased Bcl-2 protein expression; however, this decrease was also observed in IGFBP-3 knockdown cells in the presence of PC. These data suggest that IGFBP-3 affects Bcl-2 expression through a mechanism that is upstream of NFκB. Interestingly, treatment with PC increased basal expression of IGFBP-3 mRNA and protein, indicating that NFκB represses IGFBP-3 expression in the basal state. In conclusion, IGFBP-3 appears to inhibit expression of Bcl-2 protein. However, while NFκB increases Bcl-2 expression and inhibits IGFBP-3 expression, the mechanisms by which these pathways are linked remain elusive.

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List of Abbreviations

ANS	Anisomycin
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
BH	Bcl-2 homology
Bim	Bcl-2 interacting mediator of cell death
CD95	Fas receptor
CD95L	Fas ligand
DIABLO	direct IAP- DIABLO binding protein with low PI
DISC	death-inducing signaling complex
DON	Deoxynivalenol
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
FBS	Fetal Bovine Serum
FLIP	FLICE-Like Inhibitor Protein
IAP	Inhibitors of Apoptosis
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 Receptor
IGFBP-3	Insulin-like growth factor binding protein 3
IKB	Inhibitor of Kappa B
IKK	Inhibitor of Kappa B Kinase

JNK	c-Jun N-terminal kinase
LBD	Light chain binding domain
MAC-T	Mammary Alveolar Cell
Mcl-1	Myeloid cell leukemia sequence 1 ix
MEC	Mammary Epithelial Cell
PRF	Phenol Red Free
Mule	Mcl-1 ubiquitin ligase E3
NEMO	NFκB Essential Modulator
NFκB	Nuclear Factor Kappa B
NIK	NFκB-Inducing Kinase
RIP	Ribosome Inactivating Protein
MAPK	Mitogen-Activated Protein Kinase
NLS	Nuclear Localization Sequence
PARP	poly ADP ribose polymerase
PC	Phenethyl Caffeate
PC-12	pheochromocytoma cells
PC-3	prostate cancer cells
RXRα	retinoid-x-receptor-α
SAPK	Stress Activated Protein Kinases
SF	Serum Free
siRNA	small-interfering ribonucleic acid
SMAC	second-mitochondria-derived activator of caspase
TAD	Transcriptional Activation Domain
tBid	Truncated Bid
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

CHAPTER 1: REVIEW OF LITERATURE

Introduction

Dairy milk is a significant agricultural resource and according to the world health organization milk demand is expected to increase by 25% within the next ten years. However, there is a significant environmental impact of raising cattle including but not limited to methane gas emission and waste excretion which could lead to potential eutrophication of water supplies (1). Ideally expansion of the industry will include an increase in total milk output per dairy cow instead of an increase in the size of the dairy herd. Milk output of the dairy cow follows a typical lactation curve whereupon after parturition there is a sharp increase in milk output for 6-8 weeks until peak milk yield is reached. This increase in milk production is a function of both an increase in the number of milk secretory mammary epithelial cells (MEC) as well as an increase in the milk secretory capacity per MEC (2). After peak milk output is reached there is a gradual decline in milk production despite no appreciable changes in secretory capacity per cell (2). Instead, this decrease is largely caused by an increased rate of apoptosis in the mammary gland resulting in a net decline in total MEC number (3).

Several methods and technologies have been explored to prolong lactation persistency such as administration of bovine somatotropin, a growth hormone which decreases the rate of apoptosis in the mammary gland (4). These anti-apoptotic effects of somatotropin are thought to be mediated through an increase in circulating insulin-like growth factor 1 (IGF-1) signaling (5). In circulation IGF-1 is bound by one of six members of the IGF binding protein family (IGFBP), the most common of which is IGFBP-3 (6). IGFBP-3 is able to regulate the half-life and availability of IGF-1 by sequestering it and preventing it from interacting with the IGF receptor, thus inhibiting itsmitogenic and pro-survival effects (6). In addition an apoptotic role independent of IGF function has been

demonstrated for IGFBP-3 (7). The overall goal of this thesis is to determine mechanisms by which IGFBP-3 may regulate apoptosis in an IGF-independent manner. Understanding how IGFBP-3 is involved in apoptosis of the mammary gland may ultimately lead to practices that can prolong secretory cell survival and thereby increase lactation persistency and total milk yield.

Apoptosis

Apoptosis, or programmed cell death, is an evolutionarily conserved self-destruction mechanism without an associated inflammatory response (8, 9). It is a vital aspect of maintaining healthy cells and plays an essential role in cell development, maintaining cell numbers within specific tissues and as a natural defense mechanism against toxic, disease causing agents (10). Examples of apoptosis-inducing agents include growth factor withdrawal and starvation, oxidative stress, UV irradiation, ribotoxic stress, tumor necrosis factor alpha (TNF-α), and binding of Fas ligand (11-15). Dysregulation of apoptosis may result in various disease states. For example, too little apoptosis may lead to cancer and tumorigenesis while situations where apoptosis is enhanced may lead to autoimmune and neurodegeneration diseases (16).

Apoptosis is generally characterized by distinct morphological and biochemical characteristics including mitochondrial outer membrane permeabilization, cytochrome c release, caspase cleavage, and DNA fragmentation. As apoptosis progresses the plasma membrane undergoes a process called blebbing where the cell becomes abnormally shaped and breaks off into small apoptotic bodies which are quickly phagocytosed by macrophages and degraded within phagolysosomes (17-19). Unlike

necrosis the cytosolic components of the cell are never released into the extracellular environment, thereby preventing the inflammatory or cytokine response (9).

There are two main pathways of apoptosis, referred to as the extrinsic and intrinsic pathway (Figure 1). The extrinsic, death receptor-mediated pathway, is activated by binding of ligands to specific cell surface receptors and consequent activation of the associated death receptors leading to an internal signaling cascade (20). The intrinsic, or mitochondrial pathway, responds to changes or damage to intracellular components resulting in a shift in the ratio of pro-apoptotic to anti-apoptotic proteins leading to the loss of mitochondrial membrane integrity and the subsequent release of intra-mitochondrial proteins (21, 22). Both pathways culminate with activation of a family of proteases called caspases which ultimately carry out the function of apoptosis through cleavage of cytoskeletal proteins, nucleic acids, and nuclear proteins (14).

Extrinsic Pathway of Apoptosis

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These “death receptors” are members of the TNF-α receptor gene superfamily and share a cysteine rich extracellular domain as well as an evolutionarily conserved cytoplasmic region referred to as the death domain. There are several members of the death receptor signaling family, including Fas ligand receptor (CD95L), TNF-α receptor 1 (TNFR1), DR3, DR4 and DR5 (14, 23, 24). The sequences of events that define extrinsic apoptosis are best characterized with the Fas ligand/Fas receptor and TNF-α/TNFR1 models.

Fas ligand binding to the Fas death receptor causes the intracellular death domain to trimerize allowing the cytoplasmic adaptor protein Fas-associated death domain (FADD) to bind to this region via homophilic interactions mediated by the death domains. This complex then recruits procaspase-8 via the death domains of FADD and

the death domain on procaspase-8. This now activated CD95 death-inducing signaling complex (DISC) acts as a scaffold to facilitate dimerization and auto-activation of caspase-8. Upon activation caspase-8 is rapidly released from the DISC complex and proceeds to activate downstream effector caspases such as caspse-3 which execute apoptosis (12, 25). When TNF-α binds to its receptor the same characteristic aggregation and trimerization of the cytosolic death domain occurs. However unlike CD95, active TNFR1 first recruits the adaptor protein TRADD which then recruits FADD (19, 26).

In addition to directly acting on caspase-3, caspse-8 interacts with the intrinsic apoptotic pathway. Activated caspase-8 cleaves the cytosolic protein Bid to its truncated form tBid. tBID is then able to translocate to the mitochondria, impinging on the intrinsic apoptotic pathway and potentiating apoptosis (27).

The extrinsic apoptotic pathway is under the regulatory control of a number of gene products. In both TNFR1 and CD95 death receptor-mediated signaling the Flice-inhibitory protein (FLIP) regulates apoptosis by blocking the interaction of FADD with the death receptor, thus preventing recruitment and activation of procaspase-8 (23). In addition to blocking the association of FADD with the death receptor, FLIP is able to recruit alternative adaptor proteins such as RIP, or TRAF-1,2,3 which lead to stimulation of the pro-survival NFκB pathway (24).

Intrinsic Pathway of Apoptosis

The intrinsic apoptotic pathway involves a diverse array of non-receptor mediated stimuli. These can include negative factors, such as withdrawal of growth factors or loss of apoptotic suppression signals, or positive factors, such as exposure to radiation, toxins, hypoxia, free radicals or viral infection (28). All of these stimuli induce loss of

mitochondrial membrane integrity facilitating the release of pro-apoptotic proteins basally sequestered within the mitochondrial interstitial space. Once in the cytosol, these released proteins, including cytochrome c, Smac/DIABLO, and Omi/HtrA2, trigger cell death by promoting caspase activation (14).

Cytochrome c binds with the cytosolic protein apoptotic protease activating factor-1 (Apaf-1) as well as procaspase-9 forming the apoptosome which leads to proteolytic cleavage and activation of the protease (19, 29). Activated caspase-9 in turn activates the executioner caspase-3 (19). Activated caspase-3 is able to cleave and activate caspase-6 and caspase-7 which then cleave key substrates in the cell to produce many of the cellular and biochemical events of apoptosis. SMAC/DIABLO and the protease HrA2/Omi support this process by binding to inhibitors of apoptosis proteins (IAPS) and inhibiting their caspase-binding abilities, thus freeing caspases to activate apoptosis (17, 30).

Bcl-2 Family of Proteins

The hallmark of the intrinsic apoptotic pathway is mitochondria integrity which is regulated at the early stages by one or more members of the B-cell lymphoma protein-2 (Bcl-2) family of proteins. This complex family consists of both pro-apoptotic and anti-apoptotic proteins, which are grouped within three sub-families, based on the number and type of BCL-2 homology (BH) domains they share (Figure 2).

All anti-apoptotic members of the BCL-2 family, including proteins such as Bcl-2, Bcl-w and Mcl-1, share multiple domains of homology among three or more of the four BH homology domains (BH 1-4) (31). The BH4 domain is conserved among anti-apoptotic BCL-2 proteins and mutations or deletions in the BH4 domain attenuates their anti-apoptotic ability, indicating that BH4 is critical for this activity. BH4 has been shown

to be critical to binding and sequestering pro-apoptotic Bcl-2 proteins which is a key feature of how these multi-domain anti-apoptotic proteins exert their pro-survival function (32, 33). In addition a role for the BH4 domain in regulating and activating the pro-survival NFκB pathway has also been reported (34, 35).

There are two groups of pro-apoptotic Bcl-2 proteins subdivided by the number of BH domains. Pro-apoptotic proteins possessing three BH (BH1-3) domains include Bax, Bak, and Bok which are referred to as multi-domain pro-apoptotic proteins. BH3-only proteins are characterized, as their name implies, by the presence of only the BH3 domain. Bax and Bak promote apoptosis by hetero- and homo-oligomerizing at the mitochondrial membrane, forming pores, and allowing inner mitochondrial proteins to be released into the cytosol (36, 37). Members of the BH3-only sub-family, represented by proteins such as Bad, Bid, and Bim, act as upstream sentinels that selectively respond to proximal death and survival signals. They can act by either directly activating Bax or Bak or by binding and suppressing specific anti-apoptotic BCL-2 proteins (38).

One of the striking features of the BCL-2 family proteins is their ability to form homodimers and heterodimers as a way of mediating their function. Oligomerization between pro-apoptotic multi-domain proteins Bax and Bak is required for permeabilization of the mitochondria outer membrane and apoptosis (36). Pro-survival proteins will bind and sequester Bax and Bak, preventing them from inducing apoptosis. However BH3-only proteins have a higher binding affinity for the pro-survival proteins and when their numbers increase they will become the dominant heterodimer, freeing Bax and Bak to induce apoptosis. (39). Hetero-dimerization between anti-apoptotic and pro-apoptotic BH3 only members in this manner inhibits the pro-survival function of their dimerization partner. This interaction is mediated by the insertion of the BH3 region of a pro-apoptotic protein into a hydrophobic pocket composed of BH1, BH2 and BH3 from

an anti-apoptotic protein (33, 39). Thus the relative ratios of pro- and anti-apoptotic Bcl-2 family proteins dictate the ultimate sensitivity or resistance of cells to various apoptotic stimuli.

The complex web of interactions between the Bcl-2 family of proteins is not completely delineated, however, there are two presiding models: the indirect activation model and the direct activation model. In the direct activation model BH3-only molecules are further classified into activators, which act directly on Bax and Bak, and sensitizers. In this model the primary function of anti-apoptotic Bcl-2 proteins is to bind and sequester the activator BH3-only molecules, preventing them from promoting apoptosis through their interaction with Bax and Bak. Sensitizers bind anti-apoptotic Bcl-2 proteins causing them to release their sequestered activator BH3-only proteins. In the healthy cell the balance is shifted towards apoptosis when a stimuli increases cellular levels of activator BH3-only proteins causing them to exceed the neutralizing capacity of anti-apoptotic members. Sensitizer molecules further lower the threshold of apoptosis by occupying the binding pocket of anti-apoptotic members and decreasing the amount of sequestered activator BH3-only proteins. (28, 34, 40)

In the indirect activation model all anti-apoptotic Bcl-2 proteins function to inhibit Bax and Bak. Apoptotic stimuli induce the BH3 proteins to bind to pro-survival Bcl-2 proteins thereby neutralizing their inhibitory effects on Bax and Bak. Free Bax and Bak are then able to oligomerize at the mitochondrial membrane and induce apoptosis (28, 34, 40). BH3-only proteins were originally thought to bind indiscriminately to pro-survival multi-domain proteins, however it was later discovered there was selectiveness amongst different proteins. For example, the BH3-only protein Bim binds indiscriminately to anti-apoptotic proteins while the BH3-only protein Bad preferably binds to Bcl-2 (41). Thus the large number of BH3-only molecules is indicative of specialization rather than

redundancy and allows the cell to respond to a larger range of input for stress signals.

Bcl-2

Bcl-2, the first identified member of the Bcl-2 family of proteins and the source of their namesake, is important for normal B cell development and differentiation. Translocations leading to constitutive expression of Bcl-2 were found in human B cell lymphoma, and the first link to a relationship between Bcl-2 signaling and regulation of cell survival (42, 43). Despite being a candidate oncogene, no specific biological effects of Bcl-2 were identified until Vaux et al. discovered that Bcl-2 overexpression promoted survival in IL-3 deprivation-induced apoptosis (44). This connection between Bcl-2 and apoptosis was further supported by later studies which showed overexpression of Bcl-2 abrogated apoptosis induced by several stimuli including serum deprivation, heat shock, chemotherapeutic agents and toxic insult suggesting Bcl-2 plays a critical role in mediating the intrinsic apoptotic pathway (45-47).

Bcl-2 functions by binding and sequestering pro-apoptotic proteins such as Bax, Bad, Bid, and Bim. Apoptotic stimuli cause conformational changes in Bcl-2 causing it to release the sequestered pro-apoptotic protein allowing them to induce apoptosis (15). These can be caused by binding of BH3-only proteins or post-translational modulations through mechanisms such as phosphorylation and mircoRNAs (48, 49). Phosphorylation of the flexible loop between the BH3 and BH4 domain significantly reduces its binding to multi-domain and BH3-only pro-apoptotic members (15). For example, TNFα-induced apoptosis is dependent on the phosphorylation of Bcl-2 which is mediated by IGFBP-3 (50). In addition to promoting cell survival through its interaction with pro-apoptotic proteins, Bcl-2 activates the pro-survival NFκB pathway through a mechanism that involves the BH4 domain of Bcl-2 (35).

Mcl-1

Myeloid cell leukemia sequence 1 (Mcl-1) is another example of an anti-apoptotic Bcl-2 protein. Like Bcl-2, Mcl-1 exerts its pro-survival function through binding and sequestering pro-apoptotic Bcl-2 proteins. Although capable of binding to both Bax and Bak, MCL-1 preferentially binds to Bak, as well as many other BH3-only proteins such as Bim and Noxa (51). Unlike most other anti-apoptotic Bcl-2 proteins, Mcl-1 lacks a BH4 domain and has a relatively short half-life which is a key feature of its regulation (52). Under basal conditions Mcl-1 is ubiquinated by the Mcl-1 Ubiquitin Ligase E3 (MULE) which marks it for proteasomal degradation. In the healthy cell the depleted Mcl-1 is rapidly regenerated through translation of new protein and the total amount of Mcl-1 protein remains at equilibrium. Disappearance of Mcl-1 is an early event in apoptosis. Apoptotic stimuli either suppress protein synthesis of Mcl-1 or increase the ubiquitination rate to exceed that of translation resulting in a rapid decrease in total protein levels (52-54).

Bax/Bak

Bax (Bcl-2 associated X) and Bak (Bcl-2 homologues killer) induce mitochondrial outer membrane permeabilization causing the release of cytochrome c and commitment to apoptosis (36). Bax and Bak can have redundant or non-redundant roles depending on the apoptotic stimuli functions and elimination of one of the two does not always prevent apoptosis. For example, Bak plays an essential role in ricin and vinblastine induced apoptosis while Bax is favored for apoptosis induced by TRAIL (55). However, double knockout of Bax and Bak in cells confers resistance to all apoptotic stimuli that activate the intrinsic apoptotic pathway, implicating these molecules as the requisite gateway to the mitochondrial apoptotic machinery (56, 57). In a cell free system the

addition of recombinant forms of Bax and Bak to isolated mitochondria is sufficient to induce loss of mitochondrial integrity and cytochrome c release (58).

In the cell Bax and Bak are carefully regulated by interaction with pro-survival Bcl-2 proteins at multiple different levels of control. Bax and Bak are held in inactive states through direct interaction with pro-survival Bcl-2 proteins. Apoptotic stimuli induce the loss of association with these pro-survival proteins, freeing Bax and Bak and allowing them to concentrate at the mitochondrial membrane (57). Bax and Bak can also be activated through direct interaction with BH3 only proteins, however the interaction is transient earning the name “hit and run” model of activation (59). The pro-survival proteins sequester BH3-only proteins to prevent them from interacting with and activating Bax and Bak (28, 34). At the mitochondrial membrane the pro-survival proteins exert an additional level of control by binding directly to the activated proteins, inducing conformation changes and preventing them from forming homo-oligomers (57).

In the healthy cell the majority of Bax exists in a latent form in the cytosol. In response to apoptotic signals, Bax undergoes conformational changes that expose its membrane-targeting domain and hydrophobic residues, increasing its affinity for biological membranes and decreasing its stability in the cytosol. These changes induce Bax to translocate to the mitochondria where it inserts its now exposed C-terminal trans-membrane domain into the mitochondrial outer membrane and firmly associates with the organelle (36). The association can be enhanced through interactions of BH3-only proteins such as the binding of Bim to the rear binding pocket of Bax, inducing conformational change that encourage the insertion of Bax into the membrane. Mitochondrial membrane-inserted Bax then undergoes further activation and conformational changes which permit its oligomerization and pore-forming activity (36, 60, 61).

Unlike Bax, Bak is constitutively inserted in the mitochondrial outer membrane in healthy cells. Protein levels of Bak do not change after exposure to apoptotic stimuli despite changes in mRNA levels suggesting that the regulation of Bak occurs at the post-translational level. The BH3-only proteins such as Bim, Bid and Puma can bind directly to Bak, inducing conformational change that expose its previously buried BH3 domain. The exposure of the BH3 domain allows Bax and Bak to form dimers and permeabilize the mitochondrial outer membrane (60, 62, 63).

Nuclear Factor-Kappa B (NFκB)

Nuclear factor-kappa B (NFκB) consists of a family of dimer-forming subunits that share a highly conserved Rel homology (RH) domain in their N-terminus within which lies the DNA-binding and dimerization domain as well as the nuclear localization signal (NLS). There are currently over 150 known genes under transcriptional control of NFκB with a majority of those genes participating in the immune response (64). In addition to mediating the immune response NFκB plays a major role in controlling a number of cellular processes including apoptosis, cell adhesion, proliferation, and inflammation (64, 65). The NFκB transcription factors are the result of combinatorial homo- and heterodimerization of different subunits, the most common being the p50/RelA heterodimer (64).

There are two main classes of subunits of the NFκB family based on structure and sequence homologies within their C-terminal. The Rel subfamily, which includes RelA (p65) c-rel, and RelB, contains a transactivation domain (TAD) near their C-terminus which enables them to induce transcription. The NFκB family has long C-terminal domains that contain inhibitory ankryin repeats. Members of this family including p105 and p100 become active only after cleavage by proteasome/ubiquitin processing to

their mature truncated form (p105 to p50 and p100 to p52) (66, 67). NFκB subunits do not contain a transactivation domain and thus are unable to positively induce transcription unless they dimerize with a member of the Rel family or other TAD-containing monomers. However p50 and p52 homodimers can play a role in regulation of NFκB activity by competing for κB promoter sites with transcriptionally active NFκB/Rel dimers (64, 67, 68).

In most cells NFκB dimers exists in the cytoplasm in an inactive form bound to the inhibitory kappa B (IκB) proteins. IκB proteins (IKBα, IκBβ, IκBε, IκBδ) are defined by the presence of multiple ankryin repeat domains which wrap around the dimeric interface of NFκB and mask the NLS (69). The large number of IκB proteins is indicative of specificity rather than redundancy as different NFκB dimer pairings have affinity for different IκB proteins and are responsive to specific stimuli (70). In addition to blocking the NLS sequence, IκB contains a strong nuclear export sequence which biases cellular localization of the complex to the cytoplasm (71). Dissociation from IκB exposes the NLS and allows the NFκB dimer to rapidly translocate to the nucleus, serving as a rapid transcriptional control mechanism without *de novo* protein synthesis (72).

NFκB Activation and Regulation

Potent inducers of NFκB include cytokines, TNFα and bacterial and viral products such as LPS or double stranded RNA. These stimuli initiate a signaling cascade leading to activation of IκB kinases (IKK) (73). The IKK complex is composed of two catalytic subunits (IKK1 and IKK2) and one scaffold protein, either IKKγ or NFκB essential modulator (NEMO). Endogenous inflammatory stimuli or exposure to pathogen-derived substances actives the canonical NFκB signaling pathway which is associated with the NEMO scaffold protein in the IKK complex and represents the general scheme of how NFκB is regulated (73, 74). Once activated the canonical IKK complex phosphorylate IκB on conserved serine residues as a signal for ubiquitination, leading to degradation of IκB

by the ubiquitin-proteasome pathway (70, 75, 76). Breakdown of IκB frees the previously sequestered NFκB dimers to translocate to the nucleus and initiate transcription (68, 76-78).

The alternative non-canonical pathway is found primarily in development of lymphoid organs responsible for the generation of B and T lymphocytes. It is activated by specific members of the TNF cytokine family, including CD40 ligand and BAFF, and predominantly targets the activation of p52/RelB NF-κB complexes. The non-canonical pathway is independent of NEMO and is instead transduced by an IKKα containing complex. After activation of this complex by NFκB inducing kinase (NIK), IKKα processes the NFκB precursor proteins p100 (IkBδ) and p105 (IkBγ) into their mature truncated forms thereby neutralizing their inhibitory effect on NFκB dimers and allowing them to dimerize with RelB subunits and translocate to the nucleus (74, 75, 79, 80).

Transient activation of NFκB is part of the normal immune response, however, malignancies in the regulation of NFκB are associated with situations of increased cell survival and chronic inflammation (81). The inhibitory IκB proteins IκBα, IκBβ and IκBε are among the NFκB targeted genes and thus serve as an auto-regulatory mechanism to temporally restrict NFκB action. Exposure of mouse embryonic fibroblasts to TNFα, a potent inducer of NFκB, results in a transient oscillation of NFκB activity regulated by cyclic degradation and re-accumulation of cytosolic IκBα. These IκBα-driven oscillations in nuclear NFκB activity are ultimately dampened by the later accumulation of IκBε protein. The combination and deferred kinetics of the two negative feedback loops are in antiphase and the late accumulation of IκBε plays a role in preventing chronic NFκB activation (70).

NFκB Crosstalk

In addition to its role in inflammation and the immune response, NFκB subunits have been shown to impinge on other signaling pathways and are important regulators

of apoptosis. A number of NFκB inducible anti-apoptotic genes have been identified including Bcl-2 which is heavily involved in mediating the intrinsic apoptotic pathway. Stimulation of NFκB signaling results in increased Bcl-2 protein levels in lymphomas and this effect was attenuated with overexpression of IκBα leading to inhibition of the NFκB pathway (82). These results were repeated with multiple studies that found suppression of NFκB signaling led to decreases in endogenous Bcl-2 protein levels (73, 83, 84).

A collection of studies has also associated IGFBP-3 with regulation of NFκB activity. In 2007 Williams et al. found that co-treatment of cells with IGFBP-3 and TRAIL enhanced apoptosis compared to TRAIL treatment alone. This effect was shown to be mediated through an IGFBP-3 dependent inhibition of TRAIL-induced NFκB activity (85). This inverse correlation between IGFBP-3 expression and NFκB signaling was confirmed by Youngman Oh's lab in a series of papers that examined the effects of IGFBP-3 on the prostate cancer cell line HT29 which is characterized by constitutive NFκB activity. Treatment of these cells with IGFBP-3 degraded key NFκB regulatory molecules p65 and IκBα, and also enhanced chemotherapeutic-induced growth inhibition and apoptosis. Furthermore, co-treatment with a caspase inhibitor attenuated IGFBP-3's effects on NFκB inhibition suggesting IGFBP-3 inhibition of NFκB signaling is mediated through cleaved caspases (85-87).

The Insulin-like Growth Factor System

The insulin-like growth factors IGF-I and IGF-II influence cell proliferation, migration, differentiation of tissues, and survival. Most of the cellular effects of the IGFs, which are structurally and functionally related to insulin, are mediated through binding of these peptides to the IGF-I receptor (IGF-IR). Unlike insulin, IGF-I and IGF-II are not stored intracellularly, but are secreted into the extracellular environment where they are

predominantly bound by IGF binding proteins (IGFBPs) (88). There are six members of the IGFBP family (1-6) with IGFBP-3 being the most predominant member of this family in adult serum and most circulating IGF-I is found bound to IGFBP-3 in ternary complexes with acid labile subunit (89, 90).

IGFBP-3 is a multi-faceted protein and can either inhibit or enhance IGF action. It has a significantly higher affinity to IGF-I and IGF-II than does IGF-IR. Therefore IGFBP-3 not only acts as a carrier for IGFs but is also able to inhibit the mitogenic action of IGFs by binding and sequestering free IGF, preventing it from interacting with its cell surface receptor (91). In addition binding of IGF to IGFBP-3 in serum prolongs its circulating half-life from 20-30 minutes for free IGF to 12-15 hours when bound by IGFBP-3. Although the specific function of the large supply of circulating IGF bound with IGFBP-3 is currently unknown, one hypothesis is that it serves as a functional pool of material that is readily available for organisms to use during times of stress (92).

IGFBP-3 and Apoptosis

IGFBP-3 has been identified as a potent anti-proliferative agent. Originally it was hypothesized IGFBP-3 influences growth inhibition and apoptosis by modulating IGF bioavailability. This was supported by a number of studies. Addition of IGFBP-3 was shown to inhibit IGF-I stimulated DNA synthesis in fibroblast cells (93). Furthermore excess IGFBP-3 was shown to inhibit IGF-I mediated glucose incorporation in BALB/c3T3 cells. Pre-incubation of these cells with IGFBP-3 was sufficient to suppress subsequent IGF-1 signaling, suggesting the mechanism of IGFBP-3 inhibition on IGF action is related to the ability of IGF-1 to bind to the IGF-I receptor (94).

Although IGF-dependent inhibition of growth is responsible for IGFBP-3 action in some situations, a role of IGF-1 independent functions of IGFBP-3 has emerged. In

1993 Oh et al. first demonstrated that IGFBP-3 was able to specifically bind the cell surface of Hs578T human breast cancer cells and inhibit cell growth by itself, suggesting existence of specific cell surface associated receptors for IGFBP-3 (95). A series of other experiments confirmed the possible connection between IGF independent actions of IGFBP-3 (7, 94). However the first true IGF free system was not examined until Valentinas et al. discovered that IGFBP-3 inhibited cellular proliferation in IGF-1R null mouse fibroblasts, providing strong support for an IGFBP-3 apoptotic function independent of IGF-1 signaling and the IGF-1R (96). Since the initial discovery further studies have corroborated the IGF-1 independent anti-proliferative and apoptotic effects of IGFBP-3 and added to our understanding of how this molecule mediates cell signaling. IGFBP-3 has been shown to play a role in the antiproliferative and pro-apoptotic effects of retinoic acid, TNFα, p53, and vitamin D. (97-101). In bovine mammary epithelial cells (MAC-T), apoptosis induced by the ribotoxin anisomycin was shown to be mediated, in part, through IGFBP-3 (101).

Nuclear Actions of IGFBP-3

While IGFBP-3 is a secreted protein, it also has a putative nuclear localization signal in the carboxyl-terminal domain and has been identified in the nucleus in several cell types (102-104). This basic domain is highly conserved in IGFBP-3 from different species, suggesting that it has functional significance (105). In 2000 Schedlich et al. showed that neutralization of importin β resulted in a significant reduction in the level of nuclear import of IGFBP-3 (106). Functional significance of IGFBP-3's presence in the nucleus was not demonstrated until several years later when Schedlich discovered IGFBP-3 could bind with the retinoid X receptor-α (RXRα) and may mediate its IGF-independent cellular effects via RXRα dependent interaction with growth-inhibitory genes, apoptotic genes, or both (65). The apoptotic effects of IGFBP-3 were abolished in

an RXRα knockout cell line indicating that RXRα is required for IGFBP-3 induced apoptosis (107). Lee et al. expanded on these studies and showed that IGFBP-3's interaction with RXRα facilitated its hetero-dimerization with the orphan nuclear receptor Nurr77. This complex then translocates without IGFBP-3 to the mitochondria to induce cytochrome c release and apoptosis. Loss of either IGFBP-3 or RXRα prevents this mitochondrial translocation of Nurr77 and protected from IGFBP-3 induced apoptosis (108). Further studies showed that endogenous IGFBP-3 associated with RXRα but not Nurr77 under basal conditions; however when the intrinsic apoptotic pathway was activated after treatment with anisomycin, IGFBP-3 co-precipitated with the phosphorylated forms of both proteins. Knockdown of IGFBP-3 prevented anisomycin induced phosphorylation and translocation of Nurr77 from the nucleus to the mitochondria suggesting IGFBP-3 plays a critical role in these events (109).

Ribotoxic Stress

Ribotoxins such as anisomycin target the ribosome, inhibit protein synthesis, and activate a conserved cellular reaction referred to as the ribotoxic stress response. The ribotoxic stress response is characterized by a strong activation of the mitogen associated protein kinases (MAPKs) pathway and the resulting signaling transduction leading to apoptosis (110). After entry into the cell, ribotoxins associate with a specific region of the 3' end of the 28S ribosomal RNA, the peptidyl transferase center in the eukaryotic 60S ribosomal subunit (111). Association or damage to this region results in inhibition of protein synthesis, but MAPK activation occurs independently of protein synthesis inhibition (110). Certain ribotoxins such as the large family of ribosome inactivating proteins (RIPs) contain inherent enzymatic activity and depurinate a specific adenine residue of the ribosome, while binding of other ribotoxins such as tricothecnes

to this center results in conformational change of the ribosome and cleavage by internal RNases (13, 112). Still other ribotoxins such as anisomycin are not known to damage the ribosomal subunit but merely inhibit protein synthesis. In contrast to genotoxic insult or hyperosmotic shock, ribotoxic stress requires ribosomes that are translationally active at the time of toxin exposure. Pre-treatment with translational inhibitors such as cyclohexamine, or emetine, abolishes the MAPK activation in response to ribotoxins (113).

MAPK Signal Transduction

MAPKs are serine/threonine kinases that interpret a wide variety of cell stimuli. Upon activation they can phosphorylate specific substrates and modulate gene expression, proliferation and programmed cell death. There are three main arms of the MAPK pathway which include the extracellular signal related kinase (ERK) 1 and 2, the stress-activated protein kinases including c-jun NH_2 terminal kinase (SAPK1/JNK) and the p38 kinase (SAPK2) (114). The p38 pathway is associated with inflammation and cytokine production while activation of the JNK pathway is associated with transcriptional activation of c-jun and fos response elements (115). Transient activation of the MAPKs is associated with pro-survival signaling but prolonged activation, as is the case with ribotoxic stress, mediates cell death. The strength and duration of MAPK activation after ribotoxic insult is cell line dependent. For example, treatment of lymphocytes with the ribotoxin deoxynivalenol (DON) results in apoptosis dependent on JNK and p38 but not ERK while anisomycin induced apoptosis in MAC-T cells is dependent on ERK and JNK but not p38 (101, 110).

The direct mechanism of action between damage to the ribosome and the prolonged activation of the MAPK signaling cascade is currently unknown. Protein kinase R (PKR) has been suggested as the missing link between damage to the

ribosomal structure and MAPK activation (116). PKR is a serine/threonine protein kinase known to associate with the ribosome and function as an intracellular sensor of stress (117). Activated by binding dsRNA, PKR monomers dimerize resulting in autophosphorylation and self-activation. The ability of the ribotoxins DON and ANS to induce apoptosis in a human monocyte cell line iscompletely attenuated in PKR-deficient cells, implicating PKR as a critical mediator of ribotoxic stress (116, 118). PKR is able to directly interact with ASK1, an upstream MAPK, and knockdown of PKR prevents p38/JNK1 activation by DON in HeLa cells (119). In addition PKR has been found to catalyze phosphorylation and inhibition of the translation initiator eIF2α (116).

Anisomycin

Anisomycin (ANS), perhaps the first identified inducer of the ribotoxic stress response, is able to passively diffuse through the plasma membrane and associate with the peptidyl transferase center of the ribosome. ANS has been used as a model in our lab to investigate the intrinsic apoptotic pathway in the mammary epithelial cell line MAC-T (114). It is able to passively diffuse through the plasma membrane and associate with the peptidyl transferase center of the ribosome. We have demonstrated that treatment of these cells with ANS activates the p38 and JNK pathway as well as IGFBP-3 mRNA and protein expression (120). Unpublished data from our lab has determined ANS-induced IGFBP-3 upregulation is downstream of p38 activation while JNK plays a role in controlling basal IGFBP-3 expression. Furthermore, knockdown of IGFBP-3 attenuates ANS-induced apoptosis implicating IGFBP-3 as a key mediator of the intrinsic apoptotic machinery (101).

Tricothecenes

The trichothecenes, a group of sesquiterpenoid mycotoxins produced by the genus *Fusarium* are activators of the ribotoxic stress response (121). Trichothecene mycotoxins are of particular concern because of their worldwide contamination of staple crops such as barely, wheat, corn, and other cereals, their resistance to degradation during food processing and their adverse effects on health as well as potential use in biological warfare (122). To date, over 200 trichothecenes have been identified with the one most commonly encountered in grain contamination being deoxynivalenol (DON) (123). DON is a type B trichothecene that targets the innate immune system. Acute low dose exposure causes immune-stimulatory effects as well as vomiting, and severe abdominal discomfort while exposure to acute high doses results in immunosuppression, apoptosis of lymphocytes and thymus tissue, and can have lethal consequences (124).

DON is a low molecular weight molecule that does not require an extracellular receptor to enter the cell. Like ANS it is able to passively diffuse through the cell membranes, bind to the ribosome and activate the ribotoxic stress response. Due to their anti-proliferative effects trichothecenes have been evaluated for potential therapeutic roles in the treatment of cancer; however, adverse health effects were reported and they were not perused past phase II clinical trials (125). Further work is needed to understand the complexities of the intracellular signaling process leading to apoptosis after treatment with these compounds. Given that DON behaves similarly to ANS we hypothesized that IGFBP-3 may also play a critical role in mediating DON-induced apoptosis in MAC-T cells.

CHAPTER 2: ROLE OF NFκB AND BCL-2 IN IGFBP-3 MEDIATED INTRINSIC APOPTOSIS

Introduction

Efficiency of milk production is key to the sustainability of the dairy industry. In the dairy cow lactation output is a function of the number of secretory mammary epithelial cells (MEC) and the secretory capacity per cell (2). Peak milk yield occurs 6-8 weeks after calving followed by a gradual decline in milk production despite no appreciable changes in secretory capacity per cell (2). Instead, this decrease is largely caused by an increased rate of apoptosis in the mammary gland resulting in a net decline in total MEC number (3). Once milk production drops below a certain daily output, costs associated with animal maintenance outweigh profit from sale of the milk. Understanding the basic mechanisms involved in apoptosis of the mammary gland may ultimately lead to practices that can prolong secretory cell survival and thereby increase lactation persistency and total milk yield.

The loss of MEC by apoptosis after peak lactation likely occurs through the intrinsic pathway, which is dependent on mitochondrial integrity. This is controlled by members of the B-cell lymphoma protein-2 (Bcl-2) family of proteins. This complex family consists of both pro- and anti-apoptotic members that are able to form homo- and heterodimers as a way of mediating their function (34). Oligomerization of the pro-apoptotic proteins Bax and Bak on the mitochondrial surface induces pores in the outer mitochondrial membrane, disrupting the mitochondrial integrity and facilitating the release of pro-apoptotic proteins basally sequestered within the mitochondrial interstitial space thereby committing the cell to apoptosis (36). In the healthy cell Bax and Bak are held bound and sequestered by pro-survival Bcl-2 proteins such as Bcl-2 and Mcl-1, preventing them from interacting with the mitochondrial cell surface and inducing

apoptosis. Exposure to apoptotic stimuli activates pro-apoptotic BH3-only proteins either through transcriptional or post-translational mechanisms, which competitively bind to the pro-survival Bcl-2 proteins, neutralizing their inhibitory effects on Bax and Bak (28). Thus, the relative ratio of pro- and anti-apoptotic proteins dictates the ultimate sensitivity or resistance of cells to various apoptotic stimuli.

Several of the anti-apoptotic members in the Bcl-2 family are under the transcriptional control of nuclear factor kappa B (NFκB) (83) NFκB is a pro-survival transcription factor whose activity is tightly regulated by interaction with the inhibitory kappa B (IκB) proteins (78). In most cells NFκB is bound and sequestered by IκB in a latent form in the cytosol. Upon stimulation IκB is phosphorylated by the IKK complex which marks it for degradation via the ubiquitin-proteasome pathway. Dissociation from IκB allows the NFκB dimer to translocate to the nucleus, serving as a rapid transcriptional control mechanism without *de novo* protein synthesis (64).

IGF binding protein-3 (IGFBP-3) is a multi-factorial protein with an established IGF-independent apoptotic role and it has been shown to have inhibitory effects on NFκB. (85, 86). Our lab has previously demonstrated that IGFBP-3 plays a role in apoptosis induced by the ribotoxic stressor anisomycin (ANS) in the bovine MEC cell line MAC-T. Knockdown of IGFBP-3 attenuates ANS-induced apoptosis; however, the molecular mechanism that underlies these effects is not well-delineated (101). Like ANS the ribotoxin deoxynivalenol (DON) also activates the ribotoxic stress response; however, a role for IGFBP-3 in mediating DON-induced apoptosis has not been explored. The goals of the present work were to determine if IGFBP-3 is a critical component of a global ribotoxic stress pathway and to determine the cellular mechanisms by which IGFBP-3 mediates ribotoxic-stress induced apoptosis

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium with high glucose (DMEM-H), penicillin, and streptomycin were purchased from Life Technologies (Carlsbad, CA). Phenol Red-free (PRF) DMEM with low glucose, gentamicin, bovine insulin, Anisomycin (ANS), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against the following proteins were purchased as indicated: Cleaved caspase-3 and -7, total JNK, phosphorylated p38, total p38, PARP, and Mcl-1 (Cell Signaling Technology, Inc., Danvers, MA), Bcl-2, phosphorylated JNK, Bax and Bak (Santa Cruz, Dallas, TX). anti-rabbit IgG (GE, Pittsburgh, PA) anti-mouse IgG (Vector, Burlingame, CA). Custom SmartPool siRNA for bovine IGFBP-3 and scramble siRNA were purchased from Dharamacon, Inc (Lafayette, CO). IGFBP3 and cylcophilin rtPCR primers were purchased from Sigma-Aldrich. Bcl-2 rtPCR primers were purchased from Sigma-Aldrich. Deoxynivalenol was kindly provided by Dr. Rong Di, Rutgers University.

Cell Culture

The bovine mammary epithelial cell line MAC-T was maintained in DMEM-H supplemented with 4.5 g/L D-glucose, 20 U/mL penicillin, 20 µg/mL streptomycin, 50 µg/mL gentamicin, 10% fetal bovine serum (FBS), and 5 µg/mL bovine insulin. For experiments cells were plated at a concentration of 1x104 cells/cm^2 and grown to confluence in PRF DMEM-H containing 10% FBS and antibiotics and without insulin. Cells were washed twice with phosphate buffered solution (PBS) incubated overnight in serum free (SF) PRF DMEM-H supplemented with 0.2% BSA and 30 nM sodium selenite prior to exposure to treatment in PRF SF DMEM-H with antibiotics and without insulin.

Western Immunoblotting

Cells were washed twice with ice 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. Total protein content of lysates was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5 or 15% gels and transferred to nitrocellulose membranes (0.2 µm; Bio-Rad Laboratories) or PVDF (0.45 µM; Millipore, Billerica, MA) membranes. PVDF membranes were incubated in methanol for 15 sec and dried at room temperature for 15 min while nitrocellulose 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) prior to incubation 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).

siRNA Experiments

MAC-T cells were plated at $3x10^4$ cells/cm^2 in PRF DMEM-H media containing 10% FBS and antibiotics. After reaching ~70% confluence cells were transfected with 50 nM bovine IGFBP-3 siRNA as well as corresponding concentrations of scrambled control siRNA using Mirus Transit TKO reagent (Mirus Bio LLC, Madison, WI) according to

manufacturer's specifications. After reaching confluence, cells were washed twice with 1x PBS and incubated overnight in SF PRF DMEM-H supplemented with BSA, sodium selenite and antibiotics and without insulin prior to treatment in SF PRF DMEM-H media. Gene knockdown was verified by Western Immunoblotting as described above.

Reverse Transcription Quantitative PCR (qRT-PCR)

For determination of mRNA expression cells were lysed following treatment in TRizol Reagent (Life Technologies, Carlsbad, CA) and RNA was isolated using Nucleospin RNA II Kit a (Machery-Nagel, Bethlehem, PA) according to manufacturer's specifications. RNA concentration was measured using a Nanodrop 1000 spectrophotometer and integrity was assessed via visualization of the 18S and 18S ribosomal RNA bands by gel electrophoresis. For cyclophilin primers were forward = 5'-GAGCACTGGAGAGAAAGGATTTGG-3'; reverse = 5'-TGAAGTCACCACCCTGGCACATAA-3'. Final primer concentration was 0.125µM. For IGFBP-3 primers were forward = 5'-CAGAGCACAGACACCCAGAA-3'; reverse = 5'-GGAACTTGAGGTGGTTCAGC-3'. Final primer concentration was 0.25µM. For Bcl-2 primers were forward = 5'-TGTGGATGACCGAGTACCTGAA-3'; reverse = 5'-AGCCTCCGTTGTCCTGGAT-3'. Final primer concentration was 0.25µM. Samples were diluted 1:4 for IGFBP-3 mRNA analysis and 1:100 for BCL-2 mRNA analysis. 5 µl of diluted samples were amplified in a 20 µl reaction containing 10 µl SYBR green (Applied Biosystems), 4 µl water, and 0.5 µl of each gene-specific primer. Reactions were run on the ABI 7300 system using cycle parameters of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with cyclophilin as the housekeeping gene. PCR products were verified by melt curve analysis.

Measurements of Caspase Activation

Caspase-3/-7 cleavage was determined using the Sensolyte Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA) according to manufacturer's specifications.

Statistical analysis

Densitometry data were analyzed by two-way ANOVA followed by a post hoc Tukey's multiple comparison tests with differences considered significant for $p<0.05$. Data from caspase assays were analyzed by one-way ANOVA followed by a post hoc Tukey's multiple comparison tests with differences considered significant for $p<0.05$. Data from qRT-PCR were analyzed by either unpaired t-test or one-way ANOVA followed by a post hoc Tukey's multiple comparison test with differences considered significant for $p<0.05$. Analyses were performed with GraphPad Prism (La Jolla, CA).

Results

ANS and DON induce prolonged activation of SAPK signaling

A hallmark of ribotoxic stress is activation of the SAPK pathway. Previous work in our lab has determined treatment of MAC-T cells with ANS induces robust activation of both p38 and JNK signaling that is maintained for at least 3 h following treatment although the strength of activation was slightly decreased at 3 h compared to 1 h of treatment (120). However, the time course of SAPK signaling by DON in MAC-T cells has not been determined. To explore the time course of DON-induced JNK and p38 phosphorylation in comparison to ANS treatment, MAC-T cells were treated with 1 µg/ml DON or 0.1 µM ANS for 1 to 6 h. As expected, JNK and p38 signaling were activated at 1 h with ANS treatment (Figure 3A). However, both JNK and p28 declined between 4 to 6 h. Exposure of MAC-T cells to DON also stimulated robust activation of the SAPK

pathways; however, activation of both pathways was sustained through 6 h of treatment (Figure 3B).

ANS and DON both induce apoptosis and IGFBP-3 expression

Our lab has previously demonstrated that treatment with the ribotoxin ANS activates the intrinsic apoptotic pathway and induces IGFBP-3 expression in MAC-T cells (101, 120). To determine if DON produced a similar response, MAC-T cells were exposed to 0.1 µM ANS or 1.0 µg/mL DON for 6 h and immunoblotted for apoptotic markers. As shown in Figure 4A both ribotoxins induced an increase in cleaved PARP, as well as cleaved caspase-3 and -7 compared to serum-free controls. To quantify the increase in caspase-3/7 activation a fluorometric caspase assay was performed. ANS and DON both significantly increased caspase-3/7 cleavage compared to serum free (SF) control ($p<0.05$; n=4; Figure 4B).

In order to determine if treatment with DON induces IGFBP-3 expression, cells were treated with 0.1 µM ANS or 1.0 µg/mL DON for 3 h and qRT-PCR was performed. As expected, exposure of cells to ANS stimulated a large increase in IGFBP-3 mRNA expression compared to SF controls (Figure 5A; n=2; $p<0.05$). A similar increase of approximately 30-fold was observed with DON treatment. As shown in Figure 5B the abundance of IGFBP-3 protein in whole cell lysates was also substantially increased following exposure to ANS or DON for 6 h, indicating that the enhanced IGFBP-3 mRNA levels translated into increases in IGFBP-3 protein. These data confirmed our previous observations with ANS and also demonstrate that the ribotoxin DON similarly induces apoptosis and IGFBP-3 expression in MAC-T cells.

IGFBP-3 plays a role in ANS and DON-induced apoptosis

To determine if IGFBP-3 plays a role in mediating DON-induced apoptosis in MAC-T cells as it does in ANS-induced apoptosis, IGFBP-3 was knocked down using siRNA. Analysis of IGFBP-3 protein by western blot confirmed successful knockdown of IGFBP-3 expression in ANS- and DON-treated cells (Figure 6). As previously reported, treatment of cells with IGFBP-3 siRNA effectively attenuated ANS-induced apoptosis as shown by a reduction in PARP cleavage and cleavage of caspase-3 and -7 compared to control cells transfected with scramble siRNA. Similar results were obtained when cells were treated with DON. These data indicate that IGFBP-3 mediates DON-induced apoptosis as it does ANS-mediated cell death in MAC-T cells.

IGFBP-3 knockdown modulates mRNA and protein expression of Bcl-2

To investigate a possible intracellular mechanism for the observed attenuation in apoptosis in IGFBP-3 knockdown cells, we examined the expression of members of the Bcl-2 family, which are key proteins in the intrinsic apoptotic pathway. As shown in Figure 7A, expression of the pro-survival Bcl-2 protein increased in cells treated with IGFBP-3 siRNA compared to cells treated with scramble siRNA. This effect was independent of toxin treatment with a maximum induction in Bcl-2 protein of approximately 4-fold compared to SF conditions. (Figure 7B; $n=4$; $p<0.05$).

To determine if the modulation of Bcl-2 protein expression occurred at the level of mRNA Bcl-2 mRNA levels were evaluated by qRT-PCR. Since toxin treatment had no effect on Bcl-2 expression, only the serum-free condition was examined. As can be seen in Figure 7C, IGFBP-3 knockdown induced a significant increase in Bcl-2 mRNA ($n=3$; $p<0.05$).

No significant differences were observed in expression of Mcl-1 protein as a result of IGFBP-3 knockdown although a slight decrease in Mcl-1 expression was

observed in SF and DON treated IGFBP-3 knockdown samples compared to relative scramble siRNA transfected controls. Although not a large difference, this change in Mcl-1 expression as a result of IGFBP-3 knockdown was consistent in multiple experiments and may warrant further investigation. Steady state levels of Bax and Bak protein were not affected by toxin treatment or IGFBP-3 knock-down.

In summary, these data show that IGFBP-3 knockdown induces a significant increase in Bcl-2 protein. While overall protein levels of Bak and Bax were unchanged, the increase in Bcl-2 could affect the ratio between pro- and anti-apoptotic proteins and decrease the sensitivity to apoptotic-inducing agents thus contributing to the observed attenuation of ANS- and DON-induced apoptosis in MAC-T cells when IGFBP-3 is knocked down.

The increase in Bcl-2 protein is still observed with IGFBP-3 knockdown when NFκB is blocked

Bcl-2 is under transcriptional control of the pro-survival NFκB signaling pathway (83). To determine if changes in NFκB activity (possibly induced by IGFBP-3 knockdown) play a role in mediating the increased Bcl-2 expression, cells in which IGFBP-3 had been knocked down were treated with phenethyl caffeate (PC), a specific and potent inhibitor of NFκB. As indicated above, since the increase in Bcl-2 protein was observed with or without toxin, these experiments were performed in untreated MAC-T cells without subsequent exposure to ANS or DON.

As shown in Figure 8A, inhibition of NFκB by PC in cells transfected with scramble or IGFBP-3 siRNA decreased Bcl-2 protein expression compared to non-PC treated lysates. When quantified this PC-induced decrease in Bcl-2 protein expression was found to be significant supporting a role for NFκB in mediating Bcl-2 protein expression in MAC-T cells (Figure 8B; $n=3$; $p<0.05$). As expected Bcl-2 protein

expression was upregulated in cells transfected with IGFBP-3 siRNA compared to scramble-transfected control. However, there was no significant interaction between the main effects of IGFBP-3 siRNA and PC, indicating that Bcl-2 protein expression was still increased by IGFBP-3 knockdown whether or not cells were treated with PC. These findings indicate that NFκB activity is not directly responsible for the observed increase in Bcl-2 protein expression. Further work is needed to elucidate the significance of Bcl-2 expression in attenuation of ANS- and DON-induced apoptosis by IGFBP-3 knockdown and to understand the mechanisms that underlie the observed effect.

Inhibition of NFκB increases basal IGFBP-3

An interesting finding was that inhibition of NFκB with PC resulted in an increase in IGFBP-3 protein expression (Figure 9A). To determine if the upregulation of IGFBP-3 expression occurred at the mRNA level cells were treated with or without PC and IGFBP-3 mRNA was analyzed using qRT-PCR. This increase in protein correlated with a significant increase in IGFBP-3 mRNA in cells treated with PC compared to non-PC treated controls (Figure 8B; $n=3$; $p<0.05$). Although further studies are needed to expand on the mechanism between NFκB inhibition and IGFB-3 expression the data suggests that IGFBP-3 expression may be reciprocally regulated by NFκB.

Discussion

The present results indicate that IGFBP-3 plays an important role in mediating the ribotoxic stress pathway in response to the ribotoxins ANS and DON. Both ribotoxins are low molecular weight molecules capable of passively diffusing through cell membranes, interacting with the ribosome at the peptidyl transfer center of the 28S rRNA and activating the SAPK signaling pathways which are evolutionarily conserved in all eukaryotic cells (112, 118). Previous data from our lab has demonstrated treatment of

MAC-T cells with ANS results in rapid and robust activation of JNK and p38 which is maintained through 3 h; however, we were interested in seeing if this activation extended beyond this time point (120). As trichothecene-induced apoptosis in vitro is dependent on JNK and p38 activation we examined the signaling of these two MAP kinases in response to prolonged DON treatment (126, 127). We show here that both toxins resulted in sustained activation of the MAPK signaling pathways. However, DON treatment resulted in a more robust and prolonged activation of JNK that was maintained through 6 h of treatment whereas ANS-induced JNK activation was attenuated at 2 h.

Although both toxins induce ribotoxic stress, there are differences in how they interact with the ribosome that may account for the more sustained activation observed with DON. A prominent consequence of DON exposure is ribosomal RNA cleavage which has been suggested to result from upregulated RNase expression (118). However, there are no reports of ribosomal cleavage or loss of structural integrity after ANS treatment. Instead this compound is thought to associate with the ribosome at the peptidyl transferase center which is sufficient for activation of the ribotoxic stress response (128). Our lab has previously reported that ricin A chain, a ribosomal inactivating protein that specifically and irreversibly cleaves the 4323 and 4324 adenine residues of the 28S rRNA, induces prolonged p38 and JNK activation that is maintained throughout 8 h of toxin exposure (129). It may be that the duration and intensity of MAPK activation correlates with the severity of damage to the ribosomal subunit.

We have previously documented a role for IGFBP-3 in ANS-induced apoptosis and shown that this ribotoxic stressor induces both IGFBP-3 mRNA and protein expression in a time frame that coincides with maximal apoptosis (101). There have been several studies documenting the ability of anti-proliferative agents such as retinoic acid, TNFα, and the tumor suppressor p53 to stimulate IGFBP-3 protein (90); however, this is the first report implicating the trichothecene DON as a potent inducer of IGFBP-3

expression. As ribotoxins are able to inhibit protein synthesis, the upregulation of IGFBP-3 suggests it's selectively translated during this process, possibly because of its role in apoptosis. Indeed, knockdown of IGFBP-3 attenuated ANS- and DON-induced apoptosis suggesting that IGFBP-3 is a key component of the apoptotic machinery of ribotoxic stress. Based on these findings, we sought to understand the mechanism of its role in ribotoxic stress using an IGFBP-3 knockdown system.

Ribotoxic stress is mediated by the intrinsic apoptotic pathway which is regulated at the early stages by one or more members of the Bcl-2 family of proteins. This family is comprised of proteins that both up and downregulate apoptosis including anti-apoptotic members, such as Bcl-2 and Mcl-1, multi-domain pro-apoptotic members, such as Bax and Bak, and pro-apoptotic BH3-only proteins, including Bad, Bim, and Bid. The role of Bcl-2-like anti-apoptotic proteins is to inhibit their pro-apoptotic partners and the overall ratios of anti- and pro-death family proteins ultimately correlates with cell fate and sensitivity to apoptotic inducing agents (28, 62, 72). Therefore we examined IGFBP-3 knockdown cells for changes in expression of many of the key pro- and anti-apoptotic members of the Bcl-2 family.

We found that IGFBP-3 knockdown resulted in modulation of the ratio of these Bcl-2 proteins. Bcl-2 mRNA and protein were upregulated in IGFBP-3 knockdown cells without subsequent changes in Bax, or Bak. Interestingly, the pro-survival Mcl-1 did not exhibit a directional change similar to Bcl-2, indicating this effect was specific to Bcl-2 and not the pro-survival proteins in general. Bcl-2 is capable of binding and sequestering the pro-apoptotic protein Bax, preventing Bax/Bak oligomerization which would otherwise lead to the release of several apoptogenic molecules sequestered within the mitochondria and subsequent commitment to apoptosis (15). Overexpression of Bcl-2 has been associated with enhanced cell survival and is common in many types of human cancers including prostrate, colorectal and lung cancer and contributes to

resistance to chemo-toxic insult (130). It is possible that the upregulation of Bcl-2 protein after IGFBP-3 knockdown shifts the balance of the Bcl-2 family of proteins towards cell survival and confers resistance to ANS- and DON-induced apoptosis.

An association between IGFBP-3 and Bcl-2 expression is not without precedent. Overexpression of IGFBP-3 in human breast cancer cells results in down-regulated Bcl-2 protein and mRNA and a shift in the Bcl-2 to Bax ratio, leading to enhanced apoptosis (131). Conversely, downregulation of IGFBP-3 in esophageal carcinoma cells increased cell viability. This was mediated through an upregulation of Bcl-2 protein and downregulation of Bax protein which conferred resistance to ionizing radiation induced apoptosis (132). Although we did not observe a change in Bax expression in response to IGFBP-3 knockdown the effects on Bcl-2 expression mirrored the findings of these previous experiments. However there are currently no reports suggesting a potential mechanism between how the changes in IGFBP-3 protein levels modulate Bcl-2 expression.

In our study we found IGFBP-3 knockdown increased Bcl-2 mRNA. One possibility is that it may regulate Bcl-2 expression at the transcriptional level. In 2001 an NFκB binding site was identified in the p2 promoter of Bcl-2 and since then the protein has been confirmed to be transcriptionally regulated by NFκB (73, 83, 84). Recently published data suggest a possible link between IGFBP-3 expression, NFκB activity and apoptosis (86, 87). IGFBP-3 overexpression attenuates NFκB signaling in prostate cancer cell lines and enhances apoptosis (86). Furthermore, IGFBP-3 knockdown attenuates TRAIL-induced apoptosis and decreases NFκB signaling in human colorectal carcinomas (85).

Given these data we hypothesized that basal IGFBP-3 represses NFκB signaling and that removal of this inhibition with IGFBP-3 knockdown would increase NFκB activity, leading to an increase in Bcl-2 expression. To test this hypothesis we blocked

the NFκB pathway with PC. We found that inhibition of NFκB decreased Bcl-2 protein in both scramble-control and IGFBP-3 siRNA-transfected cells supporting a role for NFκB in regulating Bcl-2 protein expression. However, the addition of PC did not block the increase in Bcl-2 observed with knockdown of IGFBP-3. These data suggest the possibility that the increase in Bcl-2 protein observed in response to IGFBP-3 knockdown may not be mediated through changes in NFκB signaling but rather through other mechanisms.

A role for IGFBP-3 and direct interaction and modification of Bcl-2 family proteins has been established. Under apoptotic conditions it has been co-immunoprecipitated with the multi domain pro-apoptotic protein Bax supporting a role for direct interaction between IGFBP-3 and Bcl-2 family proteins (133). Additionally IGFBP-3 has been found to be involved in toxin induced phosphorylation of Bcl-2 which inhibits its pro-survival function (50). Thus although a role for NFκB mediated increase in Bcl-2 protein was not established in our cell system, IGFBP-3 could regulate Bcl-2 expression at the post-translational level. Further studies are needed to elucidate the specific mechanism through which IGFBP-3 knockdown increases Bcl-2 expression and to determine if the overexpression of Bcl-2 is sufficient to attenuate DON- and ANS-induce apoptosis.

An unexpected and novel observation was the increase in basal IGFBP-3 expression when NFκB activity was inhibited with PC. NFκB is a pro-survival protein and up-regulates transcription of many pro-survival protein members. It is not unreasonable to assume it may also repress pro-apoptotic proteins such as IGFBP-3 which we have previously demonstrated is a component of the apoptotic machinery in MAC-T cells. A role for NFκB-mediated transrepression of target genes has been previously reported. Steroid hormones such as glucocorticoids mediate their immuno-suppressive effects through inhibition of NFκB signaling (134). However, activation of NFκB results in repression of the glucocorticoid receptor creating a system of mutual repression (135). A

similar relationship has been observed between NFκB and progesterone receptor signaling (136). Therefore NFκB could repress expression of IGFBP-3 through a similar mechanism.

In conclusion, the ribotoxins ANS and DON associate with the 28S ribosomal subunit, active SAPK signaling pathways and induce apoptosis. We report that IGFBP-3 is involved in ANS- and DON-induced apoptosis in normal MEC and is critical for apoptotic progression. Additionally we have demonstrated that IGFBP-3 knockdown attenuates ANS- and DON-induced apoptosis and specifically upregulates Bcl-2 protein. However this effect was not found to be mediated through a change in the NFκB signaling pathway. Conversely we found that blocking NFκB signaling increased basal IGFBP-3 mRNA and protein suggesting NFκB has an upstream inhibitory role in regulating IGFBP-3 expression. Further studies are ongoing in our laboratory to determine the specific relationship between NFκB and IGFBP-3 as well as IGFBP-3 and Bcl-2 expression, and to further elucidate the role Bcl-2 upregulation has in protection from apoptosis in mammary epithelial cells.

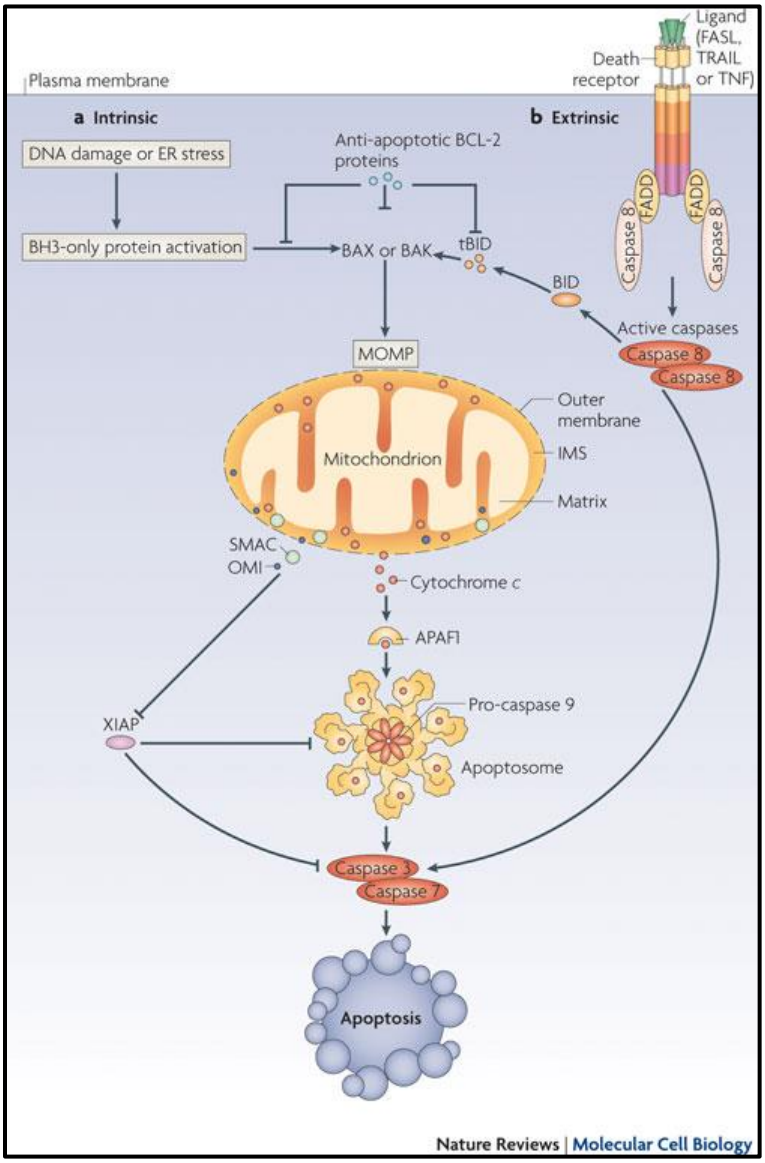

Figure 1: **Pathways of Apoptosis.**

(A) Intrinsic apoptosis is activated in response to changes in cellular homeostasis. Apoptotic stimuli induce modulation of the Bcl-2 family protein interactions resulting in loss of mitochondrial membrane integrity and subsequent release of apoptotic proteins basally sequestered in the mitochondrial intermembrane space including cytochrome c. Release of cytochrome c into the cytosol induces formation of the apoptosome, which activates caspase-9 leading to the induction of the caspase cascade and execution of apoptosis. (B) The extrinsic apoptotic pathway is activated in response to ligand binding to the extracellular death receptor which recruits and activates caspase-8 leading to the induction of the caspase cascade and execution of apoptosis. Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEW] (38), copyright (2010)

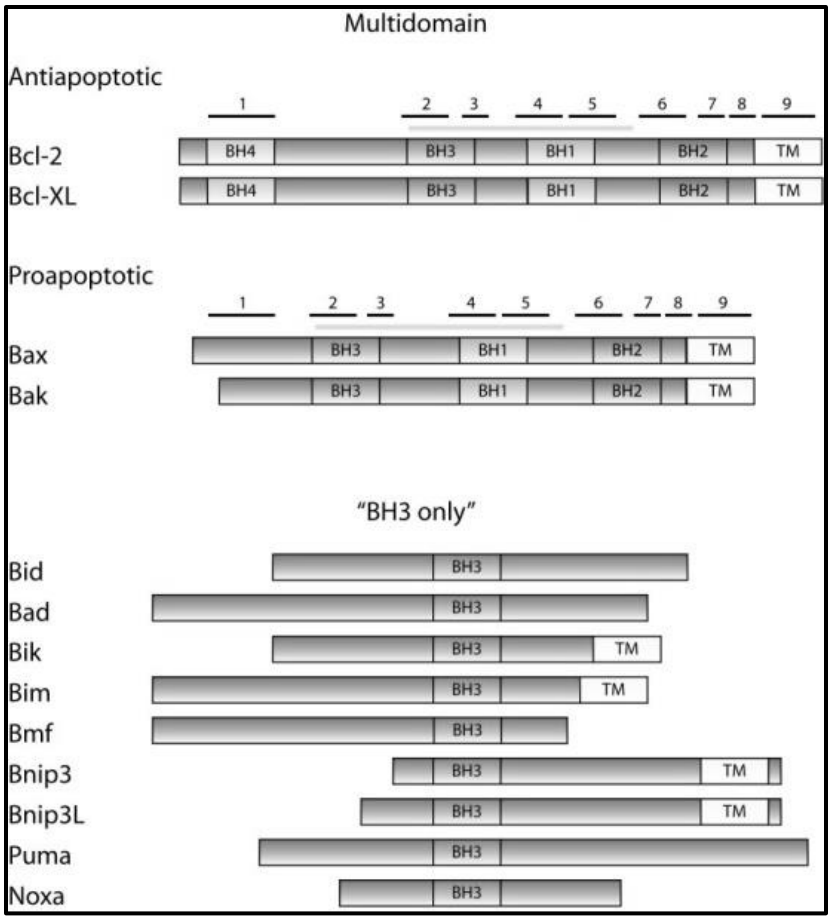

Figure 2: **Characterization of the Bcl-2 family of proteins.**

The Bcl-2 family of proteins is divided into three subcategories based on function and the presence of four different BH domains. (138)

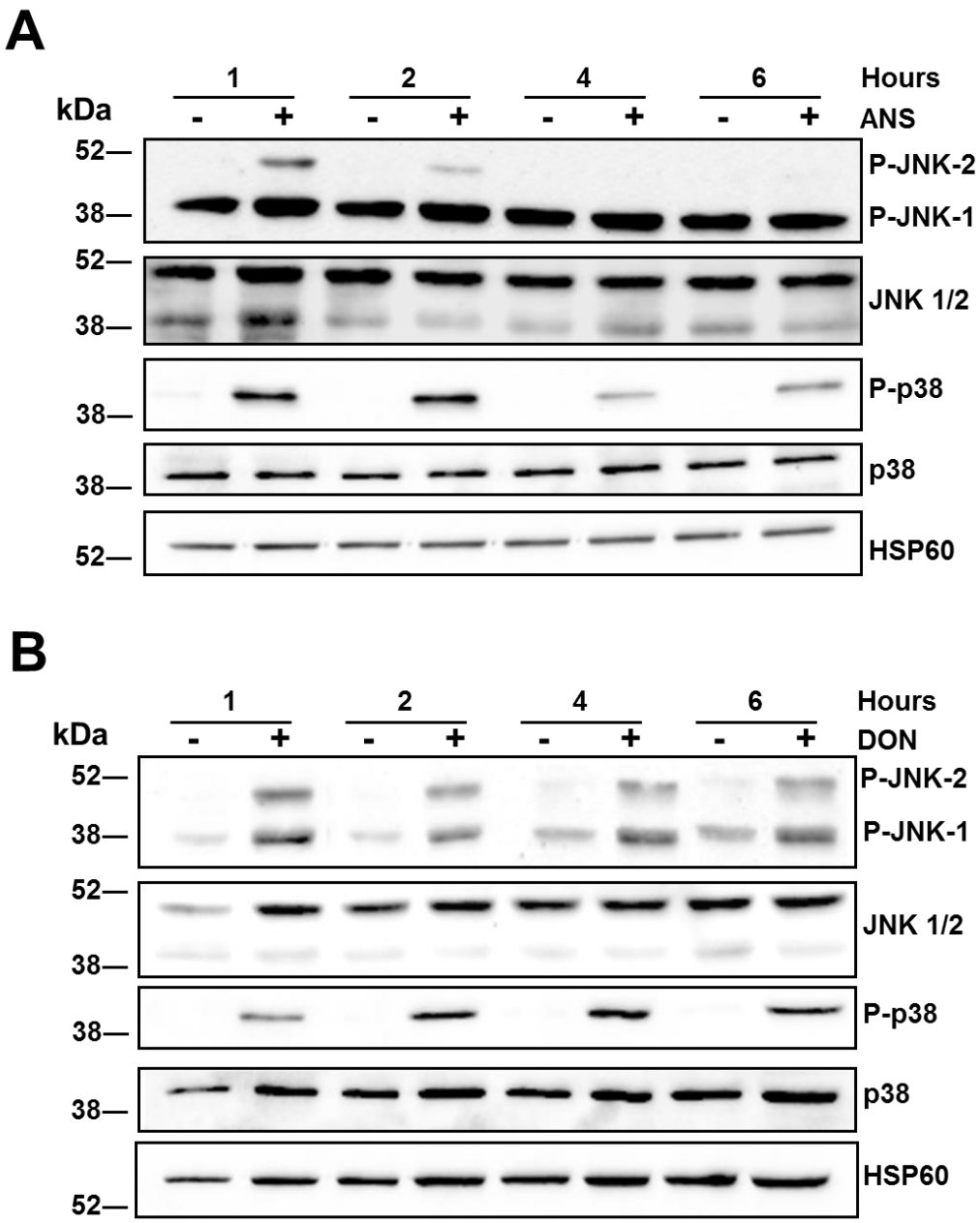

Figure 3: Anisomycin (ANS) and deoxynivalenol (DON) induce prolonged activation of JNK and p38.

Confluent MAC-T cells were serum-starved overnight and treated with 0.1 μM ANS (A) or 1.0 μg/mL DON (B) for indicated times. Whole cell lysates (40 μg) were separated using SDS-PAGE and immunoblotted for phosphorylated (p) forms of JNK and p38. HSP60 was used as a loading control.

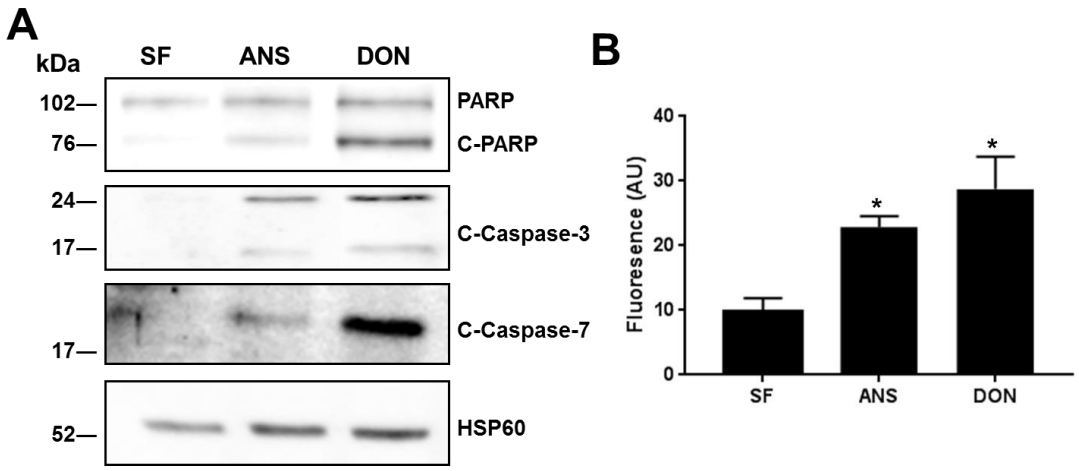

Figure 4: Anisomycin (ANS) and deoxynivalenol (DON) induce apoptosis in MAC-T cells.

Confluent MAC-T cells were serum starved overnight and treated with 0.1 µM ANS, 1.0 µg/mL DON or serum-free (SF) media for 6 h. (A) Whole cell lysates (40 µg) were separated using SDS-PAGE and immunoblotted for cleavage (c) of PARP, caspase-3, and caspase-7. HSP60 was used as a loading control. Data are representative of 3 independent experiments. (B) Caspase-3/7 activation was measured with the Sensolyte Caspase-3/7 Assay (AnaSpec) according to manufacturer's specifications. Bars represent mean ± SEM of 4 independent experiments with treatment measured in triplicate within experiment. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test. *indicates $p < 0.05$ compared to SF control.

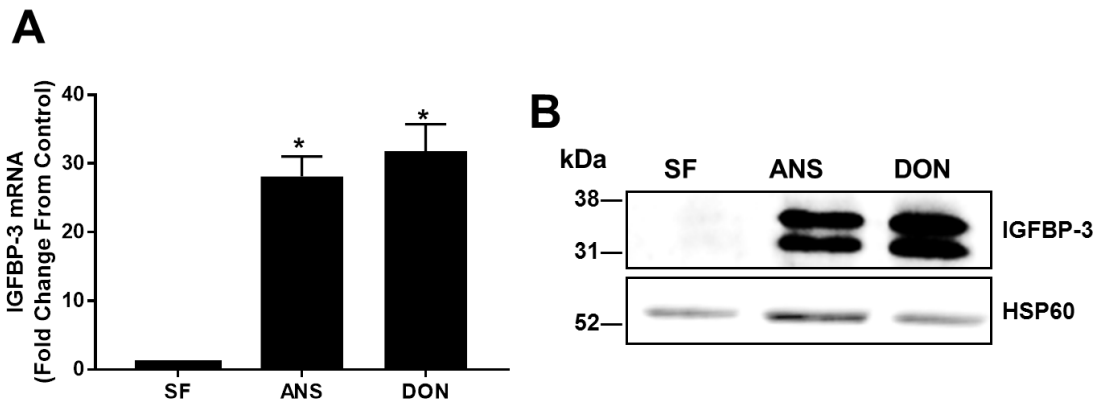

Figure 5: Anisomycin (ANS) and deoxynivalenol (DON) induce IGFBP-3 mRNA and protein and expression.

Confluent MAC-T cells were serum starved overnight and treated with 0.1 µM ANS or 1.0 µg/mL DON for 3 hours (A) or 6 hours (B). (A) Total RNA was collected and analyzed for IGFBP-3 mRNA by RT-qPCR with data corrected for cyclophilin levels. Bars represent mean ± SEM of 2 individual experiments, with treatment measured in triplicate within each experiment. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test. * indicates $p < 0.005$ compared to SF control. (B) Whole cell lysates (40 µg) were separated using SDS-PAGE and immunoblotted for IGFBP-3. HSP60 was used as a loading control. Data are representative of 4 independent experiments.

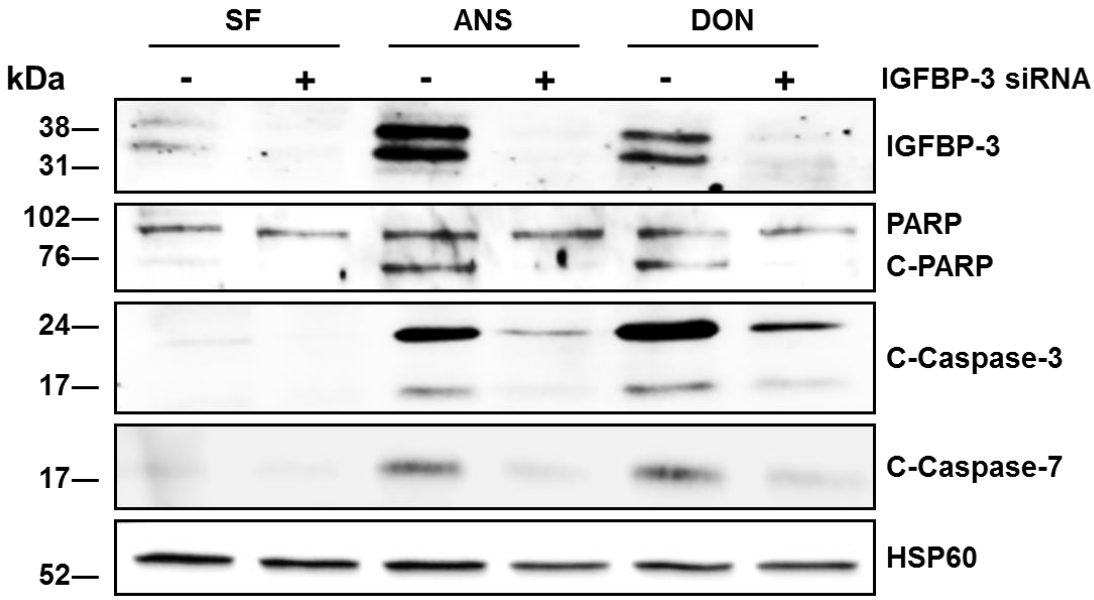

Figure 6: IGFBP-3 knockdown attenuates anisomycin (ANS)- and deoxynivalenol (DON)-induced apoptosis.

MAC-T cells were transfected with 50 nM IGFBP-3 or scramble siRNA for 48 h, serum starved overnight and treated with 0.1 μM ANS, 1.0 μg/mL DON or serum-free (SF) media for 6 h. Whole cell lysates (40 μg) were separated using SDS-PAGE and immunoblotted for IGFBP-3 and cleavage (c) of PARP, caspase-3, and caspase-7. HSP60 was used as a loading control. Data are representative of 3 independent experiments.

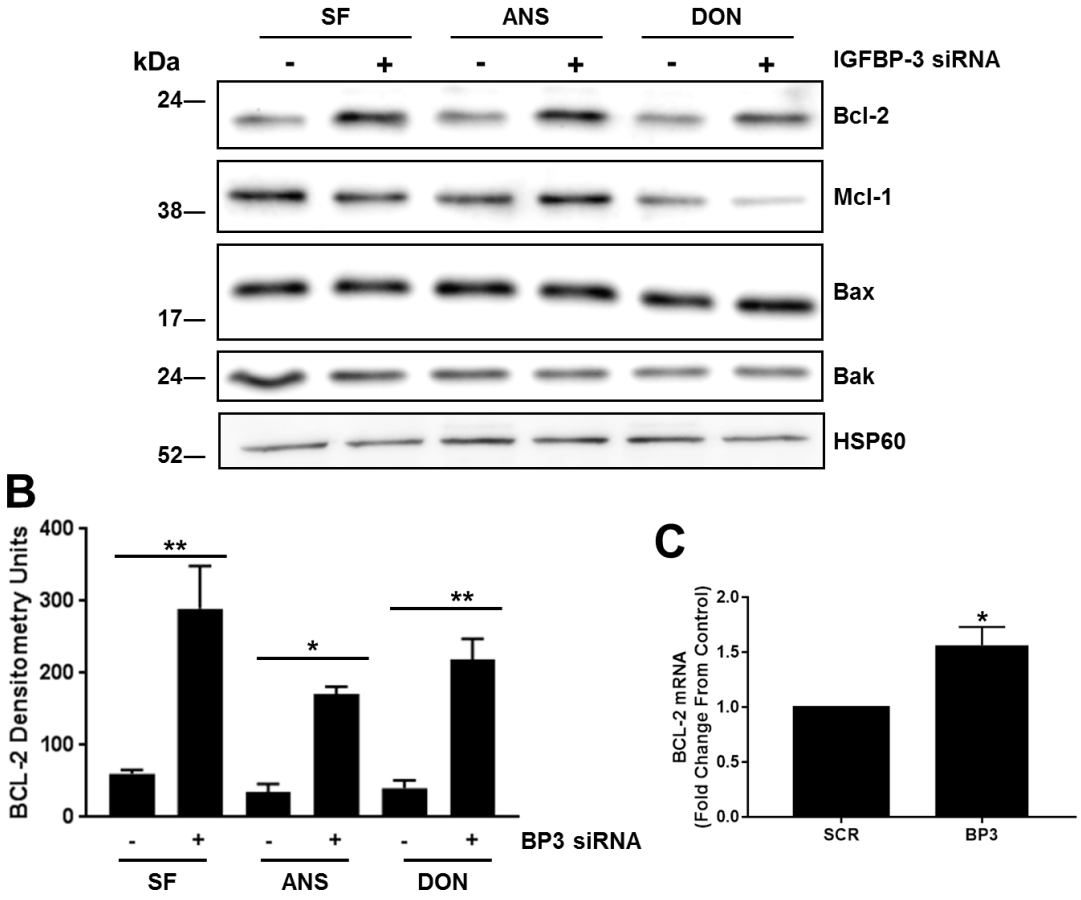

Figure 7: IGFBP-3 knockdown increases Bcl-2 protein but does not change the levels of Mcl-1, Bax or Bak.

MAC-T cells were transfected with 50 nM IGFBP-3 or scramble siRNA for 48 h, serum starved overnight, and treated with 0.1 μM anisomycin (ANS) or 1.0 μg/mL deoxynivalenol (DON) for 6 h. (A) Whole cell lysates (40 μg) were separated using SDS-PAGE and immunoblotted for Bcl-2, Mcl-1, Bax and Bak. HSP60 was used as a loading control. Data are representative of 3 independent experiments. (B) Data were quantified by densitometry. Bars represent mean ± SEM of 4 individual experiments. Data were analyzed using two-way ANOVA with Tukey's multiple comparisons post hoc test. * indicates $p < 0.05$ ** indicates $p < 0.005$. (C) Total RNA was collected from untreated cells and analyzed for Bcl-2 mRNA by RT-qPCR with data corrected for cyclophilin levels. Bars represent mean ± SEM of 3 individual experiments, with treatment measured in triplicate within each experiment. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test. * indicates $p < 0.05$ compared to Scr control.

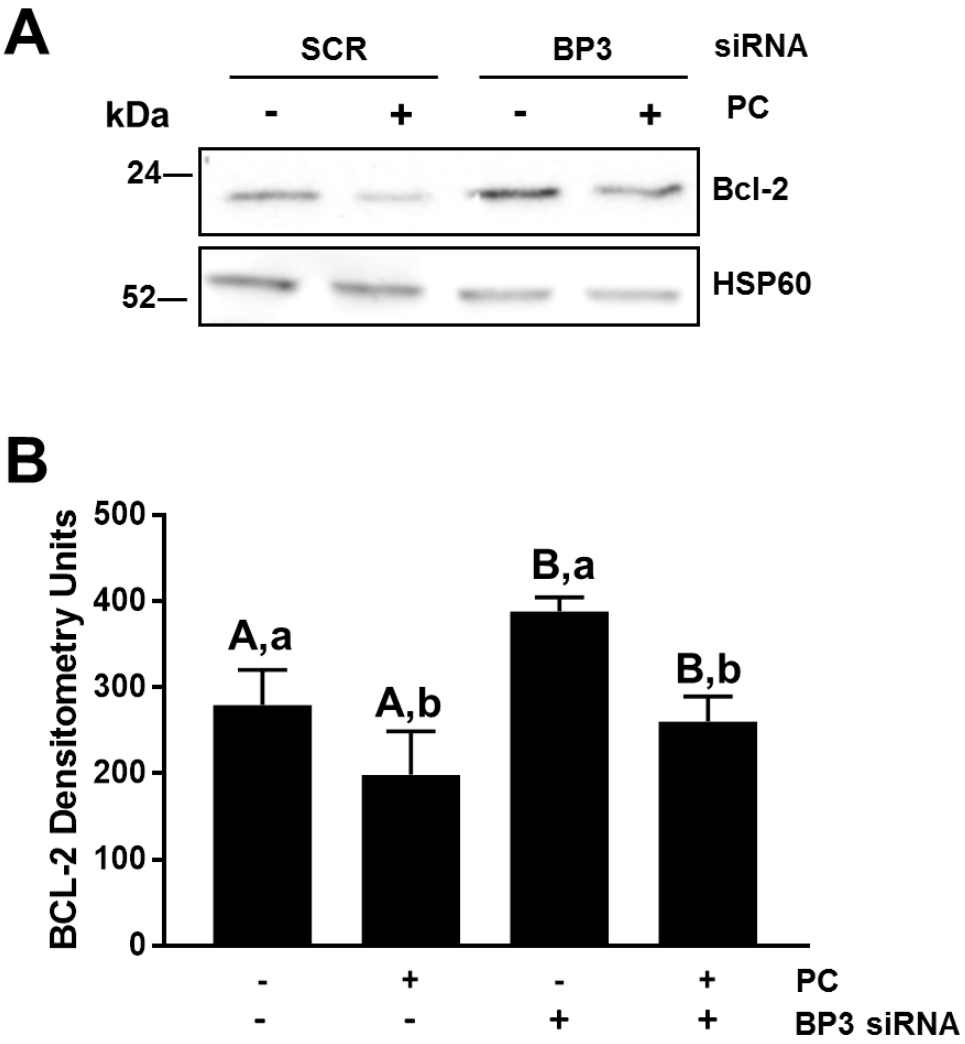

Figure 8: Inhibition of NFκB prevents the IGFBP-3 knockdown-induced increase in Bcl-2 protein expression.

(A) MAC-T cells were transfected with 50 nM IGFBP-3 (BP3) or scramble (SCR) siRNA for 48 h, serum starved overnight and treated with phenethyl caffeate (PC) for 6 h. Whole cell lysates (40 µg) were separated using SDS-PAGE and immunoblotted for Bcl-2. HSP60 was used as a loading control. Blots are representative of 3 individual experiments (B) Data were quantified by densitometry. Bars represent mean ± SEM of 3 individual experiments. Data were analyzed using two-way ANOVA with significance set at $p<0.05$. Main effects tested were ± BP3 siRNA with differences denoted by uppercase letters, and ± PC with differences denoted by lower case letters.

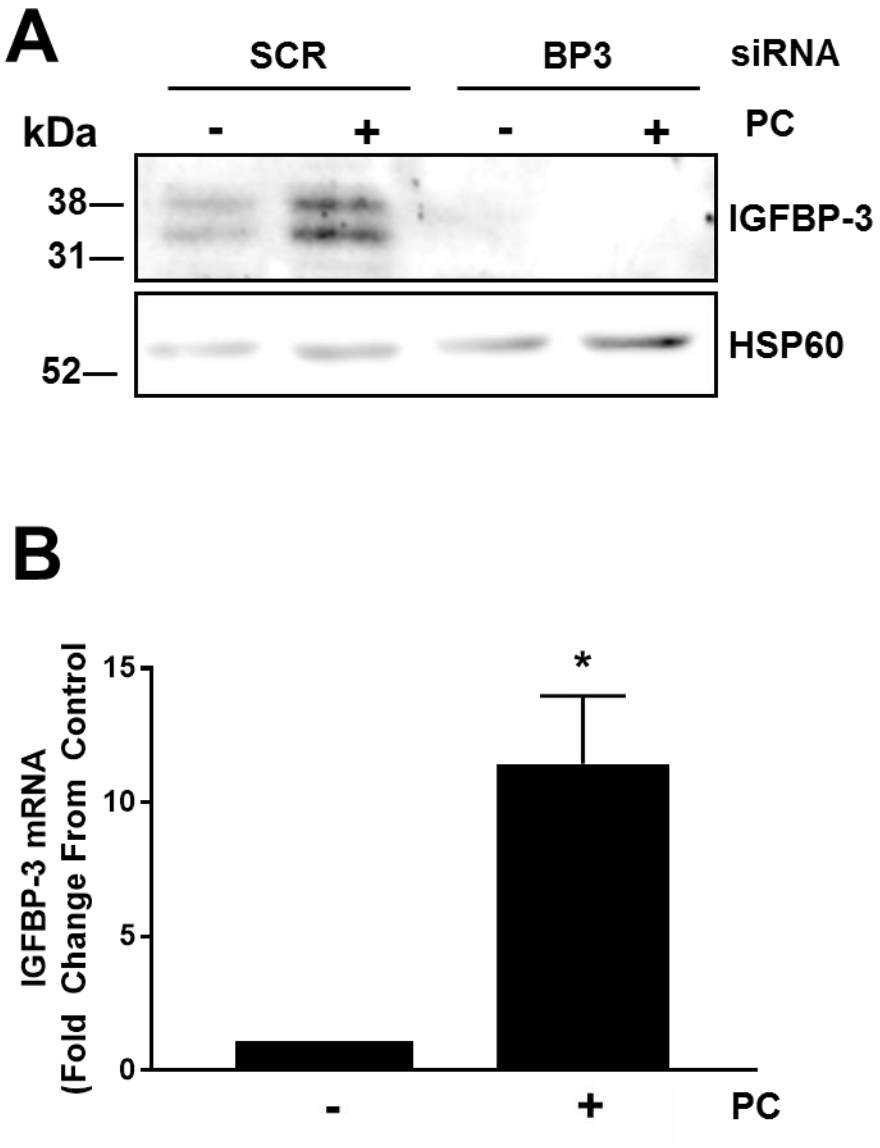

Figure 9: Inhibition of NFκB increases basal IGFBP-3 expression.

(A) MAC-T cells were transfected with 50 nM IGFBP-3 (BP3) or scramble (SCR) siRNA for 48 h serum starved overnight, and treated with phenethyl caffeate (PC) for 6 h. Whole cell lysates (40 µg) were separated using SDS-PAGE and immunoblotted for IGFBP-3. HSP60 was used as a loading control. Data are representative of three independent experiments. (B) Confluent MAC-T cells were serum starved overnight, and treated with PC for 4 h. Total RNA was collected and analyzed for IGFBP-3 mRNA by RT-qPCR with data corrected for cyclophilin levels. Bars represent mean ± SEM of 3 individual experiments with treatment measured in triplicate within each experiment. Data were analyzed using unpaired t-test. * indicates $p < 0.05$ compared to control**.**

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