RENAL INJURY, AS INDICATED BY APOPTOSIS IN THE KIDNEY, RESULTS FROM THE PRODUCTION AND RELEASE OF CYTOKINES DUE TO CARDIAC ISCHEMIA

By

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PREFACE

The purpose of these studies is to acquire a clearer understanding of cardiorenal syndrome. Observations have been made showing a small percentage of patients suffer acute renal failure after undergoing cardiopulmonary bypass as well as patients in cardiac failure. The same clinicians making these observations think the cause of the renal injury are cytokines released by the injured or ischemic myocardium, but there has been no research done to support or disprove this theory. Because TNF-α is suspected to be the most potent cytokine released from the ischemic myocardium and the most likely to induce renal dysfunction, it is the focus of this study.

The specific aims of this research is to (1) illustrate that there is an increase in serum TNF-α after cardiac ischemia, (2) find if there is injury to the kidney, measured by apoptosis (cleaved PARP), due to coronary occlusion, (3) locate the renal cells being injured by cardiac ischemia, and (4) measure renal apoptotic signaling (caspases 2, 3, and 9) after cardiac ischemia and examine the role of TNF-α in these activities. An Ultrasensitive TNF-α ELISA kit is used to measure serum levels of TNF-α and Western blot analysis is utilized to indicate apoptotic signaling in the kidney.

ACKNOWLEDGMENTS

The most important thing I have learned from this research is that you do not get it done alone. Research is a collaboration of the brightest minds in their fields. I would like to start by extending my appreciation to two very important people that without their help I could not have finished my research. First, I would like to thank my major advisor, Dr. Bruce Benjamin, for his patience, encouragement, guidance, and friendship. Secondly my thanks to Tara Kersey-Barrett, who gave me constant inspiration and support in the lab; she has become a good friend. I would also like to thank my other committee members Dr. Alexander Rouch, Dr. Randy Wymor, and Dr. James Breazile.

Further more I would like to take this opportunity to thank Wyeth for their donation of WTACE2, the TNF inhibitor used for these studies.

To do any type of research it also takes support from outside the lab. I would like to give a special thanks to my husband, Hank Bucholtz, my two daughters Danyell and Anna, and my friends. Living with me outside the lab has not always been easy but my family and friends have at all times understood how important my research is to me and have always supported me.

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Chapter I

INTRODUCTION

1.1 Background

Cardiovascular disease is a leading cause of death worldwide and remains one of the major killers in modern society [1], [2]. In recent years it has become apparent that the loss of myocardial cells may play a major pathogenic role cardiac disease [2], and current evidence has implicated proinflammatory cytokines in the pathophysiology of cell death in cardiovascular disease. Levels of these cytokines are shown to increase with cardiac ischemia during cardiopulmonary bypass (CPB) and myocardial infarction (MI) [3], [4], [5], [6], and also occurs in patients suffering from cardiac failure (CF) [7]. Studies have demonstrated the myocardium to be a major source of the cytokines tumor necrosis factor-α (TNF), interleukin (IL)-1, IL-6, and IL-8 [5], [6].

Observations of renal dysfunction or renal failure have been made in a small percentage of patients following CPB [3], [4], [6] and in patients with heart failure [7]. Some investigators feel there is a connection between heart and kidney dysfunction they call “cardiorenal syndrome,” and these researchers assume this connection is caused by the injured heart releasing cytokines that damage the kidney [3]. There has been no research done to either support or disprove this theory. Studies done in our laboratory show that with induced cardiac ischemia there are increased levels of the cell death signaling proteins in the kidney cells.

1.2 Hypothesis

Renal injury, as indicated by apoptosis in the kidney, results from the production and release of cytokines due to cardiac ischemia.

1.3 Specific Aims

1. To quantitate the levels of the cytokine TNF. ELISA will be used to measure TNF in the blood after a myocardial infarction.
2. To determine if apoptosis occurs I-n kidney cells as a result of cardiac ischemia, the cleaved form of poly ADP-ribose polymerase (PARP) will be assessed by Western blot analysis.
3. To examine which cell types in the kidney show activation of pro-apoptotic proteins following cardiac ischemia, assays will be performed using immunohistochemistry probing with specific antibodies.
4. To investigate apoptotic signaling in kidney tissue after induced cardiac ischemia as well as examine the role of TNF in these activities the following assays will be preformed:
    a. Western blot analysis will be utilized to find and measure levels of pro-apoptotic signaling of the active forms of caspase 3, caspase 2, and caspase 9; activity known for TNF receptor signaling pathways (see Figure 1.).
    b. Inhibit the release of TNF from the injured myocardium via metalloproteinase inhibitor to determine pro-apoptotic protein levels of the active forms of caspase 3, caspase 2, caspase 9, and cleaved PARP using Western blot analysis.
    c. TNF infusion studies without induced cardiac ischemia to determine levels of pro-apoptotic protein levels of the active forms of caspase 3, caspase 2, caspase 9, and cleaved PARP.

1.4 Significance

Results of this research will give a better understanding of the mechanisms involved in renal dysfunction due to ischemic injury of the heart. These studies will also show that with the increased production and release of cytokines because of cardiac ischemia, other organ systems such as the kidney can be targeted and cause further insult to an already compromised patient. To improve a patient's overall outcome from such events, further investigations as the result of these studies may produce treatments preventing the harmful effects caused by these cytokines.

Chapter II

REVIEW OF LITERATURE

2.1 Cardiorenal Syndrome

Cardiorenal syndrome is a term used to describe the connection between heart and kidney dysfunction. Investigators feel the tie between these two important organs is the release of proinflammatory cytokines from the injured myocytes. These cytokines become systemic which in turn can cause damage to various renal cells. Levels of these cytokines are shown to increase with cardiac ischemia during CPB and MI [3], [4], [5], [6], and also in patients suffering from CF [7]. Increased cytokines levels have been reflected in cardiac ischemic animal studies as well [8], [9], [10]. Research has revealed the myocardium to be a major source of the cytokines TNF, IL-1, IL-6, and IL-8 [4], [6].

The modern era of cardiac surgery began when the technique for CPB was introduced in the early 1950s [5]. CPB is a commonly used surgical heart procedures to maintain extracorporeal circulation. The procedure involves a diversion of the blood flow returning to the heart through a pump oxygenator (heart-lung machine) and then returning it to the arterial side of the circulation [11]. Most cases of CPB are associated with aortic cross-clamping, which results in global myocardial ischemia [4]. Although CPB is fundamental for cardiac surgery, it is associated with substantial postoperative morbidity. The release of cytokines during CPB can have deleterious effects on the heart and on other organs. The release of proinflammatory cytokines, such as TNF, IL-1β, and IL-8, has been associated with the development of complications after CPB. The levels of these proinflammatory cytokines have been correlated with the duration of cardiac

ischemia during CPB and may contribute to the development of multi organ failure [4] [5] .

The cytokines implicated after cardiac ischemia in renal dysfunction include IL-1, IL-6, IL-8 and TNF—the same cytokines produced and released by injured myocardium. IL-1, IL-6 and TNF can stimulate the mesangial cells of the kidney to produce its own IL-6, IL-8, and TNF [12]. IL-1 can indirectly activate apoptosis as well as activate inflammation [13], and is associated with poor outcome in several human disorders [14]. IL-1β and TNF have similar effects, despite having different receptors and different intracellular signaling pathways and activity. IL-1β elicits many of the same effects as TNF when it is infused in humans [15]. IL-6 and IL-8 are potent chemoattractants [16], [17], [5]. Increased IL-6 concentrations may mediate apoptotic signals in endothelial cells [13] and provoke cytokine cascades. Excessive expression of IL-6 is associated with morbidity and mortality [18], [19]. IL-8 as well as being a chemoattractant, it also enhances neutrophil function, expression of adhesion molecules, degranulation, and respiratory burst [15]. Finally, the cytokine TNF can be highly destructive to the kidney by reducing glomerular blood and glomerular filtration rate, inducing the synthesis of other proinflammatory mediators, increasing glomerular albumin permeability, causing glomerular fibrin deposition, and stimulating cellular infiltration. Because TNF appears to be one of the most potent inflammatory mediators in terms of its ability to induce cardiac and renal dysfunction [3] it is the focus of this study. This investigation looks at TNF and its role in cardiorenal syndrome by looking at TNF mechanisms of action that could possibly cause renal injury via the increase of apoptotic signaling.

2.2 Apoptosis

Apoptosis, or cell suicide, is a form of cell death that is morphologically and biochemically distinct from necrosis [20]—a rapid and irreversible process that occurs when cells are severely damaged [2]. Although the concept of apoptosis was introduced approximately 30 years ago, the mechanisms of how apoptosis is initiated and executed remained unclear until recently. During the past eight years, tremendous progress has been made in understanding apoptosis as a result of molecular identification of the key components of this intracellular suicide program. The core of this cell suicide program is evolutionarily conserved from worm to human [20].

Unlike necrosis, which is a passive and unregulated process, apoptosis is both energy dependent and highly regulated. Apoptosis is controlled by a complex interaction of numerous prosurvival and prodeath signals. These include the Bcl-2 family of proteins, which may be antiapoptotic or proapoptic, and exerts its effect primarily at the level of mitochondria. Other important regulators of apoptosis are enzymes called caspases. [2].

Apoptosis occurs when a cell dies through activation of an internally controlled suicide program. It is a subtly orchestrated disassembly of cellular components designed to eliminate unwanted cells during embryogenesis and in various physiologic processes. Doomed cells are removed with minimum disruption to the surrounding tissue. Apoptosis also occurs, however, under pathologic conditions, in which it is sometimes accompanied by necrosis [16]. Survival signals from the cell's environment and internal sensors for cellular integrity normally keep a cell's apoptotic machinery in check. In the event that a cell loses contact with its surroundings or sustains irreparable internal

damage, the cell initiates apoptosis. A cell that simultaneously receives conflicting signals driving or attenuating its division cycle may also trigger apoptosis. Yet another mechanism has evolved enabling an organism to actively direct individual cells to self-destruct. This kind of "instructive" apoptosis is important especially in the immune system. Death receptors—cell surface receptors that transmit apoptosis signals initiated by specific "death ligands"—play a central role in instructive apoptosis. These receptors can activate the death caspases within seconds of ligand binding, causing an apoptotic demise of the cell within hours [21].

Once initiated, apoptotic events include DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into membrane-enclosed vesicles (apoptotic bodies) [16], [2], [22], [23]. In vivo, this process culminates with engulfment of apoptotic bodies by other cells, preventing complications that would result from a release of intracellular contents, or with the loss of cells in the lumen of organ systems such as the gastrointestinal tract or renal tubules. These changes occur in a predictable, reproducible sequence and can be completed within 30 to 60 minutes [23].

Cell death, the ultimate result of cell injury, is one of the most crucial events in pathology affecting every cell type and is the major consequence of ischemia and immune reactions [16]. TNF, a component of the immune system, is a proinflammatory cytokine, which acts to increase its own production, as well as the synthesis of small inflammatory mediators. TNF can also play a key role in apoptosis via its death receptor. These receptors can activate programmed cell death within seconds of ligand binding causing an apoptotic demise of the cell within hours [21].

Myocardial TNF production is a potential source of systemic TNF affecting myocardial and renal function due to its apoptotic contribution. Renal cell apoptosis is the likely cause of renal cell death during acute renal failure following cardiac injury. Kidney biopsies of patients with acute renal failure following CPB demonstrate a substantial degree of apoptosis. TNF induces renal injury by direct and indirect mechanisms. TNF directly induces renal apoptosis and reversible dysfunction; however, concurrent TNF-induced cardiodepression via apoptosis limiting renal blood flow, further provoking a vicious cycle of renal TNF production and injury during CPB, MI, and CF [3]. A better understanding of the pathways of apoptosis and their regulation may yield novel therapeutic targets for cardiorenal syndrome [2]. Our focus is on the apoptotic pathway induced by TNF (see Figure 1). We looked at pro-apoptotic signaling (active forms of caspase 2, 3, 9—Aim 4) and apoptotic markers indicating that apoptosis is occurring (cleaved forms of PARP —Aim 2). As proposed in research Aim 3, our lab also looked at which renal cells are undergoing apoptosis in response to cardiac ischemia by performing immunohistochemistry using the specific antibodies of active caspase 3 and cleaved PARP.

2.3 TNF

The cytokine TNF elicits a wide range of biological responses, including inflammation, cell proliferation, differentiation, and apoptosis [24], [25]. Similar to growth factors, TNF can act on the same cell that produces it (an autocrine effect); on cells in the immediate vicinity (a paracrine effect); or systematically (and endocrine effect) [16]. One of the most intriguing actions of TNF is the induction of apoptosis. Apoptosis is almost certainly relevant to some of the toxic effects of TNF, such as shock

and inflammation. TNF-induced apoptosis may also have physiologic relevance, for example, mediating apoptosis of infected cells to help protect the host or down regulating inflammatory cells [26]. Increased serum levels of TNF have been observed with cardiac ischemia during CPB and MI [3], [4], [5], [6], and also in patients suffering from CF [7]. The fact that the myocardium produces as much TNF per gram of tissue as the liver or spleen, both which possess large macrophage population, which are major sources of TNF, implicates the myocardium to be a potential source of TNF in cardiac disease [3]. Levine et al. [7] research suggests that TNF plays a role in the progression of myocardial disease. They observed that circulating levels of TNF are increased in patients with chronic heart failure and that the patients with elevated circulating levels of TNF had a greater impairment of renal function [7]. As already mentioned, elevated serum levels of TNF are also observed in patients undergoing CPB and with MIs. Research on patients who underwent CPB showed a strong correlation between the levels of TNF and the duration of cardiac ischemia during CPB [5].

TNF is produced and released by both the heart and kidney [3]. It was once thought that TNF released by the kidney was via the secretory product of local macrophages but through further investigation it was discovered that the renal glomerular mesangial cells produce their own TNF in response to ischemia, exogenous TNF, and IL-1 [25]. Of all the inflammatory mediators, TNF appears to be one of the most potent in terms of its ability to induce cardiorenal syndrome via direct cytoxicity and apoptosis [3].

TNF is proposed to initiate destruction in the kidney through several avenues. TNF stimulates the production of reactive oxygen species from mesangial cells as well as recruitment of neutrophils and monocytes to the kidney enhancing their adhesion to

glomerular cells. TNF also reduces glomerular blood flow and glomerular filtration rate by stimulating renal mesangial cells to produce a variety of vasoconstrictive (platelet activating factor, endothelin-1, prostaglandins such as Thromboxane A_2) and vasodilatory (adenosine, nitric oxide, prostaglandins such as Prostaglandin E_2) mediators [3], [27]. Thus, TNF promotes renal dysfunction via direct cytotoxicity, vasoconstriction, decreased renal blood flow, the recruitment of neutrophils and monocytes, increased albumin permeability and, glomerular fibrin deposition [3], [27]. TNF has also been implicated in a variety of inflammatory diseases of the kidney, including autoimmune lupus nephritis, glomerulonephritis, diabetic nephropathy and septic acute renal failure [25]. As indicated by research, TNF can be extremely damaging to the kidney.

TNF exists as a cellular membrane bound precursor form and is processed to a soluble, secreted form. This processing of the TNF precursor is required for TNF secretion [28]. TNF exists as a 26 kilodalton (kD) cell membrane bound associated form and a 17 kD soluble form. The 17 kD TNF is released as a result of proteolytic cleavage within the cell membrane, of the 26 kD form by TNF converting enzyme (a membrane-anchored zinc protease, TACE), a member of the matrix metalloproteinase (MMP) family [29], [30], [31].

Many proteins such as TNF exist in both membrane-bound and soluble forms as a result of post-translational proteolytic processing [32]. The process of membrane proteins being released by proteolytic cleavage is referred to as ectodomain shedding. An appropriate balance between membrane-bound and soluble forms of proteins such as TNF appear to be important for their normal physiological role [33], [32]. Under physiologic conditions, the 26 kD precursor proTNF, a type II transmembrane protein, is

proteolytically cleaved by TACE. TACE is a multi-domain, type I transmembrane protein that includes three domains: pro, catalytic, and hemopexin. The pro region contains a cysteine switch, a feature that maintains the enzyme in an inactive state. The pro domain can be autocatalytically removed to become active. The catalytic domain contains a standard zinc ligand binding motif and the hemopexin domain, which is important for the recognition of membrane bound TNF [28], [33]. For the most part, TACE is constitutively expressed and becomes activated by intracellular kinase cascades upon stimulation of a cell [34]. Research has shown TACE to be highly selective for TNF [35].

The TNF receptor is referred to as the "death receptor" [21]. TNF can bind with two different receptors named TNF-R1 (55kDa) and TNF-R2 (75kDa) [21], [26], [3], [24], [27], [2], but it appears TNF-R1 is more potent than TNF-R2 [26]. In most cells TNF-induced effects are mediated by TNF-R1, and TNF-R1 is responsible for the generation of the cytotoxic death signals [21], [26], [3], [24], [27], [2]. The wide repertoire of signaling responses elicited by TNF results from the ability to recruit a range of cytosolic adaptor proteins such as TNF receptor-1-associated death domain (TRADD). The interaction between TNF-R1 and adaptor proteins is dependent on homologous regions known as a "death domain." Death domains (DD) are so named for their essential role in initiating apoptosis. These domains mediate apoptosis by recruitment of other proteins such as Fas-associated death domain (FADD) and receptor interacting protein (RIP)-associated ICH-1 death domain (RAIDD) that additionally contain a "death effector domain" (DED), which recruit and activate caspases. Once TNF binds to TNF-R1, the receptor trimerizes initiating the recruitment of the adaptor protein TRADD to the

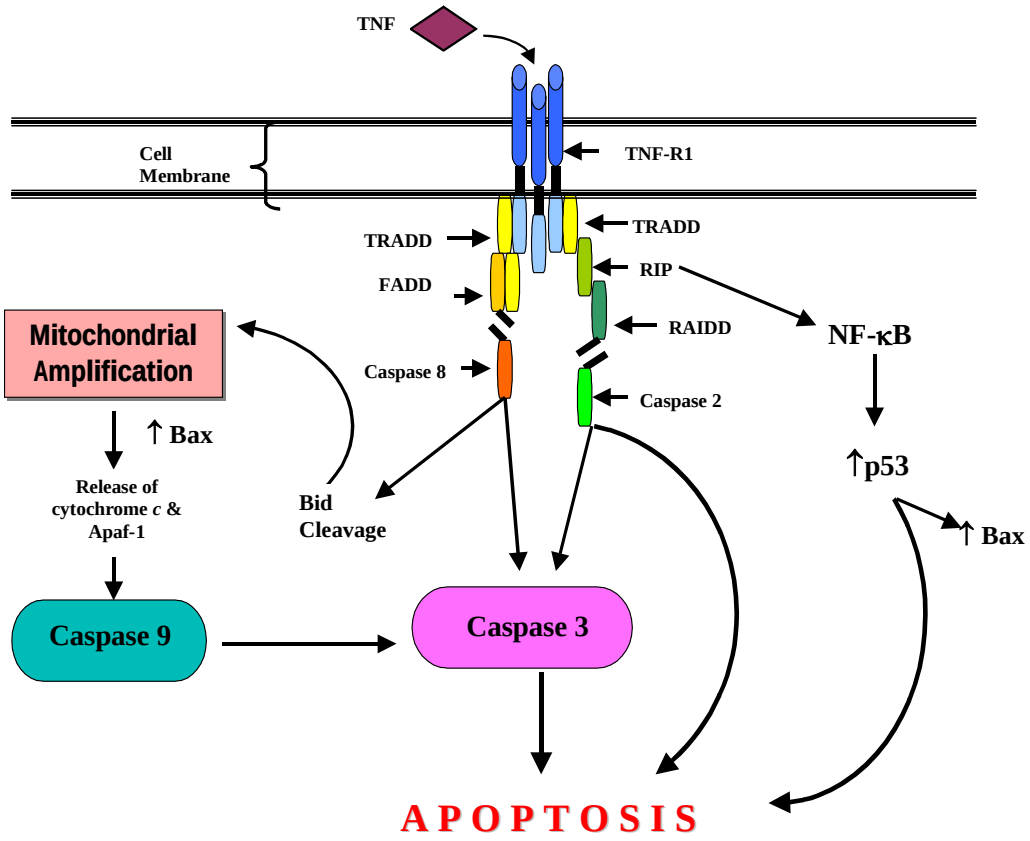

Figure 1. TNF-induced apoptosis pathways. Tumor necrosis factor (TNF) binds to and activates the tumor necrosis factor receptor (TNF-R1). Once the receptor is activated it can begin recruitment of several proteins eventually leading to apoptosis. TRADD, TNF receptor-1-associated death domain; FADD, Fas-associated death domain; RIP, receptor interacting protein; RAIDD, RIP-associated ICH-1 death domain; NF-κB, nuclear factor kappa B.

receptor's DD. TRADD uses its DD to recruit the FADD protein, which contains both a DD and a DED and initiates the pro-apoptotic activity of TNF by engaging caspase 8 (a proteolytic enzyme explained later) through its corresponding DED thereby initiating apoptosis [21] , [3], [24], [36], [27], [26]. TRADD can also recruit receptor interacting protein, which contains a DD that engages the DD of RAIDD, RAIDD in turn recruits and activates caspase 2. Caspase 2 can play two roles; one that can trigger direct apoptosis much like caspase 3, and one that can activate caspase 3 thereby causing

apoptosis. Furthermore, RIP can activate nuclear factor kappa B (NF-κB), which is a transcription factor that enters the nucleus and binds to specific promoter regions of target genes [37], [38], [39] (Figure 1).

The activation of NFκB can increase anti- or proapoptotic signaling, increase the levels of TNF, and increase inflammation. In most cells, NFκB exists in a latent state bound to its inhibitory proteins collectively called inhibitory κB (IκB) that masks the NFκB nuclear localization site. Following activation, phosphorylation of IκB results in disruption of the NFκB-IκB complex and degradation of IκB. Once liberated from IκB, NFκB translocates from the cytoplasm to the nucleus where it docks to DNA at one of four NFκB binding sites such as the TNF promoter region. It has been demonstrated that at least two of these TNF promoter regions must be bound by NFκB for TNF transcription to occur [3].

NFκB consists of two major polypeptides, p50 and p65. Both p50 and p65 homodimers and p50/p65 heterodimers bind to DNA. p65 is required for binding of the IκB inhibitor and activates transcription. p50, however does not activate transcription. It has been shown the both p50/p50 homodimers and p50/p65 heterodimers bound NFκB to the p53 promoter (p53 suppressor protein discussed later). So likewise, TNF induces NFκB activity and activates the p53 promoter. Studies showed that TNF, a specific NFκB inducer, also had an inducible effect on the p53 promotor, and activation of both required an intact NFκB site. Since NFκB is activated by signals that pose a threat to the survival of cells and p53 arrests cells in G_1 phase of the cell cycle, NFκB induction of

p53 may be a mechanism to arrest cell cycle progression and allow recovery from damage [40].

As illustrated (Figure 1), TNF can also initiate apoptosis through mitochondrial amplification by cleaving Bid (a protein of the Bcl-2 family explained later) or by increasing the levels of Bax (another protein of the Bcl-2 family via p53) implicating other pathways in which TNF can evoke cell injury. This mitochondrial amplification can be measured by increased levels of caspase 9 (mechanism explained later).

As described, stimulation of TNF-R1 can simultaneously activate several signaling pathways within the cell leading to apoptosis, but interestingly the stimulated TNF-R can also lead other pathways that can counter the death signals and result in cell survival. The balance of these two systems determines if the cell lives or dies. Most of the signaling that causes cell death via TNF has been explained and is shown in Figure 1. Several antiapoptotic mechanisms appear to begin with the protein RIP. Although RIP initiates the activation of caspase 2, which can cause apoptosis, it can also play a role in cell survival by interacting with IκB, there by activating NF-κB, and TNF-receptor associating factor (TRAF) [24], [41].

TRAF2 is characterized by the presents of an N-terminal ring and zinc finger containing domain, and a C-terminal TRAF domain. There has been six related TRAF proteins found but only TRAF1 and TRAF2 have been shown to be involved in TNF signaling. TRAF along with RIP can activate NF-κB, which in turn translocates into the nucleus and promotes the expression of antiapoptotic proteins such as cellular inhibitor of apoptosis (cIAP), TRAF1, and A20. Interestingly, a number of the NF-κB-dependent

expressed antiapoptotic proteins bind directly to TRAF2 leading to their recruitment to the TNF-R complex [24].

cIAP1 and cIAP2 are the only members of IAP family that can interact with TRAF2 [24]. cIAP works by preventing hydrolysis of caspases by sterically hindering access to the catalytic center of the protease [42]. IAPS has shown to directly bind and inhibit caspases 3, 7, and 9 [43]. A20 is an inducible cytoplasmic zinc finger protein that interacts via the N-terminal non-zinc finger-containing domain of the TRAF1/TRAF2 complex. A20 has a dual effect on TNF-mediated signaling; a potent inhibitor of NF-κB activation as well as an inhibitor of cell death. In most cells, basal expression levels of cIAPs, TRAF1, and A20 are very low or even undetectable, however, they are strongly upregulated in an NF-κB-dependent manner upon the stimulation of cells by TNF [24].

Yet another delicate balance between cell life or death stimulated by TNF is with the ceramide pathways. Sphingomyelin initiates an evolutionarily conserved and ubiquitous intracellular signaling pathway. As a major phospholipid, sphingomyelin is largely confined to the outer leaflet of cellular membranes and is hydrolyzed by sphingomyelinases (SMase, sphingomyelin-specific type C phospholipases) into phosphocholine and ceramide. The phosphocholine head group is released into the aqueous environment while ceramide diffuses within membranes, acting as an intracellular messenger. Inducible SMase activity in intact cells was initially described in response to diacylglycerols (DAG). Transient accumulation of ceramides derived from sphingomyelin hydrolysis was later recognized as a component of the signaling events that originate from different cell-surface receptors, including TNF-R1. A growing number of cell-surface receptors are now being shown to generate signals that trigger the

hydrolysis of sphingomyelin to release diffusible ceramides. Ceramides have been implicated as key mediators in signaling pathways, with outcomes as diverse as cell proliferation, differentiation, growth arrest and apoptosis. The response depends on the cell type, whether the signal is integrated with other signals originating from the same receptor or on the subcellular location of sphingomyelin hydrolysis and ceramide release [44].

Ceramide produced by SMases has been recognized as an important second messenger. By binding to its receptor, TNF rapidly activates two distinct types of SMase, a membrane-associated neutral (N)-SMase, and an endosomal acidic (A)-SMase. N-SMase and A-SMase are activated independently by different cytoplasmic domains of TNF-R [45], [41]. The activation of these two SMases through TNF-R is mediated by different adapter proteins that interact with separate cytoplasmic domains (Figure 2). The DD-associated proteins TRADD and FADD mediate A-SMase activation, and the activation of N-SMase is mediated by the novel protein factor associated with N-SMase activation (FAN) that binds to a specific binding site, N-SMase activation domain (NSD), located upstream of the TNF-R DD. Binding to the TNF-R seems to be required for the function of FAN. Each type of SMase specifically couples to select pathways of TNF signaling (Figure 2). Ceramide generated by N-SMase directs the activation of protein kinases and phospholipase A_2. In contrast, A-SMase triggers the activation of NF-κB. No apparent cross talk has been detected between N-SMase and A-SMase pathways, indicating that ceramide action depends on the topology of its production [45]. As can be seen in Figure 2 there are many ceramide pathways stimulated by the activation of the TNF-R, most of which the explanation goes beyond the scope of this investigation but

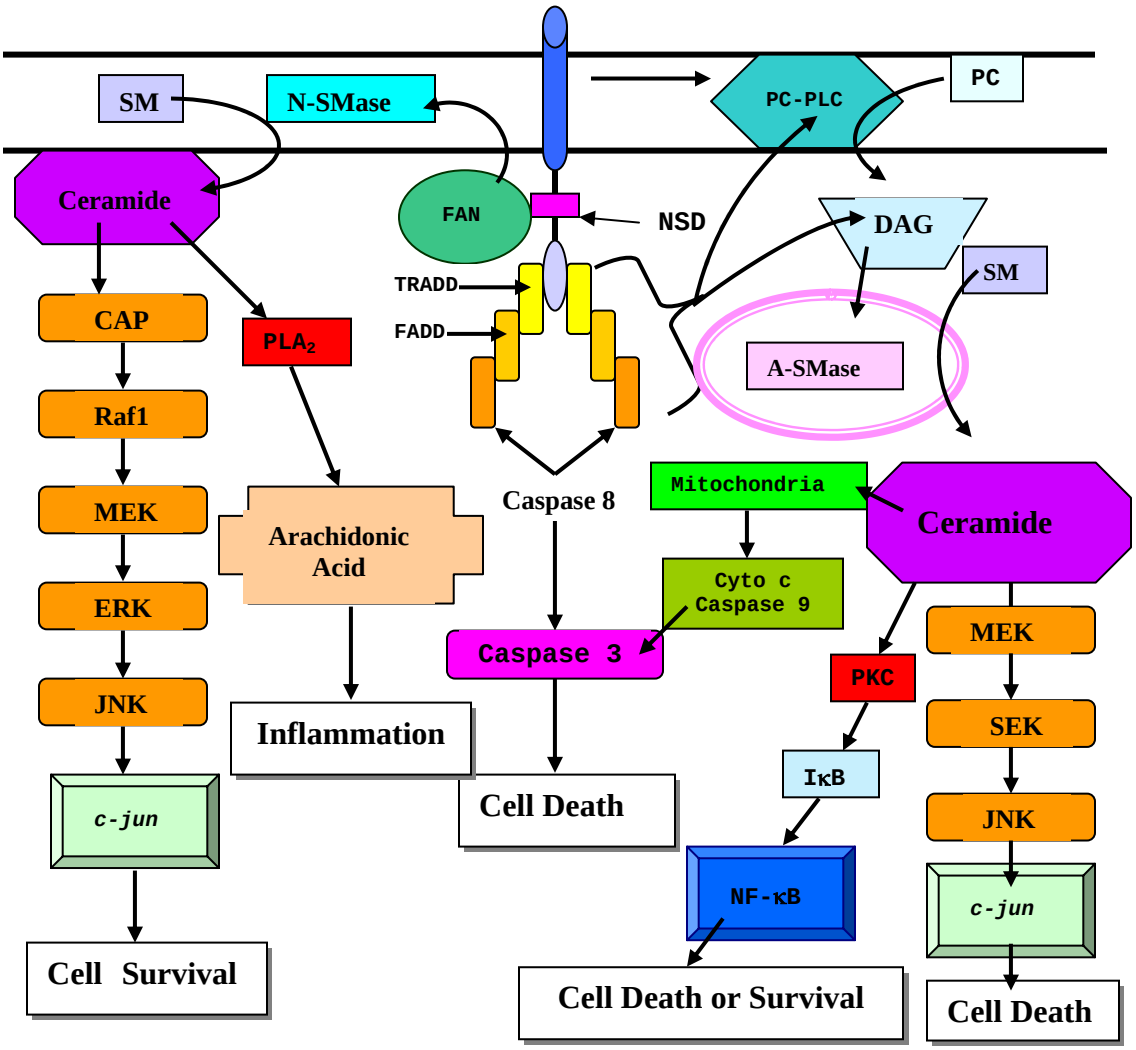

Figure 2. TNF stimulation of ceramide leading to inflammation, cell survival, or cell death. TNF activates two types of SMase (sphingomyelinases), membrane-associated neutral (N)-SMase, and an endosomal acidic (A)-SMase, mediated by different adapter proteins. The ceramide produce by Smases has been shown to be an important second messenger and a key mediator in signaling pathways that can lead to cell proliferation, differentiation, growth arrest, and apoptosis.

necessary to point out for the full ramification of the TNF-R in both cell death and cell survival.

Research to increase the understanding of the death receptor's pathways may give insight to both cardiac and renal dysfunction opening new therapeutic avenues to prevent

cardiorenal syndrome. We investigated the apoptotic pathway of TNF by looking at levels of the active forms of caspase 2, caspase 3, and caspase 9 (Aim #4a). Because caspase 9 is a direct result of mitochondrial disruption, we used it as a marker for mitochondrial apoptosis amplification. To insure that TNF is in fact one of the major players in cardiorenal syndrome we inhibited its action by inhibiting the conversion of the proTNF to its soluble form preventing the secretion of TNF (Aim 4b). Also we determined which renal cells types express apoptotic signaling due to cardiac ischemia through immunohistochemistry (Aim #3).

2.4 Caspases

Caspases are a family of proteases, which are essential molecules in pro-apoptotic pathways that are initiated in response to death stimuli such as TNF [20], [2], [24]. Caspases (cysteinyl aspartate-specific proteases) mediate highly specific proteolytic cleavage events in dying cells, which collectively initiate apoptosis. It appears that the role of these proteases in cell suicide is to disable critical homeostatic and repair processes, as well as to cleave key structural components resulting in the systematic and orderly disassembly of a cell. Caspases have been found in organisms ranging from the nematode *Caenorhabditis elegans* to humans. The 13 identified mammalian caspases (named caspase 1 to caspase 13) have distinct roles in apoptosis and inflammation. The family includes two murine homologs (11 and 12) that have no human counterparts. In apoptosis, caspases are directly responsible for proteolytic cleavages that lead to cell disassembly (effectors—caspases 2, 3, 6, 7) and are also involved in upstream regulatory events (initiators—caspases 2, 8, 9, 10) with other caspases (1, 4, 5) playing a role in inflammation [23], [20], [46].

Early observations of apoptosis found common morphological changes occurring in tissues and species, which led to the suggestion that this process is governed by a conserved biochemical system. It is now clear that these changes are due to the activities of a common set of effector caspases. The observation that distinct death signals result in the same manifestations of apoptosis is explained by the finding that effector caspases are activated by different initiator caspases, each of which is activated by a set of proapoptotic signals. Available evidence suggests that caspases are regulated by the opposing effects of activators and inhibitors. A signal apparently initiates three pathways involving cofactors, initiator caspases, and inhibitors. Activation of cofactor (for example, cytochrome *c* relocation from mitochondria to cytoplasm), modification of the caspase (for example, relocation of caspase 8 to a receptor complex), and inactivation of inhibitors together result in activation of the initiator caspase [23].

All apoptotic caspases are constitutively expressed and exist inactive in normal cells. Most of these proteases are synthesized as precursors that have little, if any, catalytic activity. The precursor is usually converted to the active enzyme by proteolytic processing mediated either by another protease or by autocatalysis, triggered by the binding of cofactors or removal of inhibitors. Hence, large amounts of a precursor can be accumulated in advance and activated on demand [23], [47]. Unlike most other postranslational modifications, proteolysis is irreversible. This implies that regulation of caspases is limited to control of their activity and availability of substrate since the only known way of correcting a cleaved protein is to make it again [23].

Two features of the proenzyme structure of caspases are central to the mechanism of activation of these enzymes. First, the NH_2-terminal domain, which is highly variable

in sequence and length, is involved in regulation of activation. Second, all domains are derived from proenzyme by cleavage at caspase consensus sites, implying that these enzymes can be activated either autocatalytically or in a cascade of enzymes with similar specificity. Caspases are among the most specific of proteases, with an unusual and absolute requirement for cleavage after aspartic acid. Recognition of at lease four amino acids at the NH_2-terminal to the cleavage site is also a necessary requirement of efficient catalysis. The strict specificity of caspases is consistent with the observation that apoptosis is not accompanied by indiscriminate protein digestion; rather, a select set of proteins is cleaved in a coordinated manner, usually as a single site, resulting in loss or change in function [23].

Caspases can regulate their own activation achieved through positive and negative feedbacks, one of which is amplification loop. In this case, the active protease can directly or indirectly activate its own precursor, resulting in an exponential rate of activation and ensuring that the caspase can be fully activated quickly. A large body of genetic and biochemical evidence supports a cascade model for effector caspase activation: a proapoptotic signal culminates in activation of an initiator caspase, which, in turn, activates effector caspases, resulting in cellular disassembly. Different initiator caspases mediate distinct sets of signals. For example, caspase 8 is associated with apoptosis involving death receptors, and in contrast, caspase 9 is involved in death induced by cytotoxic agents [23].

Activation of initiator caspases requires binding to specific cofactors, a mechanism commonly observed with proteases. This binding is triggered by a proapoptotic signal mediated through one of at least two distinct caspase prodomains and

its corresponding cofactor. Activation of procaspase 8 requires association with its cofactor FADD, while procaspase 9 activation involves a complex with the cofactor Apaf-1 along with cytochrome *c*, indicating that caspase activation may require multiple cofactors [23], [20], [24], [22].

Initial activation of caspases can involve transduction of a signal from membrane receptors belonging the TNF receptor family, such as TNF-R1. Stimulation of death receptors results in activation of caspase 8, which goes on to activate the effector caspases 3, 6, and 7, with caspase 3 being a key effector protein of the apoptotic machinery [2], [22], [48], [20], [22], [2]. The activation of caspase 8 can also trigger downstream caspases indirectly by causing the mitochondria to release cytochrome *c*. Procaspase 8 contains two tandem repeats of the DED within its N-terminal prodomain, which is involved in the recruitment of procaspase 8 to the TNF-R/FADD complex by a homotypic interaction with the DED of FADD. Binding to FADD leads to procaspase 8 oligomerization and its autoproteolytic activation, which will eventually lead to cell death [24] via two pathways: activation of downstream effector caspases or activation of Bid ultimately causing mitochondrial apoptotic amplification [22], [48], [20], [22], [2].

Multiple stimuli such as Bax, Bid, oxidants, calcium overload, active caspases, and ceramide can trigger mitochondria to release caspase-activating proteins, among which are cytochrome *c* and apoptosis-inducing factor (AIF). Two general mechanisms of release of caspase-activating proteins from mitochondria have been proposed: one involves osmotic disequilibrium leading to an expansion of the matrix space, organellar swell, with subsequent rupture of the outer membrane; the other consist of opening channels in the outer membrane (without organellar swelling), thus releasing cytochrome

c from the intermembrane space of mitochondria into the cytosol. Cytochrome *c* activates caspases by binding to the cytosolic protein of Apaf-1, inducing it to associate with procaspase 9 (this complex is often referred to as an apoptosome), thereby triggering caspase 9 activation and initiating the proteolytic cascade that culminates in apoptosis by the activation of caspase 3 [49], [20], [50], [2].

Activation of effector caspases such as caspase 3, produce the net effect of halting cell-cycle progression, disabling hemeostatic and repair mechanisms, initiating the detachment of the cell from its surrounding tissue structures, disassembling of structural components, and marking the dying cell for engulfment by other cells such as macrophages [47]. As mentioned before, this cascade of events is irreversible. Apoptotic inhibitors are not protected against this cell death cascade, and can possibly be a contributor. Caspases can cleave Bcl-2 proteins (many of which are apoptosis inhibitors), and it appears that cleavage cannot only inactivate these proteins, but also produces fragments that promote apoptosis [23]. Activation of caspase 3 in the mammalian cell has been linked to the proteolytic cleavage of cellular substrates including PARP (explained later).

Caspase 2 can be activated as well by the TNF receptor (Figure 1) via the TRADD, RIP, RAIDD pathway. Interestingly, caspase 2 can play two roles: one is by acting like an effector caspase and causing apoptosis directly, another is by acting as an initiator caspase much like caspase-8 thereby activating effector caspases like caspase-3, which initiates apoptosis directly [23], [20], [23], [37], [38], [39], or by cleaving and activating Bid thus stimulating mitochondrial apoptosis application. It has been

demonstrated recently that caspase 2 can induce the release of cytochrome *c* independent of Bid or other cytosolic factors [51].

As stated briefly above, caspases 1, 4, and 5 predominantly play a role in inflammation. Caspase 1, for example, cleaves proIL-1β generating the mature, biologically active form of this cytokine. As a consequence, this enzyme has been the subject of intense investigation as an attractive target for the treatment of IL-1-driven inflammatory diseases. Caspase 1 is synthesized as a dormant 45kD proenzyme that resides in the cell cytoplasm, and is proteolytically activated in response to various inflammatory challenges. Studies show that caspase 1 deficient mice cannot generate mature IL-1β and found these animals to be resistant to lipopolysaccharide (LPS)-induced endotoxic shock [47]. Although the caspases involved with inflammation alone are interesting, and in need of researching, the caspases of interest in this research are those involved in apoptosis induced by TNF: caspase 3, caspase 2, and caspase 9 (Aim #4).

A survey of caspases and or of their substrates suggests that caspases participate in apoptosis in a manner reminiscent of a well-planned and executed military operation. They cut off contacts with surrounding cells, reorganize the cytoskeleton, shut down DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis, and disintegrate the cell into apoptotic bodies [23]. If there is an imbalance of cell apoptotic inhibitors verses proapoptotic caspases, once the caspase cascade is initiated the cell is doomed. Our studies looked at the active forms of caspase 2, 3, and 9 (Aim #4a.) because these are the caspases that indicate the activation of TNF-R pathways.

2.5 Bcl-Family Proteins

There is a distinct genetic pathway apparently shared by all multicellular organisms that can either initiate or prevent apoptosis. The Bcl-2 family of proteins constitutes a decisional checkpoint within the apoptotic pathway. Full members of the Bcl-2 family share homology in four conserved domains designated BH1, BH2, BH3, and BH4 [46], [22]. Bcl-2 family proteins consist of both anti-apoptotic (Bcl-2 or Bcl-xL) and pro-apoptotic proteins (Bax, Bak, Bad, and Bid), which can regulate caspase activation mainly at the mitochondria level [22], [46], [48] signified by the release of cytochrome *c*, changes in the transmembrane potential, and mitochondrial permeability [48]. Many, but not all, Bcl-2 family proteins reside in the mitochondrial outer membrane anchored by a hydrophobic stretch of amino acids located within their COOH-terminal with the proteins oriented toward the cytosol. One clue to how the Bcl-protein family exerts their mitochondrial effects has come from determination of the three-dimensional structure of the Bcl-xL protein. The Bcl-xL protein is composed of seven α helices joined by flexible loops and shares striking similarity to the pore-forming domains of some types of bacterial toxins such as the diphtheria toxin. These toxins are known to insert into lipid bilayers and form channels capable of conducting ions. As predicted by their structures, Bcl-2, Bcl-xL, and Bax can form ion channels when they are added to synthetic membranes. The Bcl-xL was shown to form a cation-specific channel in both vesicles and planar lipid bilayer, whereas Bax, the pro-apoptotic counter part formed an anion-selective channel. Therefore, relative ratios of pro and antiapoptotic proteins could influence the flow of ions and, subsequently, the flow of

water causing the swelling and rupture of the mitochondrial membrane resulting in the release of cytochrome *c* [49], [20]..

The Bcl-2 protein family regulates a variety of mitochondrial events. Bcl-2 and Bcl-xL can inhibit not only caspase-dependent apoptosis but also oxidant and hypoxia induced necrosis. On the other hand, Bax, Bak, Bad, and Bid can induce cytochrome *c* release and cell death. The Bax induced release of cytochrome *c* release can be inhibited by Bcl-2 and Bcl-xL [49]. The balance in expression between Bcl-2 and Bax has been suggested as a major role in the pathway of apoptotic cell death. The downregulation of Bcl-2 and upregulated Bax proteins have been observed in ischemic heart or kidney tissue [52]. As mentioned above, the expression of Bax can be directed by TNF via simulation of NFκB by way of p53 suppressor protein [40] (Figure 1).

Interestingly, Bid is a BH3-only member of the Bcl-2 family of proteins and a substrate of caspase 8 activated by the TNF-R1 pathway (Figure 1). Bid lacks the COOH-terminal membrane anchoring domain and therefore is in the cytosol of cells as an inactive precursor [22]. Thus, Bid connects the death receptor to the mitochondria apoptosis pathway, and is responsible for the mitochondrial release of cytochrome *c* which complex with Apaf-1 and procaspase 9 subsequently activating caspase 3 (Figure 1) [48], [36],[46].

The cytosolic 22 kD Bid represents its inactive form that is proteolytically cleave by caspase 8 to generate the active 15 kD form of Bid [46] . The 15 kD form is often call the truncated Bid (tBid). The 15 kD conformation rather selectively targets the mitochondria where it resides as an integral membrane protein responsible for the release of cytochrome *c*. The rapid movement of tBid from the cytosol to mitochondrial

membranes suggest a specific mechanism of targeting. tBid by itself is sufficient to induce complete release of cytochrome *c* from the mitochondria. Bid is a much more potent cytochrome *c* releasing factor than Bax, another key cytochrome *c* releasing protein. Studies showed Bax only released up to 20% of the total mitochondrial cytochrome *c* even at a high concentration, whereas, Bid was able to release close to 100% of mitochondrial cytochrome *c* at a 500-fold lower concentration. It is not clearly understood how Bid impacts the mitochondria but it has been observed that Bid initiates the release of cytochrome *c* without evoking gross mitochondrial swelling and permeability transition; the two events that have been proposed to cause cytochrome *c* release. Bid appears to be a potent connection between the death receptor, and once more, another avenue for initiating apoptosis [22].

Fascinatingly, it has been reported that Bcl-2 and Bcl-xL, usually protective of the mitochondria, can be converted by activated caspases from anti- to pro-apoptotic molecules. Thus, caspase cleavage can upset the balance of the anti-apoptotic Bcl-2 members allowing for a feed forward loop to ensure cell death [46].

2.6 p53

The p53 suppressor protein is considered the guardian of a cell. It arrests the cell cycle allowing time for cell repairs. However, if there is too much cell damage and the repair process fails, p53 can accumulate and stimulate apoptosis [53]. Studies have revealed elevated levels of p53 in cardiac disease along with an increased incidence of apoptosis. It has also been shown that expression of p53 alone is sufficient to trigger apoptosis of ventricular myocytes [54]. Preliminary studies done in our lab show increased levels of p53 in the kidney after induced coronary ischemia and TNF infusion.

Together, these studies support a key role for p53 in cardiorenal syndrome. Donato et al. has published their data results demonstrating an increase in p53 levels after cells (ME-180S—apoptotic sensitive cell line) were exposed to TNF [55] in much the same pattern as our findings. Research has shown that NF-κB, a transcription factor activated by TNF, binds and activates the p53 promotor thus increasing p53 protein levels in a cell stimulated by TNF [40]. Once again, this indicates another route in which the cytokine TNF can induce apoptosis.

p53 is known to regulate transcription of a number of genes including the pro-apoptotic Bcl-2 family member Bax. However, recent evidence suggests that p53 may induce apoptosis through a mechanism independent of its gene activating properties. It has been reported p53 mediates apoptosis involving the disturbance of mitochondria, which includes the loss of membrane potential, release of cytochrome *c*, and increased caspase 3 like activity consistent with the mitochondrial death pathway. Although this resembles activity of Bax, increased levels of p53 initiated this pathway in the absence of Bax expression. There has been evidence supporting the ability of p53 to induce apoptosis by localizing to mitochondria in response to death signals (such as that seen with the TNF death receptor) and amplifying transcriptional dependent events [54]. High levels of p53 can indicate extreme cell stress caused by cytotoxins such as TNF and subsequent apoptosis due the stress [55].

2.7 PARP

PARP is a nuclear DNA-binding enzyme that detects DNA strand breaks and then functions in excision repair of damaged DNA. It has three functional domains, and N-terminal DNA binding domain (DBD), a C-terminal catalytic domain, and an

automodification domain in the middle of the protein. DBD of PARP contains two zinc fingers that actively binds to DNA strand interruptions. Activated PARP catalyzes the formation of long and branched polymers of adenosine diphosphate (ADP)-ribose using nicotinamide-adenine dinucleotide (NAD) as a substrate. It has been reported that at a very early stage of apoptotic cell death, while the cell continues to retain membrane integrity and the majority of the proteins are not degraded, PARP (an approximate 116 kD protein) is specifically cleaved to form two fragments of molecular mass, 89 and 24 kD (Figure 3). Cleavage of PARP has been found to be a sensitive parameter of apoptosis and can be detected within 15 minutes [56]. The cleaved forms of PARP has become the research hallmark for indicating apoptosis [20], [50], [2], [49], [56].

Since PARP is activated by DNA damage, it is suggested that DNA degradation occurring during cell death would result in massive activation of PARP. Once cleaved by caspase 3, PARP can then become a contributor to apoptosis through two possible methods. One by the 89 kD fragment, which if left relatively unaltered would permit unchecked syntheses of poly(ADP-ribose) rapidly consuming the cell of NAD and ATP (Figure 3). Secondly, the 24 kD fragment could block DNA repair at the strand breaks. This PARP fragment with its zinc fingers binds to DNA at strand breaks, and without the catalytic repair activity of the 89 kD fragment, the 24 kD fragment remains bound, thereby, freezing the DNA. Since PARP is present in large abundance, and nearly all of the PARP is stably cleaved by caspase 3 to form the 24 and 89 kD fragments, there could be a few million molecules of the 24 kD fragment in the nucleus of an apoptotic cell. Thus, the 24 kD fragment could contribute to cell death by blocking DNA repair at the strand breaks. These cleaved forms of PARP have become the research hallmark for

indicating apoptosis [20], [50], [2], [49], [56] . To show apoptosis is occurring in this study, we looked at the levels of cleaved product of PARP (Aim #2). We also looked histologically to establish which cell types where undergoing apoptosis by probing paraffin imbedded kidney tissue with cleaved PARP (Aim #3).

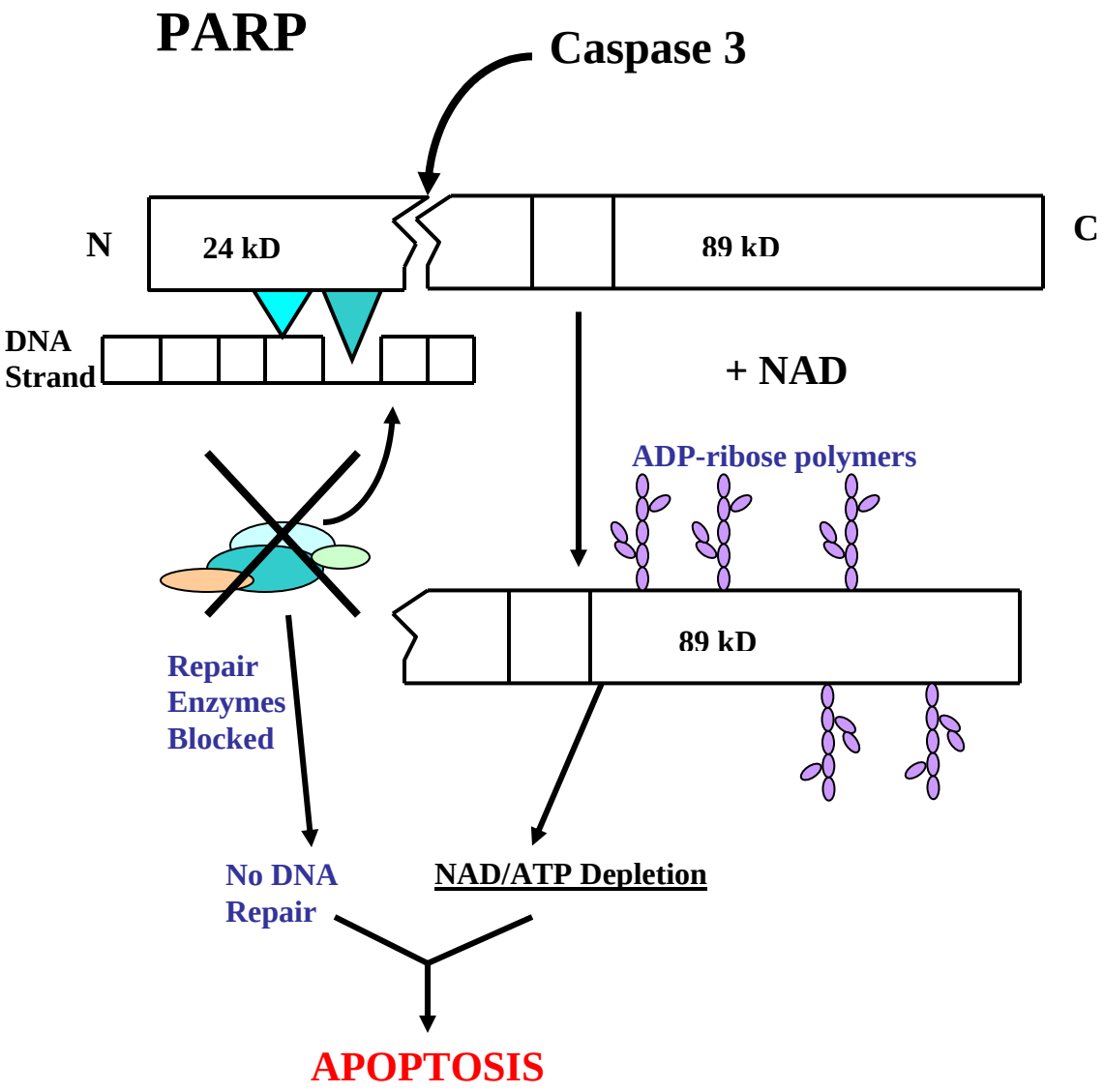

Figure 3. PARP contribution to apoptosis. Once caspase 3 is activated it can enter the nucleus of the cell and cleave PARP (poly ADP-ribose polymerase), an enzyme used to detect and repair DNA. Once PARP is cleaved it is rendered nonfunctional and can become part of the mechanism resulting in apoptosis through both segments created by caspase 3: 24 kD fragment can stick to and block DNA repair and 89 kD fragment can deplete the cell of NAD and ATP.

Chapter III

EXPERIMENTAL DESIGN

3.1 Introduction

The purpose of these studies is to acquire a clearer understanding of cardiorenal syndrome. Observations have been made showing a small percentage of patients suffer acute renal failure after undergoing cardiopulmonary bypass as well as patients in cardiac failure. The same clinicians and researchers making these observations think the cause of the renal injury are cytokines released by the injured or ischemic myocardium, but there has been no research done to support or disprove this theory [3], [4], [5], [6]. Because TNF-α is suspected to be the most potent cytokine released from the myocardium and the most likely to induce renal dysfunction [3], it is the focus of this study.

My hypothesis is renal injury, as indicated by apoptosis in the kidney, results from the production and release of cytokines due to cardiac ischemia. The way in which I plan to address my hypothesis is through the specific aims of this research: (1) illustrate there is an increase in serum TNF-α after cardiac ischemia in our cardiac ischemic model as seen in other studies; (2) find if there is injury to the kidney, measured by apoptosis (cleaved PARP), due to coronary occlusion, (3) locate the renal cells being injured by cardiac ischemia, and (4) measure renal apoptotic signaling (caspases 2, 3, and 9) after cardiac ischemia and examine the role of TNF-α in these activities. We used the following described procedures to investigate these specific aims.

3.2 Animals

Adult male Sprague-Dawley rats were obtained from the Oklahoma State University College of Osteopathic Medicine animal facility or purchased from Charles

River Company. Animals were housed in a separate room in the animal facility in plastic bins with adequate bedding, and maintained on standard rat chow and tap water. Animal facilities were under the direction of full-time animal caretakers, and the Oklahoma State University College of Osteopathic Medicine animal Use Committee approved this project. The college of Osteopathic Medicine complies with the NIH policy of animal welfare of the Animal Welfare Act and all other applicable laws.

3.3 Induced Myocardial Ischemia

Animals were first anesthetized with isofluorane/oxygen (2% volume/volume) at 2-liters per minute followed by an intraperitoneal (i.p.) injection of ketamine HCl (100 mg/kg body weight) and xylazine HCl (10 mg/kg body weight). Catheters were placed in the external jugular vein for intravenous injections of medications if necessary, and the carotid artery for monitoring the animals mean arterial pressures measured by a computerized blood pressure transducer. A tracheotomy was performed with the animal being placed on mechanical ventilation with a volume-cycled respirator. To perform coronary artery occlusion the animals' chest were opened via left thoracotomy, the pericardium was cut away, and the interventricular coronary artery was occluded using a silk suture. At the end of 15 minutes of coronary ischemia, trypan blue dye was injected into the coronary artery in order to identify the degree and the regions of myocardial ischemia. Once ischemia was determined the kidneys were harvested, rinsed in chilled normal saline and either rapidly frozen in liquid nitrogen and stored at -80°C to be further processed fromy Western blot analysis, or placed in buffered fixative for immunohistochemistry. We followed the same procedures for the control animals except there was no occlusion of the coronary artery.

3.4 Infused TNF-α

Again animals were anesthetized first with isofluorane/oxygen (2% volume/volume) at 2-liters per minute followed by an i.p. injection of ketamine HCl (100 mg/kg body weight) and xylazine HCl (10 mg/kg body weight). Catheters were placed in the external jugular vein for intravenous injections of medications if necessary, and the carotid artery for monitoring the animals mean arterial pressures. A tracheotomy was performed without the animal being placed on mechanical ventilation because animal did not need ventilation assistance since the chest was not opened to perform coronary ligation. 2ng of TNF (Sigma-Aldrich Inc., St. Louis, MO) per 1ml of extracellular fluid was diluted in 2 mls of normal saline and intravenously injected via the external jugular catheter for a period of 15 minutes. Immediately after the TNF infusion the kidneys were harvested, rinsed in normal saline, and either rapidly frozen in liquid nitrogen, and stored at -80°C for Western blot analysis or placed in buffered fixative for immunohistochemistry. We followed the same procedures for the control animals except we infused normal saline only.

3.5 Immunohistochemistry

Kidney tissue was fixed in buffered formalin for 12 to 18 hours, dehydrated in ascending series of ethanol concentrations (75%, 95%, 100%) and xylene, and then embedded in paraffin wax. Seven-micrometer sections were dewaxed in xylene and rehydrated through a descending series of ethanol concentrations (100%, 95%, 75%). Sections were then washed in deionized water before the labeling procedure. Endogenous peroxidase was blocked by placing sections in 0.3% peroxide diluted with methanol for 30 minutes and rinsed with phosphate buffered saline (PBS). Nonspecific

antibody binding was blocked by incubating sections for one hour in 2% normal blocking serum diluted in PBS (1:50 dilution). Sections were then incubated in primary antibody (active caspase 3 or cleaved PARP, Promega, Madison, WI) diluted in PBS (1:100) for 30 minutes to one hour at room temperature. After sections were rinsed of primary antibody with PBS, secondary antibody was applied to sections diluted in PBS and blocking serum incubating for 30 minutes. The sections were then stained using ABC-peroxidase substrate kit (Vectastain elite, Vector Laboratories, Burlingame, CA) and rinsed. 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, Burlingame, CA) was applied to the sections and allowed to incubate for 15 minutes or until desired stain intensity developed. The sections were rinsed with PBS, dehydrated in graded alcohols (75%, 95%, 100%), counterstained with hematoxylin (for visualization), and coverslipped.

3.6 Western Blot Analysis

Kidney tissue (stored at –80°C) was pulverized into a powder using liquid nitrogen and placed in a 50 milliliter tube containing lysis buffer (50mM Tris [pH 7.4], 2% Triton-X, 1M NaCl, 5 mM EDTA, 15% glycerol, and 1mM NaF) with protease inhibitors (PI) added (10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml trypsin type II-O, 10 μg/ml trypsin type II-S, and 10 μg/ml pepstatin, Sigma, St. Louis, MO). Tissue was then homogenized and dounced. Tissue was allowed to incubate in the lysis buffer for two hours on ice to ensure all cell membranes where adequately lysed before centrifuging (10,000 x g) for one hour to eliminate all large particles. Supernatant from centrifuged samples were decanted and place in appropriately labeled tubes. Protein levels of each sample were determined by Bradford protein assay. Protein standard curve was analyzed

by version 3.O GraphPad Prism. Proteins were resolved by 4-15% precast SDS polyacrylamide gel (Bio-Rad Laboratories, Chicago, IL) electrophoreses. After electrophoreses, samples were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) and membranes were allowed to dry. Blots were then incubated with primary antibody blocked with 5% skimmed milk mixed in tris-buffered saline tween-20 (TBST) with 0.025% tween-20, for 3 hours to overnight. The blots were than rinsed with TBST and incubated in secondary antibody blocked with 5% skimmed milk mixed in TBST with 0.025% Tween 20 for one hour and rinsed with TBST. After rinsing, the blots were developed with the enhanced chemiluminescence (Amersham Biosciences, England). Exposure time to film (Kodak Scientific Imaging film, Fisher Scientific, Houston TX) was determined by the degree of signal. Density of bands of interest from exposed film were measure by Bio-Rad G-710 Calibrated Imaging Densitometer.

3.7 Serum TNF Level Quantification

For quantification of serum TNF level a rat TNF specific Ultrasensitive ELISA kit (Bio Source International, Camarillo, CA) was utilized. Pre and post treatment blood samples were taken from adult male rats. The blood sample was allowed to clot for 30 minutes, after which they were centrifuged (1000 x g) for 10 minutes. Serum was then removed and placed in clean, appropriately labeled tubes and either stored in -4°C or immediately tested for TNF levels with the Ultrasensitive TNF ELISA kit. The ELISA assays were strictly preformed as per the ELISA kit's protocol.

3.8 TNF Inhibition

WTACE2 is a novel metalloproteinase inhibitor specific for TACE, developed and donated by Wyeth-Ayerst Research (Pearl River, NJ, USA). WTACE is a

nonpeptide sulfonamide TACE inhibitor bearing a hydorxamic acid as the requisite zinc-chelating moiety. This nanomolar level inhibitor contains a novel butynyloxybenzene P1′ group, derived from analysis for the TACE x-ray structure, which provides enhanced cellular activity. WTACE2 is an orally bioavailable, broad-spectrum inhibitor of matrix metalloproteins (MMPs) [57]. 30 minutes prior to experiment male rats were injected i.p. with either a dose of 100mg/kg of WTACE2 [57] in a vehicle containing 0.5% methocellulose and 2% Tween 80 (Fisher Scientific, Houston, TX) or with just vehicle containing 0.5% methocellulose and 2% Tween 80. After 30 minutes the rat was anesthetized and prepped for induced cardiac ischemia or sham control (described above).

3.9 Monitoring Blood Pressure

A pressure transducer connected to the carotid artery catheter continuously monitored the animals' blood pressure. Data was digitized at 500 Hz (Biobench, National Instruments) and stored for data analysis.

3.10 Statistical Analysis

Statistical analysis is preformed on the data obtained by the Ultrasensitive ELISA. Because both blood samples (pre and post treatment) were taken from the same animal, this experimental design is considered repeated-measures or pretest-posttest design. In this type of design subjects serve as their own controls, so that the variability owing to individual differences is eliminated from the error, thereby increasing the chances of observing significant differences between levels of treatment [58], [59]. The statistical analysis that was performed was two-way analysis of variance (ANOVA) with repeated measures. $P < 0.05$ is considered significant.

Statistical analysis is also performed on data obtained on mean arterial blood pressure (MAP) monitored on each animal for the ischemic, TNF infusion, TNF inhibition, and TNF ELISA studies. The MAP for each animal was monitored continuously pre and post assigned treatment. This experimental design is again considered a repeated-measures design, thus two-way ANOVA with repeated measures design is used to determine if there is statistical significance of MAP between the treatment groups and their controls ($P > 0.05$). In addition a one-way ANOVA with repeated measures is preformed to assess if there is a any significance between MAP over time by comparing all posttest MAPs to the pretest MAP for each animal. A Dunnett's test is used for comparing the pretest mean to each of the posttest pressures means across time. $P < 0.05$ was considered significant.

Chapter IV

QUANTITATE TNF LEVELS

4.1 Introduction

It was discovered by Levine et al. [7] in 1990 that patients with chronic heart failure had increased circulating levels of TNF. Serum levels of TNF were elevated primarily in the patients with the most advance disease and high levels of TNF identified the patients who were the most severely affected by heart failure and the least likely to survive. Also in the same study it was found that the patients with elevated circulating TNF levels had greater impairment of renal function [7]. More recent clinical observations have shown serum levels of TNF are increased after MI and after CPB. There were also detections of acute changes in renal function found commonly with patients after CPB procedures. The available data to date indicate that this renal dysfunction could be related to myocardial cytokine release that includes TNF [3], [25].

Because TNF is thought to generate a strong relationship between cardiac ischemia and renal injury [3], [7], this investigation will look at the TNF levels in order to elucidate clues to the role TNF may play in the injury of the kidney due to cardiac ischemia, and illustrate there is an increase in serum TNF-α after cardiac ischemia in our cardiac ischemic model as seen in other studies (Aim #1). TNF levels were measured before and after 15 minutes of induced cardiac ischemia without reperfusion.

4.2 Methods

4.2.1 Myocardial Ischemic Model

Two groups of six adult, male Sprague Dawley rats weighing between 200 to 350 grams were used as a control group or a cardiac ischemic group. Both groups were

anesthetized with isoflourane/oxygen (2% volume/volume) at two liters per minute followed by an i.p. injection of ketamine HCl (100 mg/kg of body weight) and zylazine HCl (10 mg/kg of body weight). Both groups had catheters placed in the external jugular vein for intravenous injections of medication if necessary, and the carotid artery for blood draws and monitoring the animals' arterial pressures. Both groups had a tracheotomy preformed should assisted breathing be required. For the ischemic group a control blood sample was taken before ischemia and a second sample taken 15 minutes after the interventricular coronary artery was occluded with a silk suture. To insure ischemia was accomplished, trypan blue dye was injected into the coronary artery to determine the ischemic area. With the control animals, the control blood sample was taken directly after the tracheotomy was preformed and the second blood sample (post sample) was taken 15 minutes after the control blood sample was drawn.

4.2.2 Serum Preparation

Pre and post treatment blood samples were taken off of ice and allowed to clot for 30 minutes at room temperature. After 30 minutes each sample was centrifuged 1000 x g for 10 minutes. Serum was then removed and placed in clean, appropriately labeled tubes. Serum samples were stored at –4°C or immediately tested for TNF levels with an ultrasensitive TNF ELISA Kit.

4.2.3 Ultrasensitive TNF ELISA

100 μL of each prepared serum sample was place in its designated well of a 96 well plate of the Ultrasensitive TNF ELISA Kit (Bio Source International, Camarillo, CA) along with the TNF standards provided by the kit and prepared according to the kit's protocol. After completing the ELISA protocol, the wells used in the 96 well plate were

read by Dynatech MR 700 microplate reader at the wave length of 450 nm. Statistical analysis using Two-way ANOVA with repeated measures was used to determine significance.

4.3 Results

Mean arterial blood pressures were continually monitored for all the animals in this study. Figure 4 shows a graph of the MAP for the two groups (control and ischemic). The graph shows there is a significant difference between the control group and the ischemic group at the 14 minute mark, as well as a significant difference in the 14 minute pressure compared to its control pressure within the ischemic group. While there shows to be a statistical significant difference in these two areas, we feel there is no biological significance between these MAP and were confident that animal blood pressures remained in a range where organs were properly perfused through out the assay.

The ELISA studies showed very low levels of TNF after 15 minutes of ischemia, but there was a mean increase of 9.24 pg/ml in the levels of TNF with the post treatment serum samples in the ischemic model over that of control with a mean TNF level of 1.76 pg/ml. The mean TNF levels for the post ischemia group were higher than that of the control group indicating that cardiac ischemia increases the serum levels of TNF. We also discovered the greater the area of ischemia—visually defined by the injection of trypan blue dye—the higher the levels of TNF in the serum. This phenomenon was responsible for the large standard deviation for the post ischemia serum samples. Although close to significance, this same large variability prevented our results from being significant along with reducing the power of our study. A lowered power can

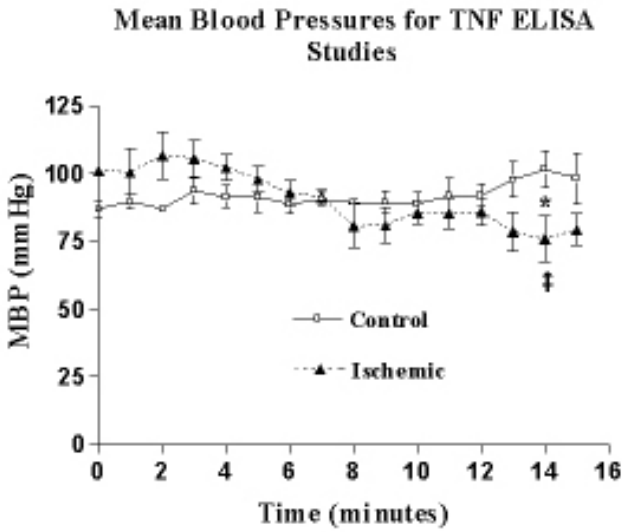

Figure 4. Mean arterial blood pressures for animals in the TNF ELISA studies showing pressures remaining at a level for the prevention of organ ischemia. ‡ indicates a statistical difference between the control and ischemic groups at the 14 minute mark. * indicates a statistical difference between the 14 minute MAP compared to its control within the ischemic group. Mean values are ± standard error of the mean (SEM).

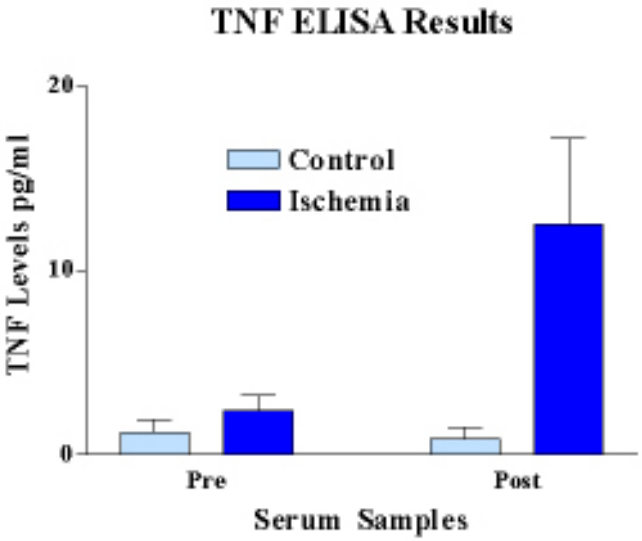

Figure 5. Bar graph representing the TNF serum levels. To increase power and avoid a Type II statistical error, data was pooled from the vehicle only ischemic studies (which mirrored that of these ischemic studies) and added to the cardiac ischemic data. TNF serum levels were statistically higher in the post ischemia group than that of the control indicating that cardiac ischemia increases levels of TNF in our ischemic model. Pre represents the serum samples that were taken prior to induced ischemia, and post represents the serum samples taken after 15 minutes of the first serum samples taken. Mean values are ± SEM, $P > 0.05$.

increase the likelihood of committing a Type II statistical error. To avoid this we pooled data from our WTACE2, TNF inhibition studies (Chapter VII) using the vehicle only pre and post cardiac ischemia data. The pooled vehicle only data mirrored that of these pre and post cardiac ischemic TNF serum levels illustrating the vehicle (0.5% methocellulose and 2% Tween 80) had no impact on the TNF serum levels. Figure 5 shows the bar graph representing TNF serum levels of the ELISA studies including pooled data. The graph shows a significant ($P < 0.05$) increase in levels of TNF after coronary occlusion with a mean increase of 15.79 pg/ml in serum TNF post treatment over that of controls with a mean value of 2.36 pg/ml.

4.4 Discussion

Although TNF levels were low and there were only small changes in pre versus post serum samples and control versus ischemic serum samples, these studies found there are increased levels of TNF over that of control in as little as 15 minutes of cardiac ischemia without reperfusion. We feel there would have been substantial increased TNF levels in the rats with induced ischemia had there been reperfusion to the ischemic site as found in other animal studies [8], [9], [10], which would be a model similar to that seen with CPB. Our model of ischemia without reperfusion probably better represents the majority of heart disease caused by that of an MI [60]. Also we feel there would be higher levels of TNF if we would have extended the time for the ischemic model, which has additionally been shown in other animal studies [8], [9], [10]. Human studies as well have noted a correlation between the levels of TNF and the duration of cardiac ischemia [5]. Observations have also shown, as our studies did, that the degree of myocardial injury determined the levels of TNF release [4]

Because of the nature of cytokines it is not surprising that even at low levels, cytokines can cause a myriad of events due to their ability to initiate a variety of cascades that can amplify their actions. The bioactivities exerted by cytokines such as TNF are extremely diverse, showing remarkable organ and cell specificity. There is strong evidence that the response of a cell type to a given cytokine is greatly influenced by its microenvironment [17]. So in essence a little can go a long way.

In conclusion, these studies show quantitatively that serum TNF does go up over control due to induced cardiac ischemia. We found only small increases, but these increases were consistent. Also found was TNF levels went up as cardiac injury increased. These studies are consistent with other research showing that the cytokine, TNF, does in fact go up due to myocardial injury [5], [3], [27], [8], [9], [10].

Chapter V

DETECTION OF PARP TO DETERMINE APOPTOSIS IN KIDNEY TISSUE AFTER CORONARY ISCHEMIA

5.1 Introduction

Apoptotic cells do not display a random or chaotic degradation of all cellular proteins. On the contrary, at an early stage of apoptosis, caspases cleave a select band of target proteins at very specific sites, often making two or more identifiable fragments. PARP, a nuclear DNS-binding protein that detects DNA strand breaks and functions in excision repairs, is among the first targeted protein shown to be purposely cleaved during apoptosis to form a signature 89 kD and 24 kD fragments (Figure 3) [56], [61]. Cleavage of PARP is one of the earliest detectable protein-degradation events and can be detected within 15 minutes of apoptotic activation [56]. Also it has recently been suggested that cleaved PARP can contribute to the machinery of apoptosis by either the 89 kD fragment exhausting the cell of needed energy or by the 24 kD fragment blocking DNA repair [56], [62], [63], [64].

The objective of this study was to examine if there is injury to the kidney after cardiac ischemia. Because PARP has become an early marker and a research hallmark indicating apoptosis [20], [50], [2], [49], [56] this investigation used Western blot analysis to detect cleaved PARP.

5.2 Methods

5.2.1 Myocardial Ischemic Model

Two groups of six adult, male Sprague Dawley rats weighing between 500 to 650 grams were used as a control group or a cardiac ischemic group. Both groups were anesthetized with isoflourane/oxygen (2% volume/volume) at two liters per minute

followed by an i.p. injection of ketamine HCl (100 mg/kg of body weight) and zylazine HCl (10 mg/kg of body weight). Both groups had catheters placed in the external jugular vein for intravenous injections of medication if necessary, and the carotid artery for monitoring the animals' arterial pressures. Both groups had a tracheotomy preformed should assisted breathing be required. With the cardiac ischemic group the animals' chests were opened by way of left thoracotomy and placed on mechanical ventilation. Once the animals' ventilation was maintained the interventricular coronary artery was occluded with a silk suture. After 15 minutes of coronary ischemia the kidneys were isolated, removed, rinsed in chilled normal saline, rapidly frozen in liquid nitrogen and stored at -80°C to be further processed for Western blot analysis. To insure ischemia was accomplished, trypan blue dye was injected into the coronary artery to determine the ischemic area. With the control animals, the kidneys were removed and handled the same as cardiac ischemic kidneys 15 minutes after the placement of carotid artery catheter.

5.2.2 Western Blot Analysis

Kidney tissue (stored at –80°C) was pulverized into a powder using liquid nitrogen and placed in a 50 milliliter tube containing lysis buffer (50mM Tris [pH 7.4], 2% Triton-X, 1M NaCl, 5 mM EDTA, 15% glycerol, and 1mM NaF) with protease inhibitors (PI) added (10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml trypson type II-O, 10 μg/ml trypsin type II-S, and 10 μg/ml pepstatin, Sigma, St. Louis, MO). Tissue was then homogenized and dounced. Tissue was allowed to incubate in the lysis buffer for two hours on ice to ensure all cell membranes where adequately lysed before centrifuging (10,000 x g) for one hour to eliminate all large particles. Supernatant from

centrifuged samples were decanted and place in appropriately labeled tubes. Protein levels of each sample were determined by Bradford protein assay. Protein standard curve was analyzed by version 3.0 GraphPad Prism. Proteins were resolved by 4-15% precast SDS polyacrylamide gel (Bio-Rad Laboratories, Chicago, IL) electrophoreses. After electrophoreses, samples were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) and membranes were allowed to dry. Blots were then incubated with primary antibody PARP (Cell Signaling Technology, Beverly, MA) blocked with 5% skimmed milk mixed in TBST with 0.025% Tween 20 overnight. The blots were than rinsed with TBST and incubated in secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) blocked with 5% skimmed milk mixed in TBST with 0.025% Tween 20 for one hour and rinsed with TBST. After rinsing, the blots were developed with the enhanced chemiluminescence (Amersham Biosciences, England). Exposure time to film (Kodak Scientific Imaging film, Fisher Scientific, Houston TX) was determined by the degree of signal. Density of bands of interest from exposed film were measure by Bio-Rad G-710 Calibrated Imaging Densitometer.

5.3 Results

MAP were continually monitored for all the animals in this study. Figure 6 shows a graph of the MAP for both groups (control and ischemic). The graph illustrates there is no statistical significance between the control and ischemic groups, but there was a statistical significance found at the 15 minute pressure compared to its control pressure within the control group. Although this one measure statistically shows significance, we feel there is no biological significance and we were confident that animal blood pressures

remained in a range where there was no organ damage due to ischemia after coronary occlusion.

Figure 7 shows the effect of cardiac ischemia on PARP cleavage in the kidney and is a Western blot that represents the data gathered in these studies. There is an increase in both the PARP fragments of 89 kD and 24 kD in cardiac ischemic renal tissue over that of control kidney tissue. The control lane of Figure 7A shows the intact 116 kD of PARP where as in the cardiac ischemic lane there are undetectable amounts of the complete protein. This blot also illustrates there is a substantial increase of the 89 kD PARP fragment in the kidney tissue due to cardiac ischemia. Figure 7B represents the increase seen in the 24 kD fragment of PARP in kidney tissue from animals that underwent cardiac ischemia, but undetectable in the control kidney tissue.

Western blot analysis of cardiac ischemic kidney tissue compared to that of control kidney tissue shows there is an increase of apoptotic signaling in the cardiac ischemic kidney tissue over that of control in as little as 15 minutes. As seen in the graph of Figure 8, PARP signaling shows a mean densitometer reading of 5.4 in the cardiac ischemic group over that of the 0.8 mean reading for the control group producing an approximate 6.75 fold increase in apoptotic signaling in the kidney due to cardiac ischemia.

5.4 Discussion

PARP is considered an early indicator and is an accepted marker for apoptosis [20], [50], [2], [49], [56]. Our lab, as did other studies [56], showed that within 15 minutes of an insult cleavage of PARP can be seen. Whether these increases in cleaved PARP shown in this study are enough to cause extensive renal damage is yet to be

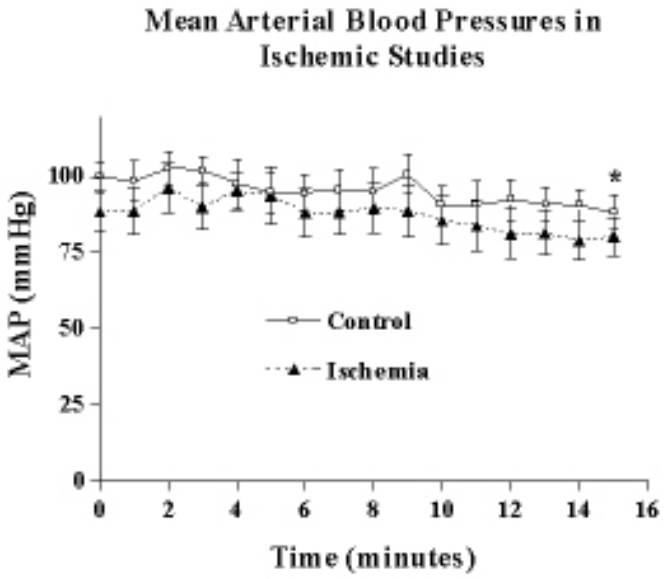

Figure 6. Mean blood pressures for animals in the PARP studies showing pressures remaining at a level for the prevention of organ ischemia. * indicates a statistical difference between the 15 minute MAP compared to its control within the control group. Mean values are ± SEM.

determined, but as indicated by this investigation there is clearly an increase in the initiation of apoptosis caused by cardiac ischemia without reperfusion.

Also seen by our Western blot analysis are both the cleaved products of PARP (89 kD and 24 kD), and if recent studies are correct in their implication of PARP fragments contributing to apoptosis [56], [62], [63], [64], the finding of both these PARP fragments would further guarantee apoptotic success in the kidney due to myocardial ischemia.

Eventual studies need to be done to look at PARP (as a sign of apoptosis) under conditions of cardiac ischemia with reperfusion. Reperfusing the myocardium after an ischemic event, such as that seen in CPB, shows considerably higher levels of cytokines, like TNF, released systemically. There are also increased levels of systemic cytokines correlated with the ischemic time span of the myocardium [4], [5]. Subsequently

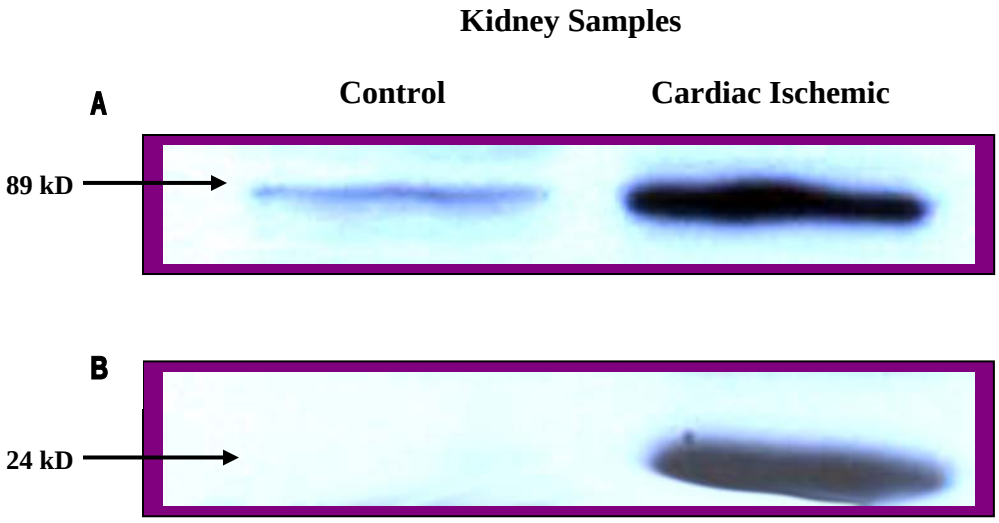

Figure 7. Western blot exposed film showing the cleaved fragments of PARP. **A** The 89 kD band is one of the cleaved products of PARP and is visibly seen at higher levels in the cardiac ischemic kidney samples then in the control. **B** The 24 kD band is the other cleaved product of PARP as a result of apoptotic activation, and is seen largely increased in the kidney tissue after cardiac ischemia where as there are undetectable levels in the control kidney. Clearly seen by this Western Blot is an increase in apoptotic signaling, determined by the early marker of cleaved PARP in the kidney after cardiac ischemia.

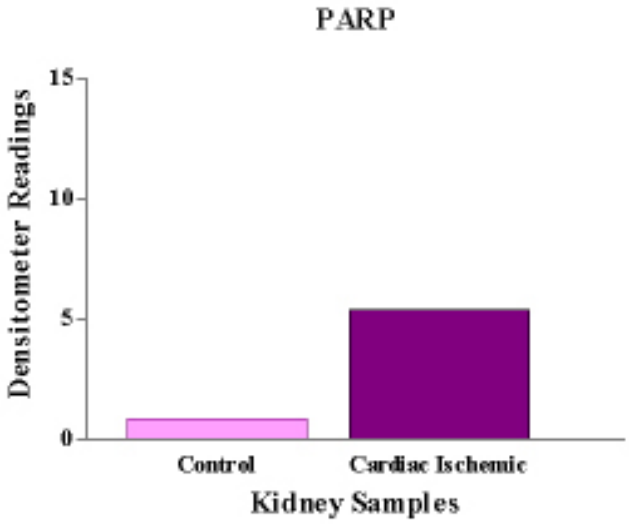

Figure 8. PARP shows a mean densitometer reading of 5.4 for the cardiac ischemic kidney tissue over that of mean readings of 0.8 for the control kidney tissue producing an approximate 6.75 fold increase of apoptotic signaling in the kidney due to cardiac ischemia.

further investigations should also be done on kidney tissue creating a time course of renal injury due to increasing the duration of cardiac ischemic events.

Chapter VI

RENAL CELLS EFFECTED BY CARDIAC ISCHEMIA

6.1 Introduction

Our investigation as well as other studies have shown increases in TNF levels as a result of cardiac ischemia [3], [4], [5], [6], [8], [9], [10]. And it is thought that the release of such cytokines can cause injury to other organ systems like the kidneys [3], [7]. To date there has been no immunohistochemistry studies on kidney tissue harvested after cardiac ischemia to determine 1) if there is injury to the kidney, and 2) which cells in the kidney are injured.

In normal kidneys the TNFR-1 is detected in the endothelium of the glomeruli with weak staining in the distal convoluted tubules (DCT). The distribution of TNFR-2 differs form TNFR-1. TNFR-2 shows no positive reaction in the glomeruli, but a weak reaction in the proximal convoluted tubules (PCT).

In the inflammatory process, it appears the TNF receptor expression in the kidney varies significantly from that of normal kidney[65], and there seems to a be disease specific expression of TNF receptors [66]. For example, in acutely rejecting renal allograft there was an increased staining for TNFR-2 on the descending loops of Henle, DCT, and collecting ducts in the medulla. In the cortex, the TNFR-1 expression was diminished in glomeruli [65]. In proliferative lupus nephritis, TNFR-1 was strongly increased in the glomeruli compared to non-diseased kidneys [66]. As the pattern of TNF receptor expression changes so should the area in which TNF will influence the kidney.

Immunohistochemistry studies have shown that different insults on the kidney show different forms of renal injury. Endotoxemia induced by lipopolysaccharide (LPS) produced glomerular injury where as renal ischemia caused injury to tubular cells [25]. Cisplatin, a chemotherapeutic widely used treatment for variety of malignancies and shown to induce renal dysfunction via TNF, was found to cause severe tubular injury reflected by cast formation, loss of brush border membranes, sloughing of tubular epithelial cells, and dilation of tubules [67].

The purpose of this study was to see histologically if the kidney does show signs of injury and demonstrate where such injury may take place after 15 minutes of induce cardiac ischemia (Aim #3). Because active caspase 3 is thought to be a key effector protein of the apoptotic machinery [2], we have chosen to use it for this investigation. Inactive forms of caspase 3 are found in all cells; therefore, in our studies the paraffin imbedded kidney tissue was probed with the active form of caspase 3. PARP, one of the most important targets of caspase 3 [49] as well as an established hallmark of apoptosis [20], [50], [56], [2], [49], was used in these studies to indicate which renal cells were undergoing injury, as signified by apoptotic signaling.

6.2 Methods

6.2.1 Myocardial Ischemic Model

Two groups of three adult, male Sprague Dawley rats weighing between 400 to 600 grams were used as a control group or a cardiac ischemic group. Both groups were anesthetized with isoflourane/oxygen (2% volume/volume), i.p. injected with ketamine HCl and zylazine HCl. Both groups were prepped as those in the prior study with placement of catheters in the external jugular vein and the carotid artery, and tracheotomy

preformed. As with the prior study, one group was subject to cardiac ischemic and the other group did not and was considered control animals. After 15 minutes the kidneys were harvested from both groups, rinsed in chilled normal saline, and place in buffered fixative for immunohistochemistry.

6.2.2 Immunohistochemistry

All kidney tissue was fixed in buffered formalin for 12 to 18 hours, dehydrated in ascending series of ethanol concentrations (75%, 95%, 100%) and xylene, and then embedded in paraffin wax. Seven-micrometer sections were dewaxed in xylene and rehydrated through a descending series of ethanol concentrations (100%, 95%, 75%). Sections were then washed in deionized water before the labeling procedure. Endogenous peroxidase was blocked by placing sections in 0.3% peroxide diluted with methanol for 30 minutes and rinsed with PBS. Nonspecific antibody binding was blocked by incubating sections for one hour in 2% normal blocking serum diluted in PBS (1:50 dilution). Sections were then incubated in primary antibody (active caspase 3 or cleaved PARP, Promega, Madison, WI) diluted in PBS (1:100) for 30 minutes to one hour at room temperature. After sections were rinsed of primary antibody with PBS, secondary antibody was applied to sections diluted in PBS and blocking serum incubating for 30 minutes. The sections were then stained using ABC-peroxidase substrate kit (Vectastain elite, Vector Laboratories, Burlingame, CA) and rinsed. 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories, Burlingame, CA) was applied to the sections and allowed to incubate for 15 minutes or until desired stain intensity developed. The sections were rinsed with PBS, dehydrated in graded alcohols

(75%, 95%, 100%), counterstained with hematoxylin (for visualization), and coverslipped.

6.3 Results

MAP were continually monitored for all the animals in this study. Figure 9 shows a graph of the MAP for the two groups (control and ischemic). The graph illustrates there is no statistical significance between the control and ischemic groups, but there was statistical significance found at the 15 minute blood pressure compared to its control blood pressure within the control group. Although this one measure statistically shows significance, we feel there is no biological significance and we were confident that animal blood pressures remained in a range where organs were properly perfused.

Kidneys from the ischemic heart group demonstrated increases in both Caspase 3 (Figure 10) and PARP (Figure 12) activity compared to kidneys from control rats (Figures 11 and 13). Active caspase 3 and cleaved PARP were localized to the cortex of the kidney with most all activity found in the proximal tubules.

The activation of caspase 3 can be executed by caspase 8 or caspase 9 complex resulting in the fragmented 17 kD active form of caspase 3 and was the form used in these studies. Figure 10 is a slide representative of kidney tissue from the cardiac ischemic group. Increases in activated caspase 3 is found to be localized in the cortex of the kidney with most of the activity found predominately in the proximal tubules. On the

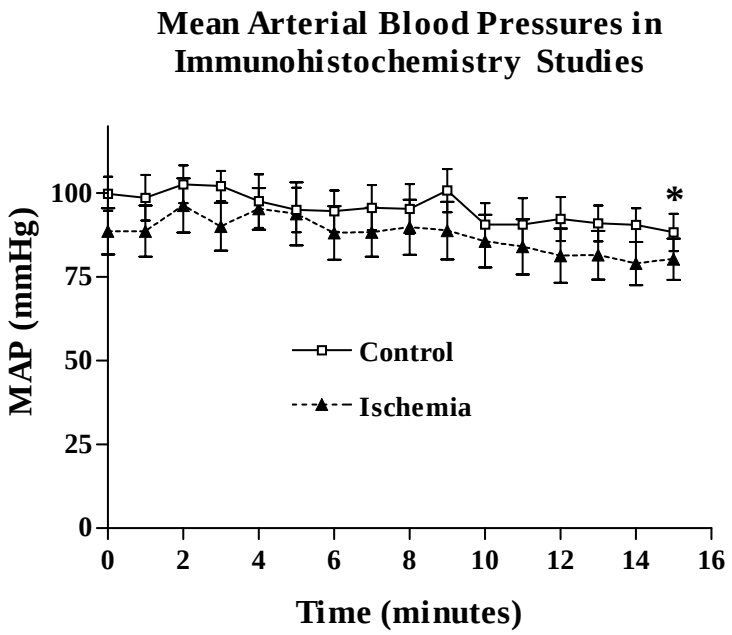

Figure 9. Mean blood pressures for animals in the ischemic studies showing pressures remaining at a level for the prevention of organ ischemia. * indicates a statistical difference between the 15 minute MAP compared to its control within the control group. Mean values are ± SEM.

other hand, Figure 11 shows kidney tissue from the control group to be mainly clear of any active caspase 3 signaling. Also seen in Figure 10 are the beginnings of renal injury illustrated by the scalloping brush border of proximal tubule epithelial cells (green arrow). This renal injury is not seen in Figure 11 of the control kidney tissue.

Because of our findings with Western blot analysis of increased levels of cleaved PARP in kidney tissue following cardiac ischemia (Chapter V), we were confident that we would see PARP signaling in the kidney tissue of the cardiac ischemic group, and we do. As with Caspase 3 the cleaved PARP staining was found largely in the proximal tubules of the renal cortex in kidney tissue from animals that underwent cardiac ischemia (Figure 12). In contrast, the renal tissue collected from animals of the control group showed a definite reduction in the amount of PARP staining (Figure 13).

Caspase 3 Found in Cardiac Ischemic Group

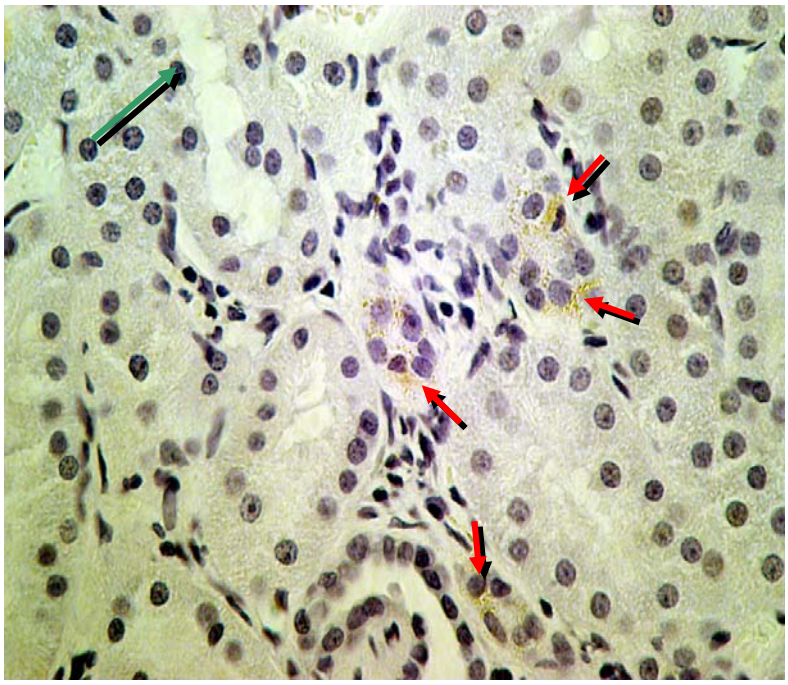

Figure 10. Active caspase 3 after 15 minutes of cardiac ischemia. The red arrows are pointing to the caspase 3 signaling localized to the cortex of the kidney with most activity found predominately in the proximal tubules. The green arrow is pointing to brush border scalloping of proximal tubule epithelial cells indicating the beginnings of renal injury.

Caspase 3 Absent in Control Group

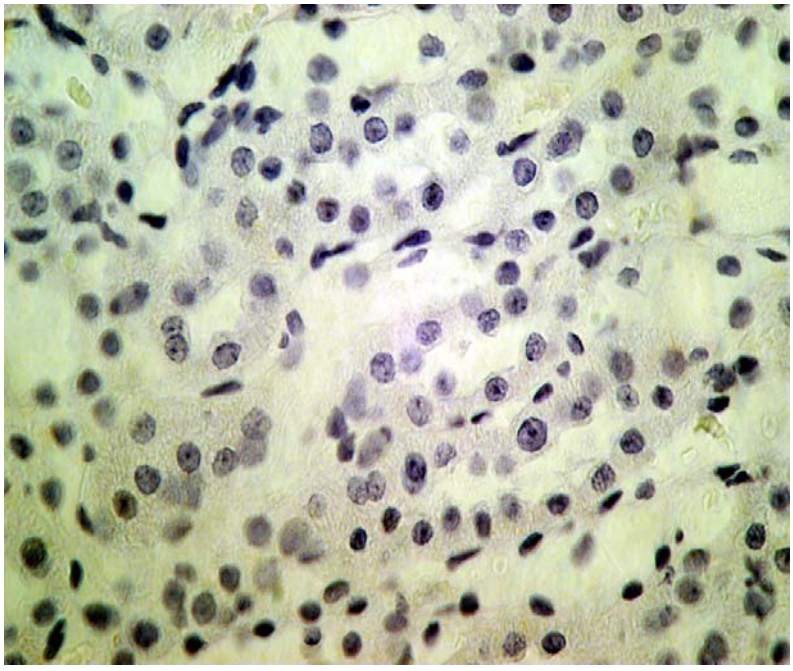

Figure 11. Control tissue stained for active caspase 3. This slide is representative of the overall kidney tissue for the control group. The brown freckling representing activated caspase 3 as seen in the cardiac ischemic kidney tissue (Figure 10) is absent in the control tissue slides. Plus the renal epithelial cells continue to look healthy; no brush border scalloping observed in the cardiac ischemic kidney tissue (Figure 10).

PARP Found in Cardiac Ischemic Group

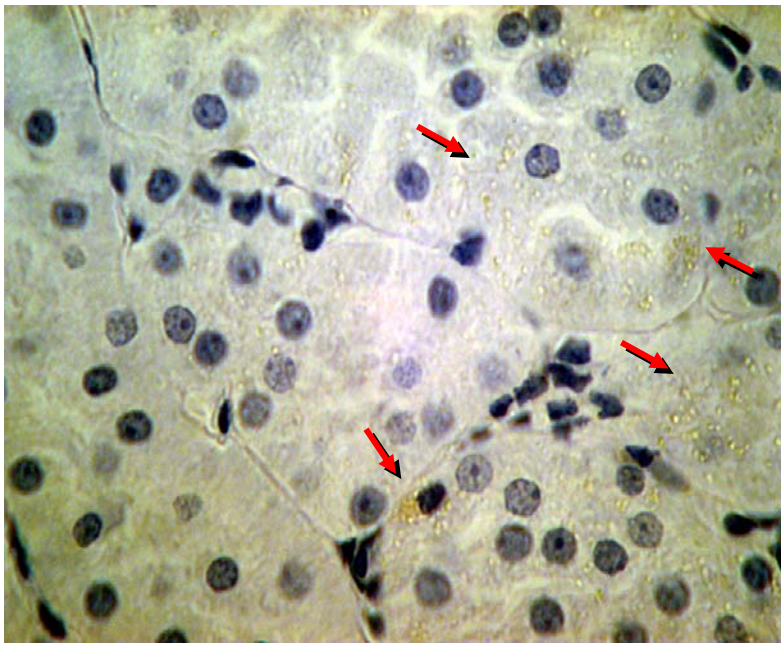

Figure 12. Cleaved PARP after 15 minutes of cardiac ischemia. The red arrows pointing to some of the PARP staining. The staining was mainly seen in the proximal tubules in much the same pattern as that of active Caspase 3 (Figure 10)

PARP Staining Absent in Control Tissue

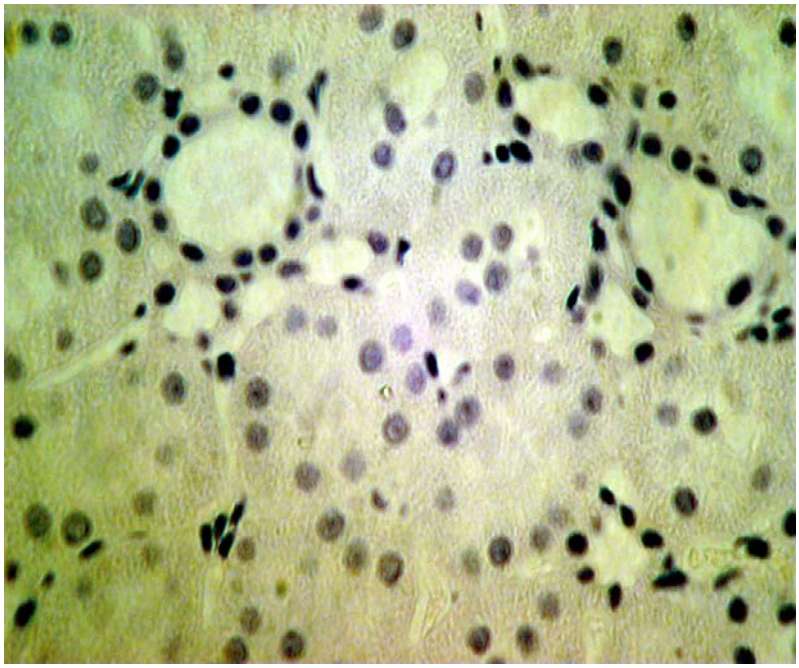

Figure 13. Control tissue stained for cleaved 89 kD fragment of PARP. This slide is characteristic of the overall kidney tissue for the control group. The brown freckling representing cleaved PARP as seen in the cardiac ischemic kidney tissue (Figure 12) is not present in the control tissue slides.

Caspase activation results in cleavage of PARP into two characteristic fragments of molecular mass 85-89 kD and 24-26 kD [20], [50], [56], [2], [49], in our study the paraffin imbedded kidney tissue was probed with the cleaved 89 kD form.

6.4 Discussion

For the first time, these immunohistochemical studies show the renal cells most effected by an injured heart and implicates the kidney being at risk for injury after acute coronary ischemia. Because of the unique ability of the myocardium to release cytokines when injured indicate that these cells can and, as shown by these studies, do increase apoptotic signaling in renal cells. These lethal signals could cause renal cell death or dysfunction.

The injury to the kidney caused by cardiac ischemia appears to focus in the proximal tubules of the renal cortex. The apoptotic signally discovered in our studies were not global but found in small patches of proximal tubules throughout the cortical portion of the kidney. The extent of the injury we see after only 15 minutes of cardiac ischemia would probably not have much of an effect on a healthy kidney, but for diseased or aged kidneys having already lost some of their function this could be critical.

In a preliminary study done in our lab, we prepped kidney tissue for immunohistochemistry harvested after one-hour post TNF infusion study. We then probed the tissue with the same active caspase 3 and cleaved PARP as in our cardiac ischemic studies. What we found was renal injury much like that described in the cisplatin study; loss of brush border, cast formation, and dilated tubules [67] (Figure 13).

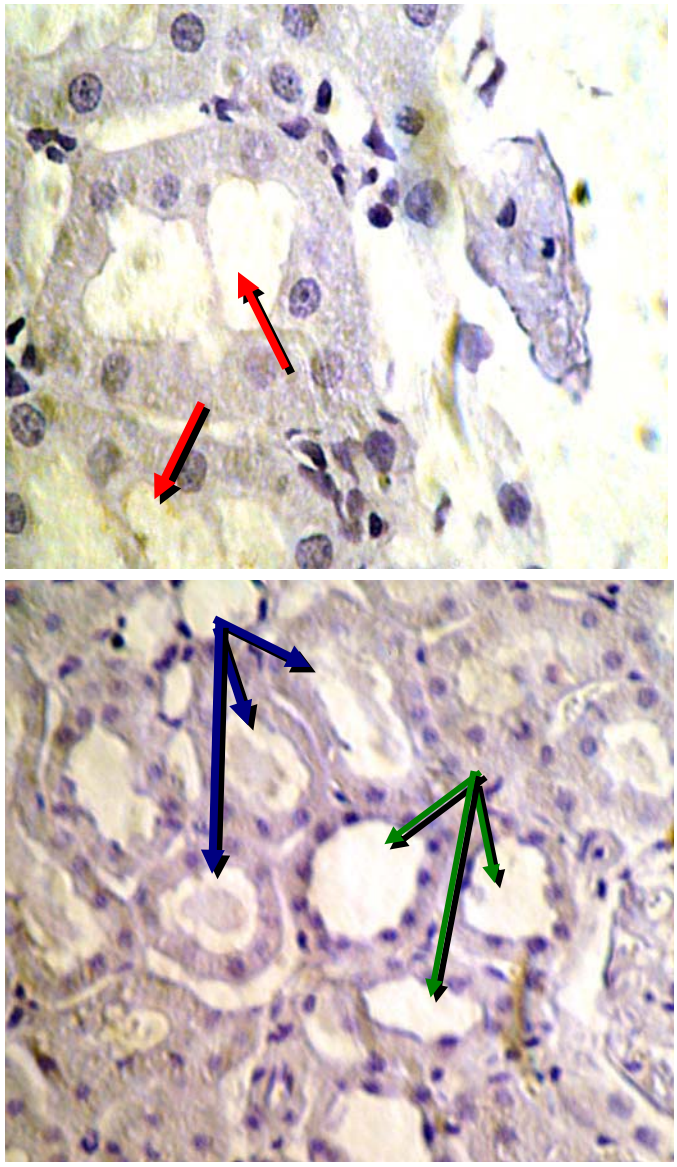

Figure 14. Immunohistochemistry slides showing renal tubular injury post one hour TNF infusion. The injury was localized to the renal cortex. **A**. The red arrows point to proximal tubules that are shedding their brush borders. **B**. The blue arrows point to cast formation and the green arrows point to tubule dilation.

Further immunohistochemical studies need to be done extending the duration of cardiac ischemia to see if it reflects the same type of renal injury our lab found with the one-hour TNF infusion histological studies. Since studies have already shown a correlation between the length of time for myocardial ischemia and the levels of harmful cytokine release [4], [5], additional studies need to be done to measure if the degree of renal injury is amplified as the result of lengthening the period of coronary ischemia.

Chapter VII

APOPTOTIC SIGNALING IN THE KIDNEY AFTER INDUCED CARDIAC ISCHEMIA AND THE ROLE OF TNF IN THESE ACTIVITIES

7.1 Introduction

Cardiorenal syndrome is a term used by clinical researchers who observed that a small percentage of patients suffered from renal dysfunction or renal failure following CPB [6], [3], [4]. This same syndrome has been seen in patients suffering from heart failure [7]. It is also the thought of these researches that TNF plays a major role in renal injury.

The heart has been found to be a major source of TNF. Cardiac myocytes themselves produce TNF, which is nearly evenly distributed between cardiomyocyte and resident cardiac macrophage cell types. Under ischemic stress, the heart can release this TNF causing potential dysfunction to other organs such as the kidneys. Once in the kidney, TNF can be highly destructive by increasing glomerular albumin permeability, causing glomerular fibrin deposition, stimulating cellular filtration, inducing the synthesis of other proinflammatory mediators, and reducing glomerular blood and glomerular filtration rate [3], [68].

To this point, our studies have shown there is a consistent increase in serum TNF levels after cardiac ischemia (Chapter IV: Quantitate TNF Levels), we have seen an approximate 6.75 fold increase over control in levels of PARP in the kidney due to cardiac ischemia (Chapter V: Detection of PARP to Determine Apoptosis in Kidney tissue After Coronary Ischemia), and we have determined histochemically the renal cells most effected by cardiac ischemia (Chapter VI: Renal Cells Effected by Cardiac

Ischemia). The purpose of this study is to look at the consequence of cardiac ischemia on renal tissue as well as investigate the role TNF plays in the process. Renal injury due to cardiac ischemia will be determined via Western blot analysis by looking at apoptotic signaling indicated by increased levels of active caspases 2, 3, and 9 (Aim #4.a.). To investigate the significance of TNF in apoptotic signaling found in the kidney following a cardiac ischemic event we will first inhibit the release of TNF from the injured myocardium via WTACE2, a metalloproteinase inhibitor specific for TACE. Inhibition of TNF is determined by serum levels of TNF tested by an ultrasensitive TNF ELISA kit comparing serum from animals undergoing cardiac ischemia that were injected by WTACE2 to those animals undergoing cardiac ischemia who were injected with the vehicle only. Kidney tissue was harvested from these same animals and prepped for Western blot analyses to measure apoptotic signaling, determined by caspases 2, 3, and 9, as well as cleaved PARP (Aim #4.b.). Comparisons in apoptotic signaling levels are done between the TNF inhibited tissue with tissue that TNF is not inhibited. If TNF has a significant role in cardiorenal syndrome we should see a substantial reduction in apoptotic signaling when TNF is inhibited.

To take the investigation of TNF and its importance in renal injury after cardiac ischemia a step further, we eliminated the cardiac ischemic event. In two groups of six animals we either infused TNF or normal saline. The kidney tissue from both groups was harvested and prepped for Western blot analysis to once again determine apoptotic signaling by measuring active forms of caspases 2, 3, and 9, and cleaved PARP (Aim #4.c). Once more, if TNF is important in the harming of the kidney after acute coronary

ischemia, the TNF infused tissue should show apoptotic signaling patterns similar to that seen in kidney tissue after cardiac ischemia.

7.2 Methods

7.2.1 Myocardial Ischemic Model

Two groups of six adult, male Sprague Dawley rats weighing between 500 to 650 grams were used as a control group or a cardiac ischemic group. Both groups were anesthetized with isoflourane/oxygen (2% volume/volume) at two liters per minute followed by an i.p. injection of ketamine HCl (100 mg/kg of body weight) and zylazine HCl (10 mg/kg of body weight). Both groups had catheters placed in the external jugular vein for intravenous injections of medication if necessary, and the carotid artery for blood draws and monitoring the animals' arterial pressures. Both groups had a tracheotomy performed should assisted breathing be required. With the cardiac ischemic group the animals' chests were opened by way of left thoracotomy and placed on mechanical ventilation. Once the animals' ventilation was maintained the interventricular coronary artery was occluded with a silk suture. After 15 minutes of coronary ischemia the kidneys were isolated, removed, rinsed in chilled normal saline, rapidly frozen in liquid nitrogen and stored at -80°C to be further processed for Western blot analysis. To insure ischemia was accomplished, trypan blue dye was injected into the coronary artery to determine the ischemic area. With the control animals, the kidneys were removed and handled the same as cardiac ischemic kidneys 15 minutes after the placement of carotid artery catheter.

7.2.2 Infused TNF-α Model

One of the methods we used to investigate the significance of TNF in the role of kidney injury due to myocardial ischemia was to infuse TNF without induced ischemia and compare the apoptotic signaling to that of the cardiac ischemic kidney tissue. Two groups of six adult mail Sprague-Dawley rats (500-650g body weight) were anesthetized first with isofluorane/oxygen (2% volume/volume) at 2-liters per minute gases at 1 atmosphere followed by an i.p. injection of ketamine HCl (100 mg/kg body weight) and xylazine HCl (10 mg/kg body weight). Catheters were placed in the external jugular vein for intravenous injections of medications if necessary, and the carotid artery for monitoring the animals mean arterial pressures. A tracheotomy was performed without the animal being placed on mechanical ventilation because animal did not need ventilation assistance since the chest was not opened to perform coronary ligation. 2ng of TNF per 1ml of extracellular fluid was diluted in 2 mls of normal saline and intravenously injected via the external jugular catheter for a period of 15 minutes. Immediately after the TNF infusion the kidneys were harvested, rinsed in chilled normal saline, rapidly frozen in liquid nitrogen, and stored at -80°C for Western blot analysis. We followed the same procedures for the control animals except we infused normal saline only.

7.2.3 Western Blot Analysis

Kidney tissue (stored at –80°C) was pulverized into a powder using liquid nitrogen and placed in a 50 milliliter tube containing lysis buffer (50mM Tris [pH 7.4], 2% Triton-X, 1M NaCl, 5 mM EDTA, 15% glycerol, and 1mM NaF) with protease inhibitors (PI) added (10 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml trypson type II-

O, 10 μg/ml trypsin type II-S, and 10 μg/ml pepstatin, Sigma, St. Louis, MO). Tissue was then homogenized and dounced. Tissue was allowed to incubate in the lysis buffer for two hours on ice to ensure all cell membranes where adequately lysed before centrifuging (10,000 x g) for one hour to eliminate all large particles. Supernatant from centrifuged samples were decanted and place in appropriately labeled tubes. Protein levels of each sample were determined by Bradford protein assay. Protein standard curve was analyzed by version 3.O GraphPad Prism. Proteins were resolved by 4-15% precast SDS polyacrylamide gel (Bio-Rad Laboratories, Chicago, IL) electrophoreses. After electrophoreses, samples were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA) and membranes were allowed to dry. Blots were then incubated with primary antibody (caspase 2, caspase 3, caspase 9—Santa Cruz Biotechnology, Santa Cruz, CA, and cleaved PARP—Cell Signaling Technology, Beverly, MA) blocked with 5% skimmed milk mixed in TBST with .025% Tween 20, for 3 hours to overnight. The blots were than rinsed with TBST and incubated in secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) blocked with 5% skimmed milk mixed in TBST with .025% Tween 20 for one hour and rinsed with TBST. After rinsing the blots were developed with the enhanced chemiluminescence (Amersham Biosciences, England). Exposure time to film (Kodak Scientific Imaging film, Fisher Scientific, Houston TX) was determined by the degree of signal. Density of bands of interest from exposed film were measure by Bio-Rad G-710 Calibrated Imaging Densitometer.

7.2.4 TNF Inhibition

Once again to substantiate the role of TNF in Cardiorenal syndrome we inhibited TNF release by way of WTACE2, a novel metalloproteinase inhibitor specific for TACE,

developed and donated by Wyeth-Ayerst Research (Pearl River, NJ). Two groups of five male Sprague-Dawley rats (200-400 g body weight) were either injected i.p. with a dose of 100 mg/kg of WTACE2 in a vehicle containing 0.5% methocellulose and 2% Tween 80 (Fisher Scientific, Houston, TX) or with the vehicle containing 0.5% methocellulose and 2% Tween 80 only 30 minutes prior to induced cardiac ischemia. After 30 minutes post designated i.p. injection, the animal was made subject to induced ischemia as described above. Also to see what affect the WTACE2 would have on kidney tissue without induced cardiac ischemia we did three control animals where we gave i.p. injection of WTACE2 with described vehicle 30 minutes prior to prepping for a sham control (without the coronary occlusion). After 15 minutes with or without cardiac ischemia, kidneys were harvested, rinsed in chilled normal saline, immediately frozen in liquid nitrogen, and stored at -80°C for future preparation for Western blot analysis.

7.2.5 Ultrasensitive TNF ELISA

To ensure the WTACE2 did in fact lower the serum levels of TNF, from all the above-mentioned animals (cardiac ischemia with WTACE2, cardiac ischemia without WTACE2, and control with WTACE2) there was a blood draw prior to treatment and 15 minutes post treatment. Pre and post treatment blood samples were allowed to clot for 30 minutes, after 30 minutes each sample was centrifuged 1000 x g for 10 minutes. Serum was then removed and place in clean, appropriately labeled tubes. Serum samples were stored at -4°C or immediately tested for TNF levels with the Ultrasensitive TNF ELISA kit. 100 μL of each prepared serum sample was place in its designated well of a 96 well plate from the Ultrasensitive TNF ELISA Kit (Bio Source International, Camarillo, CA) along with the TNF standards provided by the kit and prepared according to the kit's

protocol. After completing the ELISA protocol, the wells used in the 96 well plate were read by Dynatech MR 700 microplate reader. Statistical analysis using Two-way ANOVA with repeated measures is used to determine significance.

7.3 Results

Western blot analysis of cardiac ischemic kidney tissue compared to that of control kidney tissue shows there is an increase of apoptotic signaling in the cardiac ischemic kidney tissue over that of control in as little as 15 minutes. By constant monitoring of the animals' blood pressures throughout the entire procedure, we were confident animal blood pressures fell within parameters of safe MAP to properly maintain perfusion to the kidney. Figure 15 shows a graph of the MAP for the two groups (control and ischemic). The graph demonstrates there is no significant difference between the control group and the ischemic group, but there was a statistical difference in the 15-minute pressure compared to its control pressure within the control group. While there shows to be a statistical difference in this area, we feel there is no biological significance.

Figure 16 shows an example of a Western blot exposed film probed with the primary antibody actin to illustrate even protein loading. This procedure is universally utilized in protein studies to maintain accuracy in the amounts of protein used for each sample when measuring protein levels with Western blot analysis. We used this process for each blot in all of our protein studies. The actin primary antibody used for our studies fell within the 43-45 kD range. Once we probed each blot with actin, the blots were then developed with enhanced chemiluminescence and exposed to film. The density of the

bands of interest from exposed film were measured on a densitometer to calculate equal protein loading.

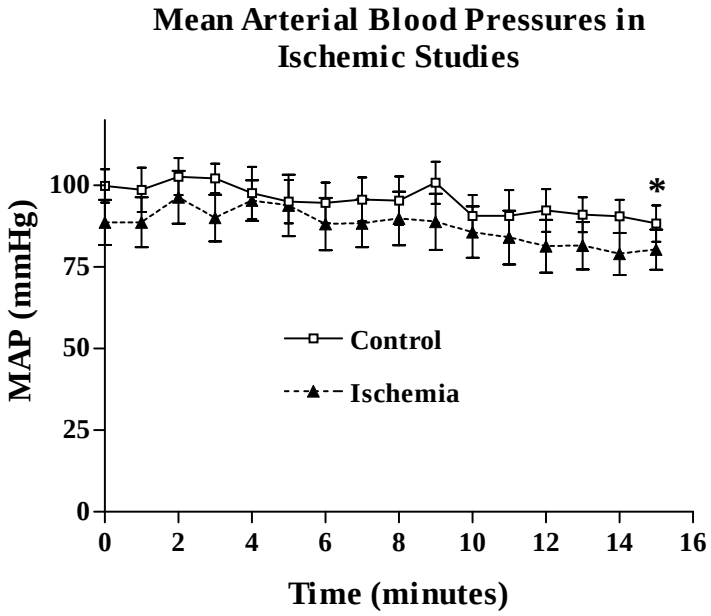

Figure 15. Mean arterial blood pressures for animals in the cardiac ischemic studies showing pressures remaining at a level for the prevention of organ ischemia. . * indicates a statistical difference between the 15 minute MAP compared to its control within the control group. Mean values are ± SEM.

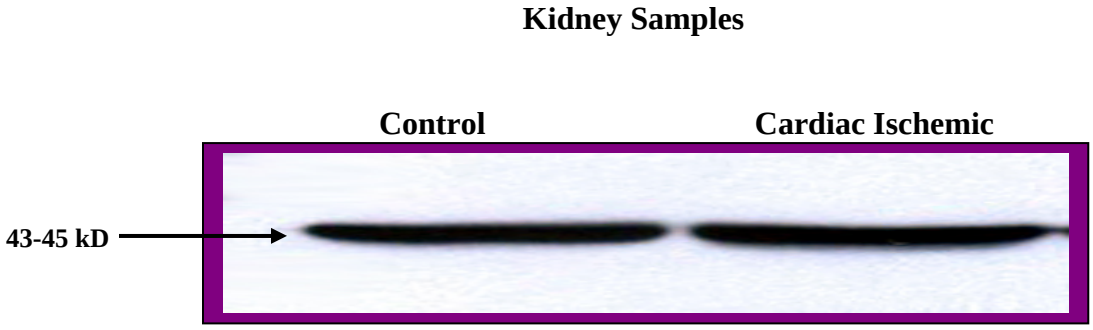

Figure 16. Actin. Western blot exposed film showing even loading determine by probing blot with the primary antibody of actin. This procedure was done for each blot to ensure even loading for the cardiac ischemic studies.

As shown in Figure 1, caspase 3 cleavage and subsequent activation is targeted by caspase 8, caspase 2, and caspase 9 complex suggesting that caspase 3 plays a dominate role in apoptosis [47], [2], [22], [48], [20]. Western blot

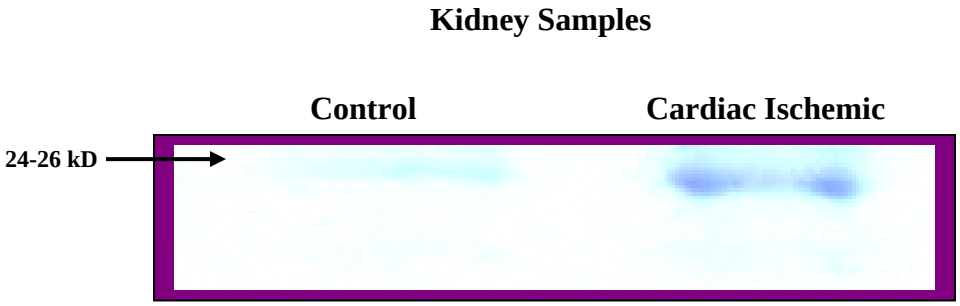

Figure 17. Caspase 2. Western blot exposed film showing the 24-26 kD band for Caspase 2 with cardiac ischemic kidney tissue signal being higher than that of control kidney tissue.

analysis of caspase 3 (Figure 19) in our investigation shows there is an increase in caspase 3 in cardiac ischemic kidney tissue over the control kidney tissue. This is a similar pattern to what we see with levels of caspase 2. Figure 17 shows an example of Western blots of caspase 2. The activated caspase 2 fragment falls in the range of 24-26 kD, and as seen in Figure 17, activated caspase 2 levels are higher in cardiac ischemic renal tissue than that of control. These blots illustrate there is an increase in caspase 2 signaling with a mean densitometer reading of 8.6 in the cardiac ischemic tissues over that of the 1.2 mean reading of control tissues, producing an approximate 7 fold increase (Figure 18).

The caspase 3 primary antibody used for these studies was the activated 17 kD caspase 3. Figure 19 shows an example of the Western blot analysis for caspase 3 where there is a distinct increase in the levels of active caspase 3 in the renal tissues from the cardiac ischemic group over that of the control group. The mean densitometer reading for cardiac ischemic kidney tissue was 4.6 compared to the mean reading for control kidney tissue of 1.4. This gives an approximate 3.3 fold increase in caspase 3 signaling in the cardiac ischemic tissue over the control tissue (Figure 20).

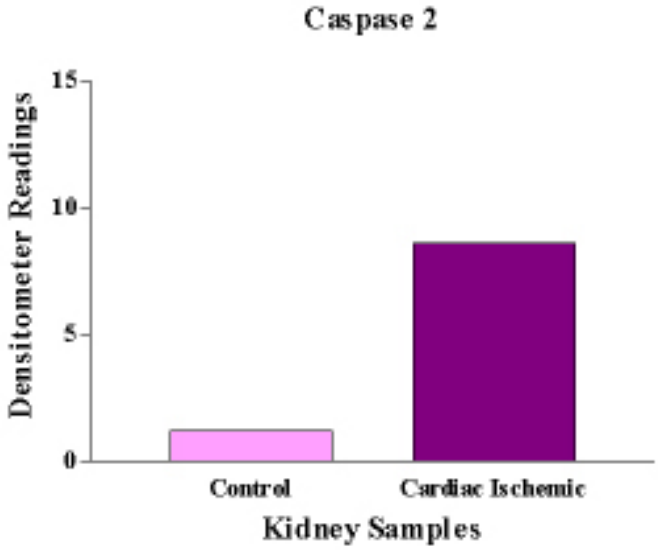

Figure 18. Caspase 2 shows a mean densitometer reading of 8.6 for the cardiac ischemic kidney tissue over that of mean readings of 1.2 for control kidney tissue producing an approximate 7 fold increase of Caspase 2 signaling in the kidney due to cardiac ischemia.

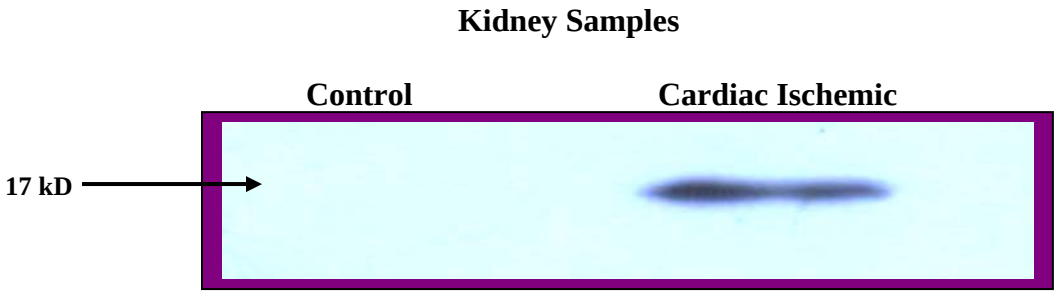

Figure 19. Caspase 3. Western blot exposed film showing the 17 kD band indicative of activated Caspase 3. The cardiac ischemic kidney samples clearly have a strong caspase 3 signal compared to that of the control kidney samples.

In order to examine if there is any mitochondrial amplification in the apoptotic signaling found in kidneys for our cardiac ischemic research model, we looked at caspase 9. For caspase 9 to be activated it must be combined with the released mitochondrial cytochrome *c* along with the cofactor Apaf-1 (Figure 1). Once caspase 9 has complexed with cytochrome *c* and Apaf-1 (also known as an aposome) it can then activate caspase 3. With Western blot analysis active forms of caspase 9 can be seen by

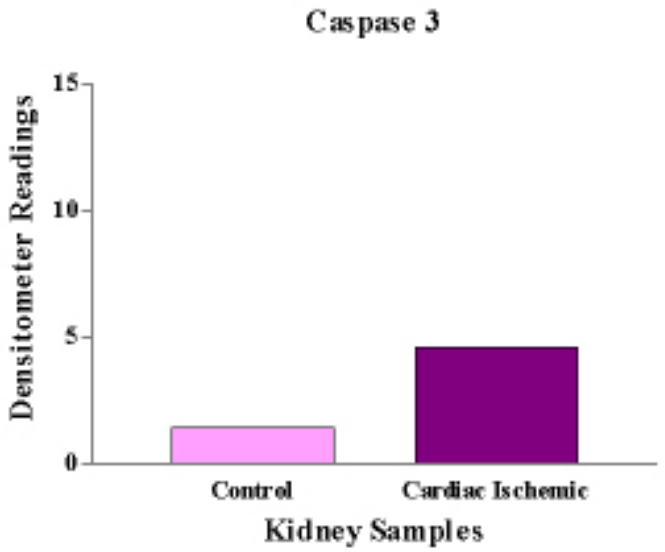

Figure 20. Caspase 3 shows a mean densitometer reading of 4.6 for the cardiac ischemic kidney tissue compared with the mean reading of 1.4 for the control kidney tissue giving an approximate 3.3 fold increase in caspase 3 signaling in the cardiac ischemic samples over that of the control samples.

bands at 10, 35, and 37 kD. Studies show the 35 kD fragment is associated with the complex of cytochrome *c* and Apaf-1 and the 37 and 10 kD fragments are generated by activated caspase 3 [69], [50].

Our studies did in fact find there is increased mitochondrial apoptotic amplification in the cardiac ischemic renal tissue over that of control as signified by the increase in activated caspase 9. Figure 21 shows an example of Western blots in our investigation demonstrating the marked increase in active caspase 9 (37 and 35 kD) signaling in the cardiac ischemic renal tissue over that of control. When these blots were read on a densitometer, the mean reading for caspase 9 for the cardiac ischemic kidney tissue is 8.4 compared to the densitometer reading of 2.6 for the control kidney tissue. This gives an approximate 3.2 fold increase in active caspase 9 for the cardiac ischemic tissue over that of the control tissue illustrated in the graph shown in Figure 22.

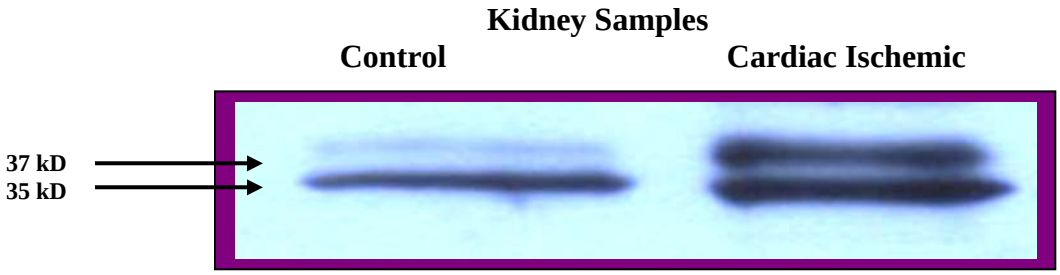

Figure 21. Caspase 9. Western blot exposed film showing the 35 and 37 kD band both active forms of caspase 9. The 35 kD active form of caspase 9 is processed by the release of cytochrome *c* from the mitochondria and complexing with Apaf-1, and the 37 kD active form is mediated by caspase 3. Both the 35 and 37 kD bands of the cardiac ischemic kidney tissue show increased signaling greater than that of control kidney tissue.

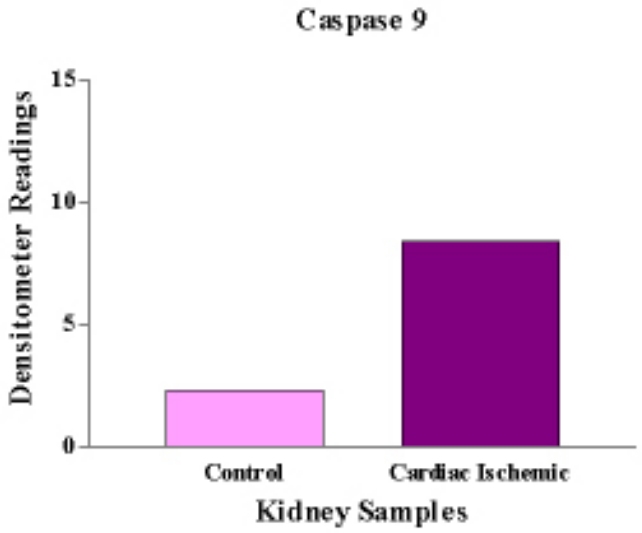

Figure 22. Caspase 9 shows a mean densitometer reading of 8.4 for the cardiac ischemic kidney tissue over that of the mean reading of 2.6 for the control kidney tissue producing an approximate 3.2 fold increase of caspase 9 signaling in the kidney due to cardiac ischemia.

In order to investigate the significance of TNF in apoptotic signaling found in the kidney following a cardiac ischemic event, we used WTACE2, a metalloproteinase inhibitor specific for TACE (the converting enzyme for TNF). To insure that the release of TNF was in fact inhibited by WTACE2, serum samples pre and post cardiac ischemia were taken and measured for levels of TNF in the same fashion as the TNF quantitative studies of Chapter IV. The ELISA studies were done prior to prepping harvested kidney

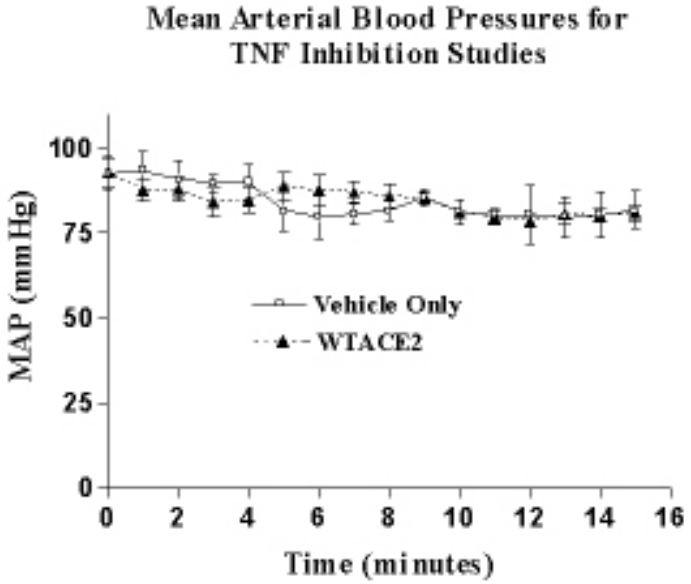

Figure 23. Mean blood pressures for animals in the WTACE2 studies showing pressures remained at a level to prevent organ ischemia. These animals were used for TNF inhibition ELISA studies and the TNF inhibition Western blot analysis. Mean values are ± SEM.

tissue for Western blot analysis to look for apoptotic signaling. During each assigned treatments there was constant monitoring of the animals' blood pressures throughout the entire procedure. Figure 23 shows a graph representing the MAP for animals in the WTACE2 studies. There is no statistical significant difference of MAP between the vehicle only or the WTACE2 groups, nor was there a significant difference within the groups. We were confident that animal blood pressures remained in a range preventing any organ damage due to ischemia.

The bar graph seen in Figure 24 represents the TNF levels found in serum collected from two groups of five rats; one group receiving i.p injection of WTACE2 and the other group receiving vehicle only. Both groups were made subject to cardiac ischemia. It is clear in Figure 24 the levels of TNF with the WTACE2 group remained low after cardiac ischemia, showing numbers similar to that of the pretreatment

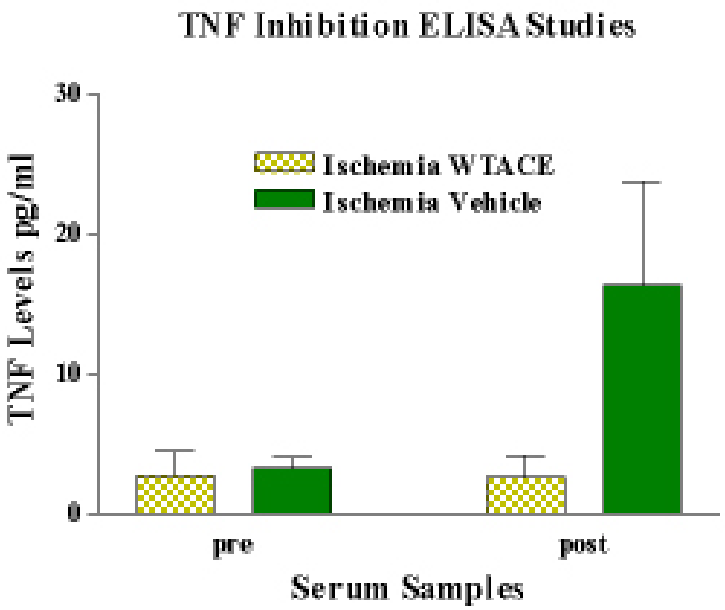

Figure 24. Bar graph representing the TNF levels found in serum collected from two groups of five rats, with one group receiving the TNF release inhibitor and the other group receiving the vehicle only. Both groups where made subject to cardiac ischemia. It is clear with these results that levels of TNF with the WTACE2 group remained low after cardiac ischemia, similar to the pretreatment serum samples. Where as levels of TNF clearly went up after cardiac ischemia in the group not receiving WTACE2. These TNF ELISA studies show that WTACE2 was successful in preventing the release of TNF after cardiac ischemia. Pre represents the serum samples that were taken prior to induced coronary ischemia, and post represents the serum samples that were taken 15 minutes after the tie off of the coronary occlusion. Mean values are ± SEM.

serum samples. On the other hand, the animals receiving the vehicle only had an increase in post cardiac ischemic TNF serum levels over the pre treatment levels. We feel confident that this study demonstrates WTACE2 inhibited the release of TNF.

These ELISA studies, like the studies in Chapter IV, showed very low levels of TNF in the serum samples, but we did find increases that were consistent in serum samples collected after cardiac ischemia with the group who received vehicle only. Also seen in these studies, which mirrored the ELISA studies in Chapter IV, was the greater the area of ischemia—visually defined by the injection of trypan blue dye—the higher the

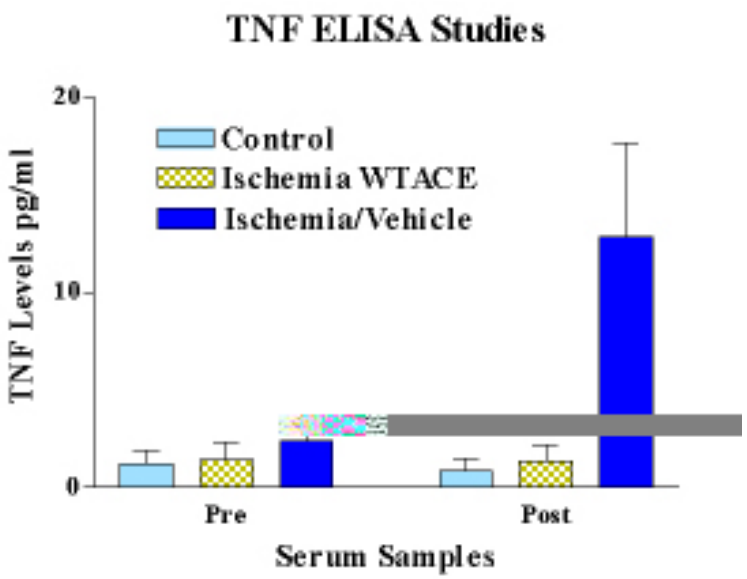

Figure 25. Graph showing the pre treatment serum samples of the WTACE2 studies resemble the TNF levels of the control serum sample from the TNF quantitative studies of Chapter IV. You can also see where the post TNF levels in the WTACE2 post cardiac ischemic samples show TNF levels to be like the control sample. The pooled data of vehicle only and ischemic (Chapter IV) studies show that the WTACE2 TNF serum levels are significantly lower than that of serum levels from cardiac ischemia without inhibition. These comparisons illustrate that indeed WTACE2 decreased the release of TNF due to cardiac ischemia**.** Mean values are ± SEM, $P > 0.05$.

levels of TNF in the serum. This was responsible for the large standard deviation seen in the post ischemia serum samples with the vehicle only group as was seen with the ischemic group in the TNF ELISA studies in Chapter IV. Again close to significance, this same large variability prevented our results from being significant along with reducing the power of our study. As explained in Chapter IV, a lowered power can increase the likelihood of committing a Type II statistical error. To once again to avoid this we pooled the data from our ischemic studies (Chapter IV) using the cardiac ischemic data that mirrored the vehicle only data. The graph of Figure 25 represents the control data of Chapter IV, WTACE2 data, and the pooled data of ischemic and vehicle only studies. This graph demonstrates that the levels of TNF in those animals receiving treatment with WTACE2 are significantly ($P > 0.05$) lower than the ischemic/vehicle

Samples	Mean Densitometer Readings			
	Caspase 2	Caspase 3	Caspase 9	PARP
w/WTACE	2.4	4.3	3.9	1.5
w/Vehicle	3.3	7.5	5.1	2.8
Ischemia	8.6	4.6	8.4	5.4
Control	1.2	1.4	2.6	.8
Con/WTACE	1.7	2.2	2.5	.8

Table 1. Mean densitometer readings of Western blot analysis of cardiac ischemic kidney tissue with WTACE2 (w/WTACE), cardiac ischemic kidney tissue with vehicle only (w/Vehicle), and the regular cardiac ischemic kidney tissue (ischemia). The table illustrates there is a decrease in apoptotic signaling due to TNF inhibition (w/WTACE), but not as low as either the control kidney tissue for the ischemic studies (Control) or the control with WTACE2 (Con/WTACE). This table also points out that the two control tissue mean densitometer readings are very similar, with PARP being the same indicating WTACE has no effect on renal tissue alone

TNF serum levels. Also WTACE2 TNF serum levels are very similar to that seen with TNF levels found in the control animals shown in the TNF quantitative studies in Chapter IV.

Convinced that indeed TNF was inhibited, Western blot analysis studies were done on the kidney tissue harvested from the same animals in the TNF inhibition ELISA studies. To ensure the WTACE2 had no effect on kidney tissue, assays were done on a group of three rats that were i.p. injected with WTACE2. After 15 minutes post placement of carotid artery catheter, without inducing cardiac ischemia, the kidneys were harvested for Western blot analysis to be included with the other animal tissues in the inhibition studies.

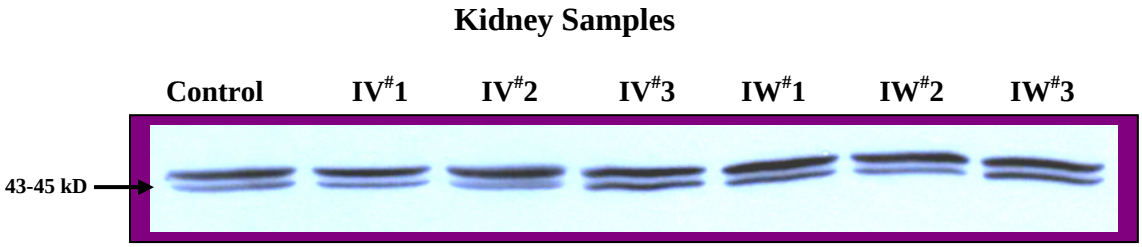

Figure 26. Western blot exposed film showing even loading determined by probing blot with the primary antibody of actin. This procedure was done for each blot to ensure even loading for the WTACE2 studies. IV—cardiac ischemic kidney tissue with i.p. injected vehicle only; IW—cardiac ischemic kidney tissue with i.p. injected with WTACE2.; Control—kidney tissue i.p. injected with WTACE2 without induced cardiac ischemia.

Interestingly what we found was that although the release of TNF was inhibited there was however, an increase in the levels of apoptotic signaling, but according to densitometer readings of these Western blots seen in Table 1 the signaling, did not reach those levels seen with the cardiac ischemic animals receiving vehicle only or the animals from the cardiac ischemic studies. Also notable, are the comparisons of the densitometer readings for control kidney tissues from the ischemic studies (of this chapter) and the control kidney tissues with WTACE2. The mean densitometer readings were very similar, with PARP readings being the same. We feel these readings indicate that WTACE2 had no direct effect on the kidney tissue.

Figure 26 shows an example of a Western blot exposed film probed with the primary antibody actin to illustrate even protein loading. This procedure was done on each blot for the WTACE2 studies. The actin primary antibody used for our studies fell within the 43-45 kD range. Once we probed each blot with actin, the blots were then developed with enhanced chemi-luminescence and exposed to film. The densities of the bands of interest from exposed film were measured on a densitometer to calculate equal protein loading.

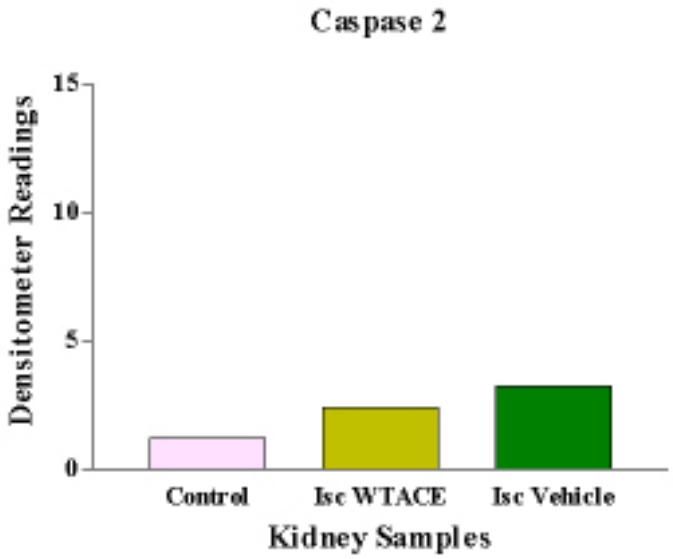

Figure 27. Bar graph representing the mean densitometer readings of Western blots for caspase 2. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of WTACE2 (Isc WTACE) had a mean densitometer reading of 2.4, an approximate 2 fold increase of caspase 2 levels over control. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of vehicle only (Isc Vehicle) had a mean densitometer reading of 3.3, an approximate 2.7 fold increase in caspase 2 levels over control. This indicates there is still apoptotic signaling even though TNF release has been inhibited, but not to the degree seen when TNF release is not restricted.

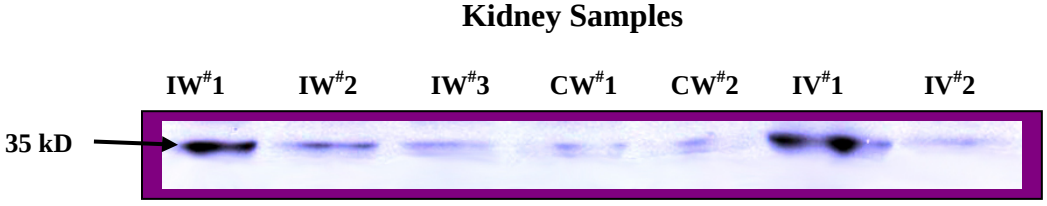

Figure 28. Caspase 2. Western blot exposed film showing the 35 kD band for active caspase 2. This blot illustrates there is still apoptotic signaling in the kidney after only 15 minutes of cardiac ischemia although the release of TNF has been inhibited. This blot is also a good example of how apoptotic signaling increased due to a greater area of cardiac ischemia—visually defined by the injection of trypan blue dye. IW#1 and IV#1 had a large area of cardiac ischemia where IV#2 had a much smaller area and the caspase 2 levels look much like the controls. IW—cardiac ischemic kidney tissue with WTACE2; CW—kidney tissue i.p. injected with WTACE2 without induced cardiac ischemia; IV—cardiac ischemic kidney tissue with vehicle only.

Caspase 2 Western blot analysis (Figure 27) showed a mean densitometer reading of 2.4 for cardiac ischemic tissue with WTACE2 producing an approximate 2-fold increase over that of control from the ischemic studies with a mean densitometer reading of 1.2. We continued to use the densitometer readings from the controls of the ischemic studies to create a standard by which to measure the densitometer readings for all the WTACE2 Western blot assays. There was a mean densitometer reading of 3.3 for caspase 2 with the cardiac ischemic kidney tissue i.p. injected with just vehicle only producing an approximate 2.8 fold increase over control kidney tissue. Western blot analysis of caspase 2 shows that although the release of TNF was inhibited, there is still apoptotic signaling over that of control. But as can be seen by comparing the WTACE2 kidney tissue with the vehicle only kidney tissue, there is not as great a caspase 2 signal when the release of TNF is inhibited. Figure 28 shows an example of the Western blots done on caspase 2 for this study. Seen with this example, there is still apoptotic signaling in the kidney although the release of TNF has been inhibited. The blot in Figure 28 demonstrates how apoptotic signaling increases due to a greater area of cardiac ischemia—visually defined by the injection of trypan blue dye. Tissue from kidney samples IW$^{\#}$1 and IV$^{\#}$1 had a large area of cardiac ischemia where IV$^{\#}$2 had a much smaller area, and therefore, the caspase 2 levels look much like the controls. Similar occurrences happened with the other apoptotic signaling indicators of caspase 3, caspase 9, and cleaved PARP.

Figure 29 gives an example of Western blots using the primary antibody active caspase 3 showing a band at the level of 17 kD. This blot, like that of the blots of caspase 2, illustrates there is still apoptotic signaling in the kidney after cardiac ischemia although

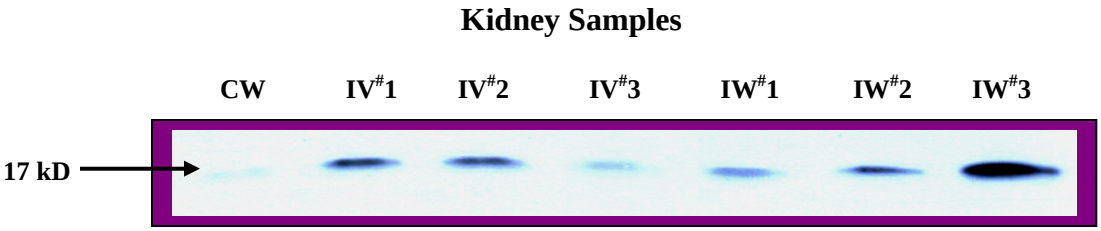

Figure 29. Caspase 3. Western blot exposed film showing the 17 kD band for active caspase 3. This blot illustrates there is still apoptotic signaling in the kidney after only 15 minutes of cardiac ischemia although the release of TNF has been inhibited. IW—cardiac ischemic kidney tissue with WTACE2; CW—kidney tissue i.p. injected with WTACE2 without induced cardiac ischemia; IV—cardiac ischemic kidney tissue with vehicle only.

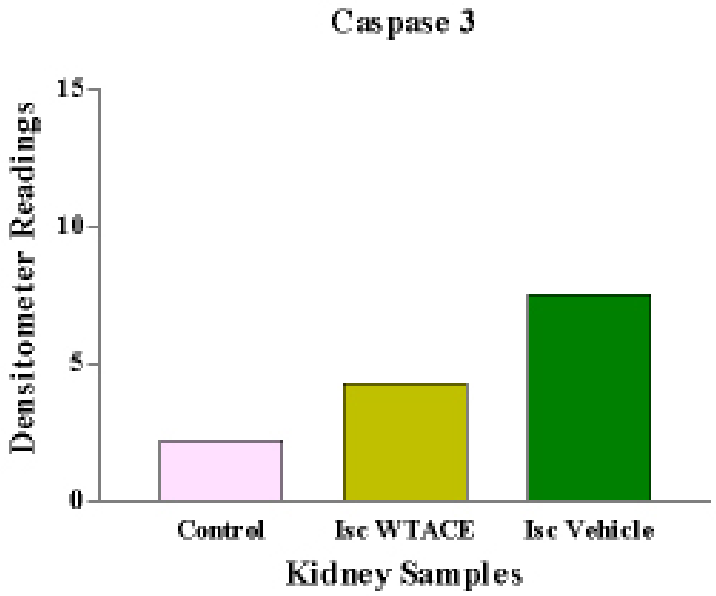

Figure 30. Caspase 3. Bar graph representing the mean densitometer readings of Western blots for caspase 3. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of WTACE2 (Isc WTACE) had a mean densitometer reading of 4.3, an approximate 3 fold increase of caspase 3 levels over control. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of vehicle only (Isc Vehicle) had a mean densitometer reading of 7.5, an approximate 5.4 fold increase in caspase 3 levels over control. As with caspase 2, results of Western blot analysis of caspase 3 indicates there is still apoptotic signaling even though TNF release has been inhibited, but not to the degree seen when TNF release is not restricted.

the release of TNF has been inhibited with WTACE2. And as seen in Figure 30 the bar graph of densitometer readings of Western blots probed with active caspase 3, while there is an increase in active caspase 3 levels in the TNF inhibited group over control, it does not equal that of the vehicle only group. The mean densitometer reading for WTACE2 injected group is 4.3; an approximate 3 fold increase of caspase levels over the mean control densitometer reading of 1.2. The levels of TNF were higher in the vehicle only group showing a mean densitometer reading of 7.5, and approximate 5.4 fold increase in caspase 3 levels over control.

The Western blot of caspase 9 shown in Figure 31 is an example of the Western blots ran and probed with activated caspase 9 for these studies. Much like that seen with both caspase 2 and 3, regardless of TNF inhibition with WTACE2, there is still active caspase 9 as indicated by the signaling seen at 37 and 35 kD. The bar graph in Figure 32 shows the densitometer readings of these same Western blots. The kidney samples from animals that underwent induced cardiac ischemia after WTACE2 injections had a mean densitometer reading of 3.9, an approximate 1.5 fold increase of the control densitometer reading of 2.6. Kidney samples from animals that underwent induced cardiac ischemia after injections of vehicle only had a mean densitometer reading of 5.1, and approximate 2 fold increase in caspase 9 levels over control.

An example of Western blots probed with cleaved PARP (an early marker for apoptosis) is seen in Figure 33. As mentioned in Chapter V and shown in Figure 3 of Chapter II, PARP is cleaved during apoptosis to form 89 kD and 24 kD fragments. The PARP Western blot example in Figure 33 shows the 24 kD band. This blot, as well as the other blots done in these studies, illustrates there is apoptotic signaling in kidneys after 15

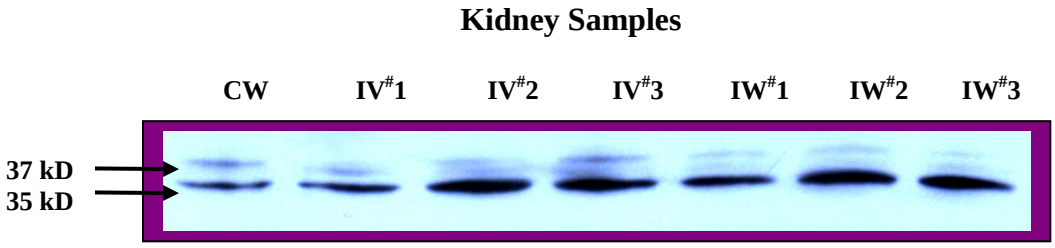

Figure 31. Caspase 9. Western blot exposed film showing the 37 and 35 kD bands for active caspase 9. This blot as well illustrates there is apoptotic signaling in the kidney after only 15 minutes of cardiac ischemia regardless of the inhibition in the release of TNF. Also the presence of caspase 9 indicates the apoptotic amplification via the mitochondria. IW—cardiac ischemic kidney tissue with WTACE2; CW—kidney tissue i.p. injected with WTACE2 without induced cardiac ischemia; IV—cardiac ischemic kidney tissue with vehicle only.

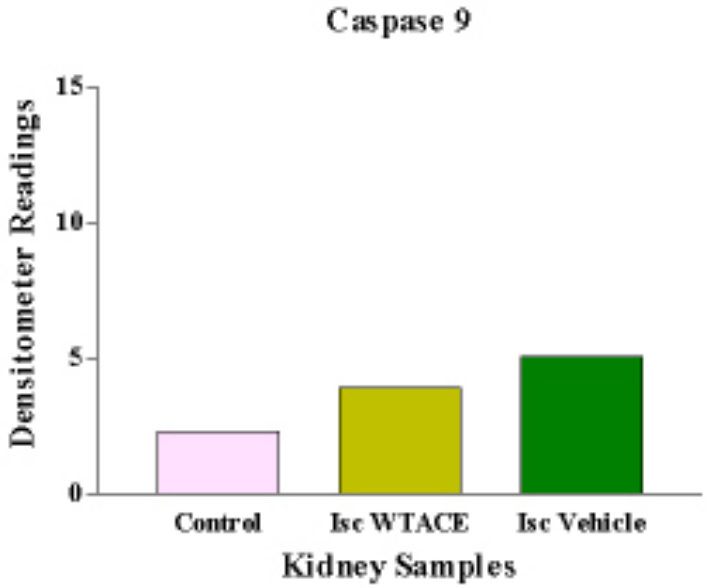

Figure 32. Bar graph representing the mean densitometer readings of Western blots for caspase 9. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of WTACE2 (Isc WTACE) had a mean densitometer reading of 3.9, an approximate 1.5 fold increase of caspase 9 levels over control. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of vehicle only (Isc Vehicle) had a mean densitometer reading of 5.1, an approximate 2 fold increase in caspase 9 levels over control. As with caspase 2 and 3, results of Western blot analysis of caspase 9 indicates there is still apoptotic signaling although TNF release has been inhibited, but not to the degree seen when TNF release is not restricted.

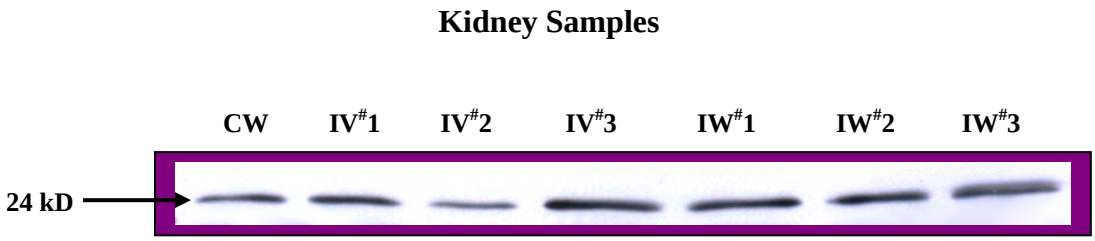

Figure 33. PARP. Western blot exposed film showing the 24 kD band for the cleaved fragment of PARP. This blot as well illustrates there is apoptotic signaling in the kidney after only 15 minutes of cardiac ischemia regardless of the inhibition in the release of TNF. IW—cardiac ischemic kidney tissue with WTACE2; CW—kidney tissue i.p. injected with WTACE2 without induced cardiac ischemia; IV—cardiac ischemic kidney tissue with vehicle only.

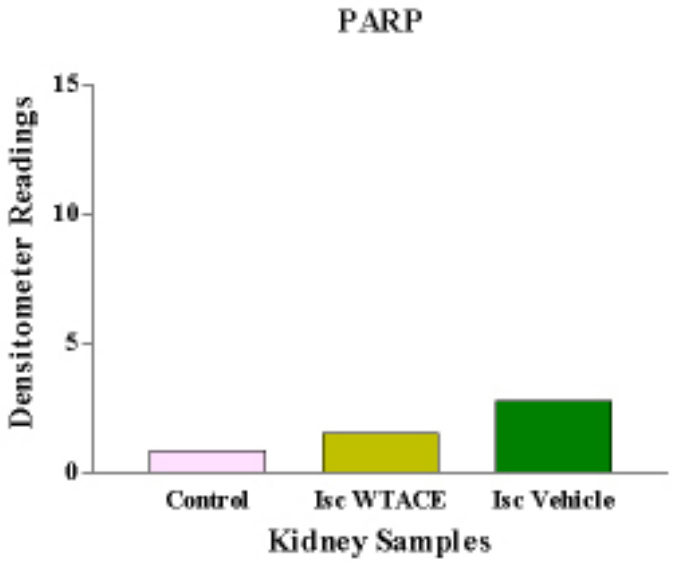

Figure 34. Bar graph representing the mean densitometer readings of Western blots for PARP. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of WTACE2 (Isc WTACE) had a mean densitometer reading of 1.5, an approximate 2 fold increase of cleaved PARP levels over control. Kidney samples from animals that underwent induced cardiac ischemia after i.p. injections of vehicle only (Isc Vehicle) had a mean densitometer reading of 2.8, an approximate 3.5 fold increase in cleaved PARP levels over control. As with caspase 2, 3, and 9 results of Western blot analysis of PARP indicates there is still apoptotic signaling although TNF release has been inhibited, but not to the degree seen when TNF release is not restricted.

minutes of cardiac ischemia regardless of the inhibition of TNF release. The mean densitometer readings of these Western blots are represented in the bar graph in Figure 34. Kidney samples of animals that experienced cardiac ischemia after WTACE2 injections had a mean densitometer reading of 1.5, an approximate 2 fold increase of cleaved PARP levels over the control mean densitometer of .8. Kidney samples from animals that experienced cardiac ischemia after injections of vehicle only had a mean densitometer reading of 2.8, and approximate 3.5 fold increase in cleaved PARP over control. These results of PARP reflect that seen with caspase 2, 3, and 9 where apoptotic signaling is still apparent after the inhibition TNF, but not to the degree seen when TNF release is not restricted.

To continue the investigation on the importance of TNF in cardiorenal syndrome, we eliminated the cardiac ischemic event and i.p. injected TNF with two milliliters of normal saline in one group of rats, or i.p. injected simply two milliliters of normal saline in a second group of rats. It was decided to do this study because the myocardium is known for releasing more then just the cytokine TNF [3], [4], [5]. The infusion of TNF with out an ischemic event will give a clearer picture of the role TNF plays in injury to the kidney since none of the other cytokines will be released as a result of cardiac ischemia.

MAP were continually monitored on all the animals in the TNF infusion studies. Figure 35 shows the graph of the MAP for the two groups (control and TNF infused) showing there is no statistical significant difference between or within the two groups. Blood pressures in these animals stayed at a level that we were confidant organ ischemia was prevented.

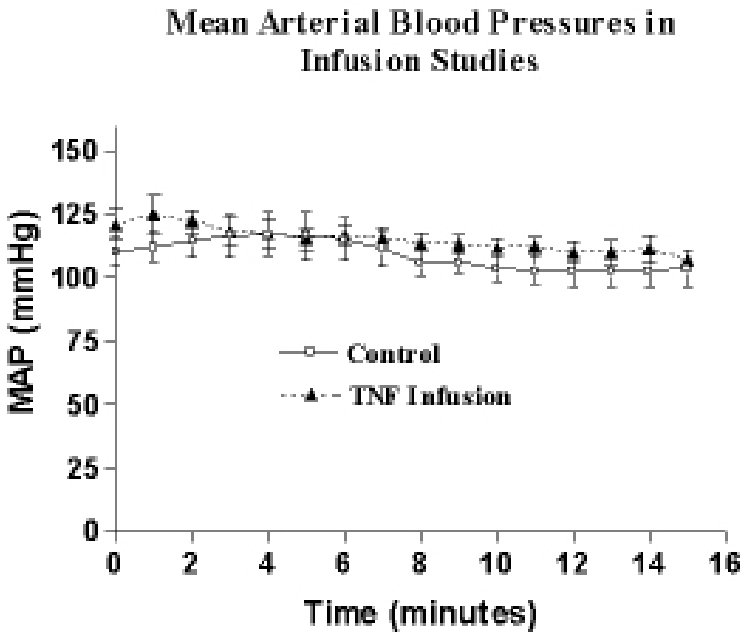

Figure 35. Mean blood pressures for animals in the TNF infusion studies. As can be seen there is no significant difference between the control animal and the TNF infused animals. MAP remained in a range where there was proper perfusion to the kidneys. Mean values are ± SEM.

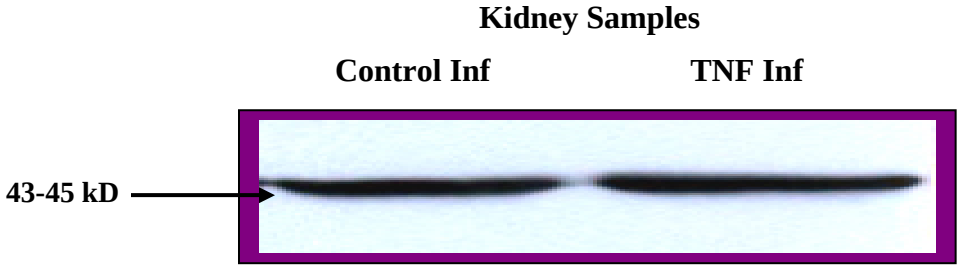

Figure 36. Western blot exposed film showing even loading determine by probing blot with the primary antibody of actin. This procedure was done for each blot to ensure even loading for the TNF infusion studies.

Samples	Mean Densitometer Readings			
	Caspase 2	**Caspase 3**	**Caspase 9**	**PARP**
Control	1.2	1.4	2.6	.8
Inf Control	1.6	.9	3.9	1.6
Ischemic	8.6	4.6	8.4	5.4
TNF Inf	4.0	1.3	4.7	3.4

Table 2. Mean densitometer readings of Western blot analysis of kidney tissue with no treatment (Control), kidney tissue from animals infused with normal saline alone (Inf Control), cardiac ischemic kidney tissue (Ischemic), and kidney tissue from animals infused with TNF (TNF Inf). This table illustrates there is an increase in apoptotic signaling due to TNF infusion over control, but not to the degree seen with kidney tissue from animals who underwent coronary occlusion.

Figure 36 shows an example of a Western blot exposed film probed with the primary antibody actin to illustrate even protein loading. As with our other studies, the density of the bands of interest from exposed film were measured on a densitometer to calculate even loading. This procedure was done on each blot for the TNF infusion studies.

Western blot analysis of kidney samples from the TNF infusion studies showed there is some apoptotic signaling due to TNF alone, but not to the degree found in the cardiac ischemic kidney tissues seen in the cardiac ischemic assays of this chapter. These results suggest that TNF is not the only source for increased apoptosis in the kidney due to cardiac ischemia. Table 2 shows the mean densitometer readings from both cardiac ischemia and TNF infusion studies. Interestingly, these results closely resemble that of the WTACE2 studies (Table 1): when TNF is inhibited there is a decrease in apoptosis activity again indicating that TNF is not the only influence driving apoptotic signaling in the kidney after cardiac ischemia.

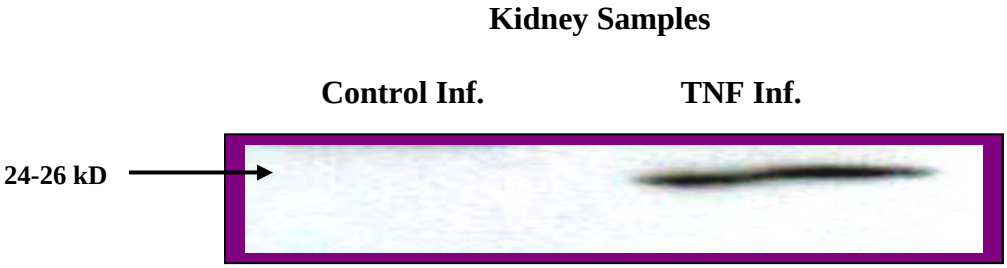

Figure 37. Western blot exposed film showing the 24-26 kD band for active caspase 2. This blot illustrates there is apoptotic signaling in the kidney tissue from animals infused with TNF (TNF Inf.), and it is clear that the caspase 2 signaling is greater than that of the animals infused with normal saline alone (Control Inf.).

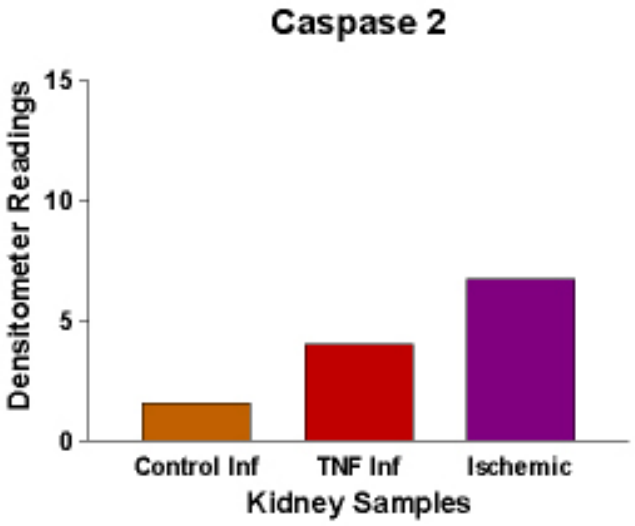

Figure 38. Bar graph representing the mean densitometer readings of Western blots for caspase 2. Kidney samples from animals that were infused with TNF (TNF Inf) had a mean densitometer reading of 4.0, an approximate 2.5 fold increase of caspase 2 levels over control kidney tissue (Control Inf) of animals infused with normal saline alone with a mean densitometer reading of 1.6, but there is not the degree of caspase 2 signaling as that seen in cardiac ischemic kidney samples (Ischemic) showing a mean densitometer reading of 8.6. These results indicate that although TNF does play a role in apoptotic signaling in the kidney after cardiac ischemia it is not the sole participant.

An example of a caspase 2 Western blot exposed to film is shown in Figure 37. The activated fragments of caspase 2 fall between 24-26 kD, and as illustrated in this Western blot, there is a distinct band with TNF infusion compared to an undetectable band with the kidney tissue taken from animals injected with normal saline alone. The caspase 2 Western blots of the TNF infusion studies look very similar to that found in the caspase 2 Western blots of the ischemic studies of this chapter, but the caspase 2 signal read by the densitometer is not as strong with the TNF infusion studies as that of the ischemic studies shown in Figure 38. Caspase 2 Western blot analysis for the TNF infusion studies shows an increase in a mean densitometer reading of 4.0 compared to the control mean densitometer reading of 1.6, producing an approximate 2.5 fold increase in caspase 2 levels in the TNF infused kidney samples over that of the infusion control tissues. This caspase 2 increase did not equal the 7-fold increase in the cardiac ischemic kidney tissue over control.

As mentioned before, caspase 3 plays a dominate role in apoptosis. It is the target of caspase 8, caspase 2, and caspase 9 complex shown in Figure 1 [47], [2], [22], [48], [20]. Figure 39 illustrates an example of a Western blot exposed film showing the 17 kD band for active caspase 3, and like caspase 2, this blot indicates a substantial increase in caspase 3 levels in the kidney tissue from animals infused with TNF over that of tissue from animals infused with normal saline alone. Mean densitometer reading of the Western blots for caspase 3 with these same kidney tissue samples is 1.3. This is an approximate 1.5 fold increase of caspase 3 levels over kidney tissue of animals infused with normal saline alone having a mean densitometer reading of 0.9 (Figure 40). But

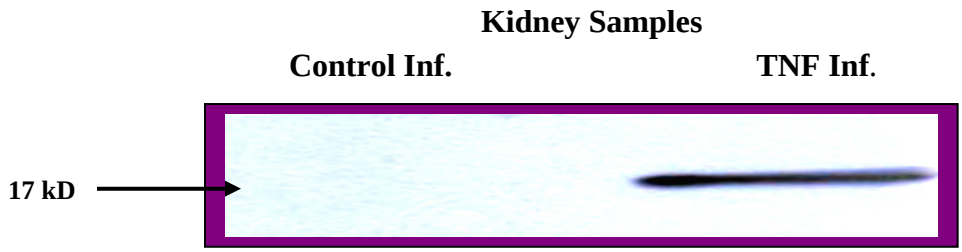

Figure 39. Western blot exposed film showing the 17 kD band for active caspase 3. Like that of caspase 2, this blot illustrates there is apoptotic signaling in the kidney tissue from animals infused with TNF (TNF Inf.), and it is clear that the caspase 3 signaling is greater than that of the animals infused with normal saline alone (Control Inf.).

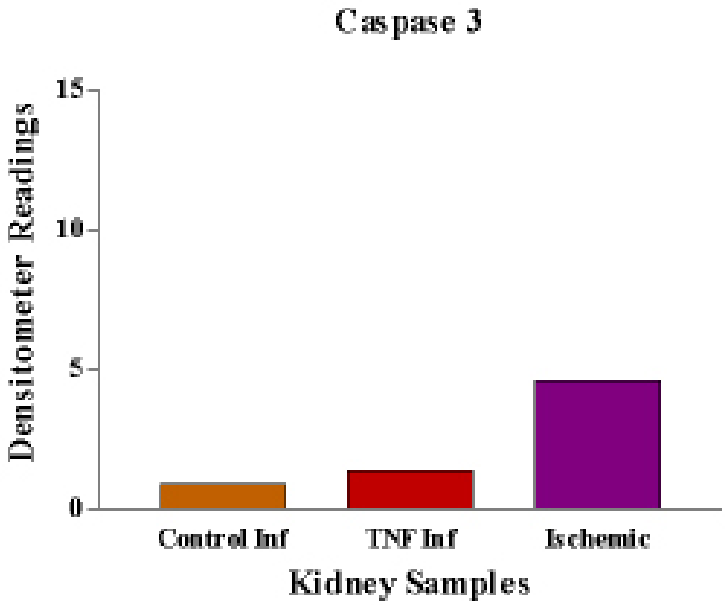

Figure 40. Bar graph representing the mean densitometer readings of Western blots for caspase 3. Kidney samples from animals that were infused with TNF (TNF Inf) had a mean densitometer reading of 1.3, an approximate 1.5 fold increase of caspase 3 levels over control kidney tissue (Control Inf) of animals infused with normal saline alone with a mean densitometer reading of .9, but again as there is with caspase 2, caspase 3 signaling is not as great as that seen in cardiac ischemic kidney samples (Ischemic) showing a mean densitometer reading of 4.6.

again as reflected in Figure 40, caspase 3 signaling, like caspase 2, is not as great as that seen in cardiac ischemic kidney samples with a mean densitometer reading of 4.6.

In our studies we chose caspase 9 to indicate mitochondria amplification of apoptosis via the TNF pathway shown in Figure 1—caspase 8 activation → Bid cleavage → mitochondrial release of cytochrome *c* complexing with Apaf-1 and pro-caspase 9 → activation of caspase 9 → caspase 3 activation [48], [36],[46]. Figure 41 shows an example of Western blot exposed films probed with active caspase 9. This blot illustrates that in fact there is mitochondria amplification of apoptosis indicated by the increase in levels of active caspase 9 from kidney tissues harvested from animals who under went TNF infusion over the control animal tissue. Figure 42 demonstrates however, that this amplification is not as great as that seen in the kidney tissue from cardiac ischemic kidney samples. The mean densitometer reading of TNF infused tissue for caspase 9 was 4.7, an approximate 1.2 fold increase over control kidney tissue of animals infused with normal saline alone having a mean densitometer reading of 3.9, but not as high as the kidney tissue from cardiac ischemic tissue which show a densitometer reading of 8.4.

The cleavage of PARP has been found to be a sensitive parameter of apoptosis and can be detected within 15 minutes [56]. Therefore, the cleaved form of PARP has become the research hallmark for indicating apoptosis [20], [50], [2], [49], [56]. It has also been suggested that cleaved PARP can contribute to the machinery of apoptosis by either the 89 kD fragment exhausting the cell of needed energy or by the 24 kD fragment blocking DNA repair [56], [62], [63], [64]. Figure 43 is an example of Western blot exposed film showing the small-cleaved fragment of 24 kD band of PARP. This blot illustrates there is an increase in apoptotic signaling in the kidney tissue from animals

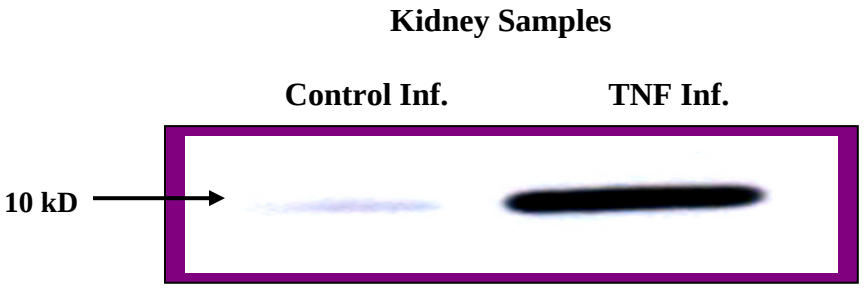

Figure 41. Western blot exposed film showing the 10 kD (the fragment cleaved by caspase 3) band for active caspase 9. Like that of caspase 2 and 3, this blot illustrates there is apoptotic signaling amplified via the mitochondria in the kidney tissue from animals infused with TNF (TNF Inf.), and it is apparent that the caspase 9 signaling is greater than that of the animals infused with normal saline alone (Control Inf.).

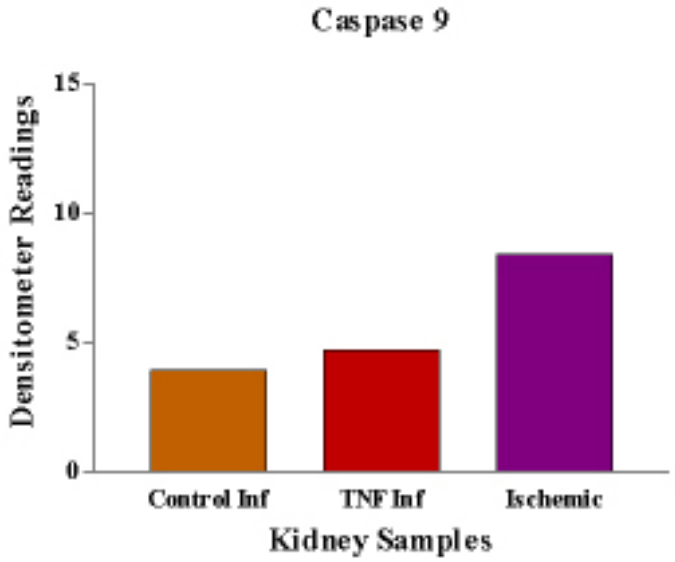

Figure 42. Bar graph representing the mean densitometer readings of Western blots for caspase 9. Kidney samples from animals that were infused with TNF (TNF Inf) had a mean densitometer reading of 4.7, an approximate 1.2 fold increase of caspase 9 levels over control kidney tissue (Control Inf) of animals infused with normal saline alone with a mean densitometer reading of 3.9, but again as there is with caspases 2 and 3, signaling is not as great as that seen in cardiac ischemic kidney samples (Ischemic) showing a mean densitometer reading of 8.4.

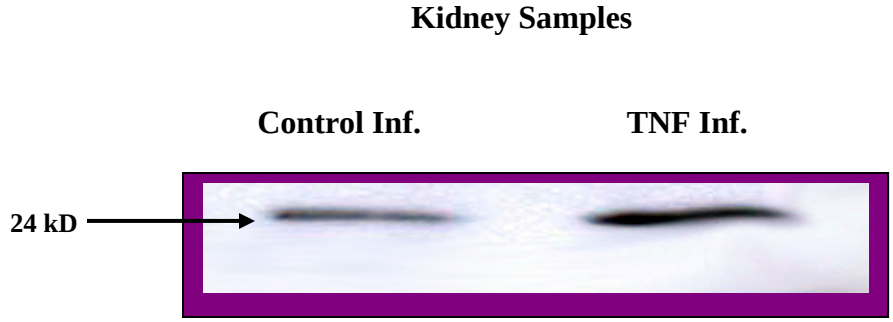

Figure 43. Western blot exposed film showing the small cleaved fragment of 24 kD band of PARP. Once again, this blot illustrates there is apoptotic signaling in the kidney tissue from animals infused with TNF (TNF Inf.), and the PARP signaling is greater than that of the animals infused with normal saline alone (Control Inf.).

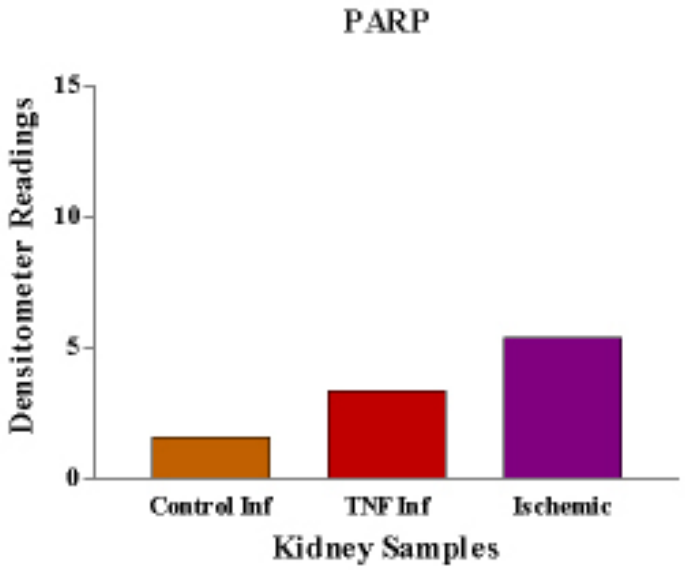

Figure 44. Bar graph representing the mean densitometer readings of Western blots for PARP. Kidney samples from animals that were infused with TNF (TNF Inf) had a mean densitometer reading of 3.4, an approximate 2.1 fold increase of cleaved PARP levels over control kidney tissue (Control Inf) of animals infused with normal saline alone with a mean densitometer reading of 1.6. The TNF Inf. kidney tissue PARP signaling is not as great as that seen in cardiac ischemic kidney samples (Ischemic) showing a mean densitometer reading of 5.4. Yet again, PARP signaling shows the same pattern as that of the other apoptotic indicators used in these studies (caspases 2, 3, and 9) demonstrating that there is more than just TNF causing injury to the kidney in acute coronary ischemia.

infused with TNF over that of the animals infused with normal saline alone. This is much like the results seen with Western blots of caspase 2, caspase 3, and caspase 9. Figure 44 is a bar graph representing the mean densitometer readings of Western blots for PARP. As shown, the kidney samples from animals infused with TNF had a mean densitometer reading of 3.4, an approximate 2.1 fold increase of cleaved PARP levels over control kidney tissue from animals infused with normal saline alone having a densitometer reading of 1.6. Once again the same pattern seen with caspase 2, caspase 3, and caspase 9, demonstrates apoptotic signaling, indicated here by PARP, is not as great in TNF infused kidney tissue as that seen in cardiac ischemic kidney samples showing amean densitometer reading of 5.4. These TNF infusions studies along with the TNF inhibition studies (of this same chapter) are indicative that TNF does cause injury to the kidney but there is participation of other players causing a magnification of renal injury over that seen with just TNF.

7.4 Discussion

The purpose of this study was to look at the consequence of cardiac ischemia on renal tissue as well as investigate the role of TNF in this process. We have demonstrated through Western blot analysis for the fist time that cardiac ischemia can cause renal injury determined by the levels of apoptotic signaling. These cardiac ischemic studies illustrate that in as little as 15 minutes of coronary occlusion, without reperfusion, there are increased levels of the apoptotic indicators active caspases 2, 3 and 9. These same indicators are found in TNF receptor pathways leading to apoptosis (Figure 1). Caspase 2 can act either as an initiator activating caspase 3 and Bid, or act as an in effector and

directly cause apoptosis. Because caspase 3 is targeted by caspases 8, 2, and 9 it is thought to play a dominant role in the apoptotic machinery [47], [2], [22], [48], [20]. Finally, the activation of caspase 9 can intimate a few things in the TNF-R pathway. Caspase 9 is an indicator of the mitochondria amplification of apoptosis, and with the pathway stimulated by TNF this can imply there is activation of caspase 8, caspase 2, and Bid. Once caspase 8 or 2 is activated they can cleave Bid, which rapidly travels to the mitochondria causing the release of cytochrome *c* a factor necessary for the activation of caspase 9 [51], [47], [2], [22], [48], [20]. With that being said, we see that there is injury to the kidney after cardiac ischemia as indicated by the apoptotic signaling, but the question still remains as to how important TNF is in the development of this renal injury.

Confident via TNF inhibition ELISA studies that TNF release was suppressed with the novel metalloproteinase inhibitor, WTACE2, after cardiac ischemia, we continued with the Western blot analysis comparing tissue that TNF was inhibited to that tissue where it was not. We find that although TNF is inhibited, there appears to be continued apoptotic signaling in the kidney as indicated by increased levels of active caspases 2, 3, and 9, as well as the early marker for apoptosis cleaved PARP. But as reveled by these investigations, the levels of these apoptosis indicators did not increase to the same degree as that seen in the cardiac ischemic kidney tissue. These TNF inhibition studies imply that TNF does play a role in renal injury after cardiac ischemia, but is not the sole participant.

The myocardium is known to be a major source of the cytokines IL-1, IL-6, and IL-8 along with TNF [6], [5]. Release of these cytokines under myocardial stress such as ischemia, can cause renal injury [4], [5]. So carrying the TNF investigation a step

further, our lab eliminated the cardiac ischemic event and infused TNF alone to give a clearer picture of the function of TNF in renal injury due to cardiac ischemia by reducing the possible influence of other cytokines. Through Western blot analysis we discovered once more that TNF alone can cause the rise in apoptotic signaling as determined by increased levels of caspases 2, 3, and 9, as well as cleaved PARP, but again, the increase in signaling is not to the extent as that seen in renal tissue from animals who underwent 15 minutes of cardiac ischemia.

In summary, these investigations show that cardiac ischemia can cause renal injury and that in 15 minutes of cardiac ischemia TNF does not act alone in injuring the kidney. TNF may become a bigger player in renal injury if the duration of the ischemic event is increased or the myocardium is reperfused. It has been shown in both these situations that TNF levels go up [3], [4], [5], [6], [8], [9], [10], but could it be that other cytokines go up as well? Further research needs to be done to determine if in fact TNF is the major participant in the cause of renal dysfunction in cardiorenal syndrome. Investigations such as expanding the duration of cardiac ischemia as well as reperfusing the myocardium need to be done due to correlations made between the length of ischemia and the levels of cytokine release and myocardial reperfusion and the levels of cytokine release [6], [3], [4], [5]. Other infusion studies such as the ones done in our lab with TNF need to be done with other cytokines. Elimination of cardiac ischemia and infusing one cytokine per assay could elucidate which cytokine plays a key role in renal injury or show renal injury is a combined effort of all cytokines as we find in our 15 minute cardiac ischemic model. These continued studies are imperative for answers key to developing proper therapeutics to prevent renal injury due to myocardial ischemia.

Chapter VIII

SUMMARY AND CONCLUSIONS

The purpose of these studies was to acquire a clearer understanding of cardiorenal syndrome. Cardiorenal syndrome is a term created and used by clinicians who observed a connection between renal injury and an injured heart. These same clinicians feel the cause of the renal injury is due to cytokines being released by the injured or ischemic myocardium [6], [7], [3], [4], but not until these studies has there been any research to support or disprove this theory. Furthermore, it is thought that TNF is the most likely to induce renal dysfunction [3], and so, was the cytokine of interest in these studies.

The specific aims of this study were:

Aim #1 – To quantitate the serum levels of the cytokine TNF after a myocardial infarction using an ultrasensitive TNF ELISA (Chapter VI).

Aim #2 – To determine if apoptosis occurs in kidney cells as a result of cardiac ischemia by looking at the early marker of apoptosis, PARP, using Western blot analysis (Chapter V).

Aim #3 – To examine which cell types in the kidney show activation of pro-apoptotic proteins following cardiac ischemia assessed by immunohistochemistry probing for cleaved PARP and activated caspase 3 (Chapter VI).

Aim #4 – To investigate apoptotic signaling in kidney tissue after induced cardiac ischemia, as well as examine the role of TNF in these activities (Chapter VII), accomplished by performing:

a. Western Blot analysis on kidney tissue measuring the apoptotic proteins of caspases 2, 3, and 9
b. Inhibition of TNF release via a metalloproteas inhibitor and determining apoptotic signaling by looking at caspases 2, 3, 9, and PARP using Western blot analysis
c. TNF infusion studies without induced cardiac ischemia to determine levels of pro-apoptotic protein levels of caspases 2, 3, 9, and PARP.

One of our major concerns with inducing cardiac ischemia was to ensure the animals' kidneys were properly perfused for the entire 15 minutes. To make certain there was proper renal blood flow we continually monitored arterial pressure in the animals of all groups (controls, ischemic, TNF inhibition, and TNF infusion studies) by placing a catheter in the carotid artery attached to a computerized blood pressure transducer. Figures 4, 6, 9, 15, 23, and 35 are graphs of MAPs in these studies. There was no evidence that arterial blood pressures decreased to a point where there was not sufficient renal perfusion throughout all the studies.

Since TNF became the focus of these studies, our first aim was to quantitate the serum levels of TNF after induced coronary ischemia (Chapter VI). These studies were successful in measuring TNF pre and post 15-minute cardiac ischemia without reperfusion. It was shown quantitatively that serum TNF levels consistently increase after cardiac ischemia was induced. Our studies were consistent with other animal research showing the cytokine TNF does in fact go up due to myocardial infarction [8], [9], [10]. Interesting, we found the greater the area of ischemia the higher the levels of

TNF in the serum. Wan et al. were also able to correlate the serum TNF levels with the extent of myocardial ischemia [4].

PARP is considered an early indicator and is an accepted marker for apoptosis [20], [50], [2], [49], [56]. Studies have shown that PARP cleavage can be seen within 15 minutes of an insult [56]. Due to this early cleavage we used cleaved PARP as our indication of apoptosis for Aim #2 (Chapter V). Via Western blot analysis we did indeed find an increase in both the cleaved products of PARP (89 kD and 24 kD) in renal tissue after 15 minutes of cardiac ischemia (Figure 7 and Figure 8); an approximate 6.75 fold increase of apoptotic activity. It is yet to be determined if the increases in cleaved PARP found in these studies is enough to cause extensive renal damage, but there is clearly an increase in the initiation of apoptosis caused by cardiac ischemia without reperfusion.

These studies also demonstrated for the first time those renal cells most affected by an injured heart. Using immunohistochemistry on renal tissue specially prepared, we looked at both kidneys from animals of the control group and ischemic group to achieve Aim #3 (Chapter VI). Using the active forms of caspase 3 and PARP, we found that harm to the cardiac ischemic kidney samples appears to focus in the proximal tubules of the renal cortex (Figures 10, 11, 12, 13). The apoptotic injury was not global but found in small patches of the proximal tubules throughout the cortical portion of the kidney. This finding would probably not have much effect on a healthy kidney, but for diseased or aged kidneys this loss of proximal tubules could be critical.

The final portion of these studies was to examine the role of TNF in the apoptotic activity found in Chapters V and VI (Aim #4). In order to assess TNF we first looked at apoptotic signaling using Western blot analysis specifically looking at active forms of

caspases 3, 2, and 9 (activity known for TNF receptor signaling pathways [Figure 1]). Secondly, we inhibited the release of TNF and again looked at apoptotic activity via protein levels of active forms of caspases 3, 2, 9, plus cleaved PARP using Western blot analysis. And finally, to carry the TNF investigation a step further, our lab eliminated the cardiac ischemic event and infused TNF alone to give a clearer picture of the function of TNF in renal injury due to cardiac ischemia. It was our belief this procedure would reduce the possible influence of other harmful cytokines, which has been shown to be increase with the injured or ischemic myocardium. Once again through Western blot analysis we looked at TNF apoptotic activity via caspases 2, 3, and 9, as well as cleaved PARP.

We found that there is an increase in apoptotic activity as seen with the increase levels of caspases 2, 3, and 9 in the cardiac ischemic kidney samples over that of control samples. Table 3 shows mean densitometer readings of our Western blot analysis findings on apoptotic signaling. This table shows a clear picture of apoptotic activity with increase levels of caspases 2, 3, and 9. Caspase 2 had an approximate 7 fold increase over controls, caspase 3 shows an increase of approximately 3.3 fold over control, and caspase 9 activity increased approximately 3.2 fold over that of control.

Our investigation on the role of TNF in renal injury after a cardiac ischemic event shows that after 15 minutes of cardiac ischemia, TNF does not act alone in harming the kidney. We found that although the release of TNF is inhibited, there is still an increase in apoptotic activity over that of controls (Table 1, Chapter VII). This signaling did not, however, reach those levels seen with either the cardiac ischemic animals receiving vehicle only or the animals from the cardiac ischemic studies. These findings were seen

with levels of caspases 2, 3, and 9, as well as levels of cleaved PARP. Also reflected in Table 1 of Chapter VII is the control without TNF inhibitor and the control with TNF inhibitor showing very similar mean densitometer readings indicating the TNF inhibitor had no direct effect on the kidney tissue.

Finally, in order to investigate the importance of TNF in cardiorenal syndrome, we eliminated the cardiac ischemic event and i.p. injected 2ng of TNF for every 1ml of extracellular fluid diluted in two milliliters of normal saline in one group of rats, and i.p. injected two milliliters of normal saline only in a second group of rats. By eliminating the release of other cytokines, known to be increased in serum after cardiac ischemia, we could center our research findings on just the effects of TNF on renal tissue. What we found was very similar to that seen in the TNF inhibition studies. There was an increase in apoptotic signaling (measured by levels of active caspases 2, 3, 9 as well as cleaved PARP) but not to the extent seen in the renal tissue harvested from animals that underwent coronary occlusion (Table 2, Chapter VII).

The investigations for Aim #4 show that cardiac ischemia can cause renal injury and that in 15 minutes of cardiac ischemia TNF does not act alone in injuring the kidney. We find this to be true by looking at the inhibition studies as well as the TNF infusion studies, neither of which showed apoptotic activity at the scale shown in the ischemic renal tissue.

In conclusion, our investigation of cardiorenal syndrome has given a better understanding of its mechanism of action. 1) We know that serum TNF goes up after an ischemic event as seen in Chapter IV; 2) we know there is an increase in apoptosis in renal tissue after cardiac ischemia measured by the early marker of cleaved PARP

Samples	Mean Densitometer Readings		
	Caspase 2	Caspase 3	Caspase 9
Ischemia	**8.6**	**4.6**	**8.4**
Control	**1.2**	**1.4**	**2.6**

Table 3. Mean densitometer readings of Western blot analysis of cardiac ischemic kidney tissue and controls (kidney samples of animals who did not undergo coronary artery occlusion). This table demonstrates the increase in TNF activity as illustrated by the rise in levels of active caspases 2, 3, and 9.

(Chapter V); 3) for the first time we now know were the injury takes place in the kidney due to coronary occlusion as seen in our immunohistochemistry studies (Chapter VI); 4) and finally, we know now that TNF is not the only participant in renal injury (Chapter VII). We feel that although we have enhanced our knowledge of cardiorenal syndrome there is much more research that needs to be done.

Our study protocol called for 15 minutes of ischemia without perfusion. We feel a clearer story of the observed cardiorenal syndrome may arise should time for the coronary occlusion be extended. It has been shown in other animal and human studies that the greater the duration of cardiac ischemia the higher the levels of serum TNF [8], [9], [10], [5]. Preliminary studies done in our lab found some increased damage to the kidney when we extended the duration of our study protocol to one hour (Figure 13). The damage was still in the cortical proximal tubules as found in the 15-minute protocol. The 15-minute method showed an increase in apoptotic activity, but actual damage was seen in the one-hour tissue slides.

Also, we feel that studies should be done examining renal tissue after reperfusion of the heart following coronary occlusion. This type of procedure would be more

representative of clinicians' findings with cardiorenal syndrome after CPB. Research findings have shown a substantial increase in serum TNF levels after coronary reperfusion [8], [9], [10]. Furthermore, we believe with the extension of coronary occlusion with reperfusion that TNF may become a bigger participant in renal injury then what we measured in our studies (Chapter VII). But with this in mind, there could also be an increase in serum levels of other harmful cytokines. Additional research needs to be done to determine if TNF is in fact the major injury causing cytokine seen in cardiorenal syndrome, and further research needs to be done measuring other participating cytokines in this renal injury.

As mentioned in Chapter I, TNF-R activity is very complex. Not only can the TNF-R activate apoptosis, but it can also initiate antiapoptotic activity [24], [43], [41], [70], [71], [42], [72]. Although there are many CPB procedures done, clinicians have only observed a small percentage of their patients who experience acute renal failure [3]. It is our feeling this low percentage is due to the protective aspect of TNF. Figure 45 is a simple illustration on the TNF-R pathway leading to cell life instead of cell demise. Literature shows that antiapoptotic proteins IAPs, A20, TRAF1 and TRAF2 are related to the TNF-R. TRAF2 is activated by both TRADD and RIP, which then stimulates the activation of NF-κB. The initiation of NF-κB can then upregulate IAPs, TRAF1, and A20. TRAF1 is thought to enhance the recruitment of IAPs, and A20 can bind to and inhibit NF-κB as well as inhibit apoptosis by mechanisms still unclear [24]. IAPs prevent apoptosis by breaking into fragments that bind specifically to certain caspases [24], [43], [41], [70], [71], [42], [72]. In our lab, we did some preliminary studies on one of the IAP proteins, XIAP, and of particular interest in our research was the interaction of

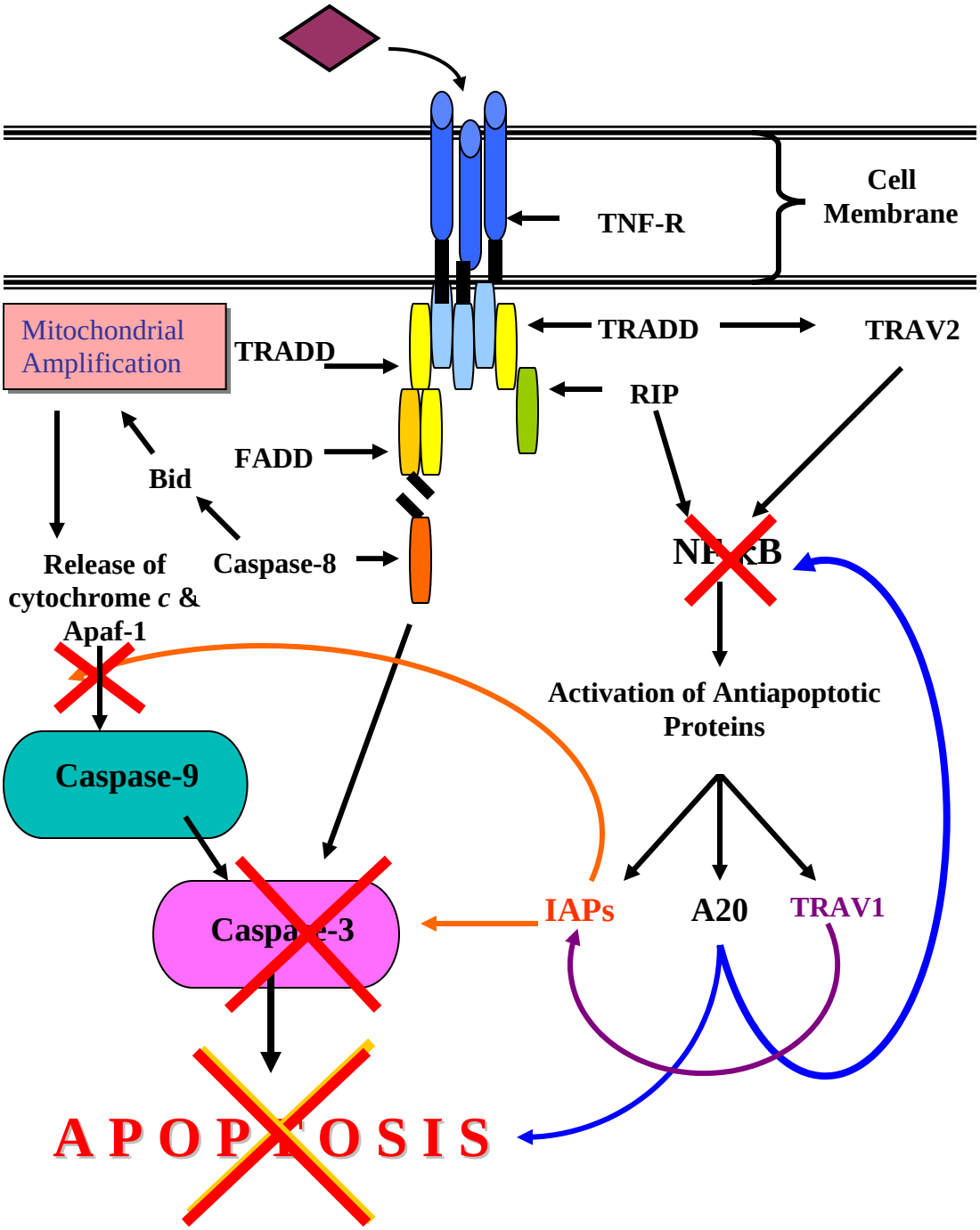

Figure 45. TNF induced antiapoptotic pathway. TNF binds to and activates the TNF-R. Once the receptor is activated not only can it initiate apoptosis but also it can prevent it. The recruitment of TRADD activates TRAV2 and RIP. RIP and TRAV2 both activate NF-κB, which initiates the upregulation of IAPs, A20, and TRAV1. TRAV1 enhances the recruitment of IAPs, and IAPs inhibit caspases such as caspases 3 and 9. A20 inhibits NF-κB as well as directly inhibiting apoptosis

XIAP with caspases 3 and 9. Using the same cardiac ischemic kidney tissues as used in the ischemic studies in Chapter VII, we investigated protein levels of XIAP by means of Western blot analysis. What we found was, as the levels of caspase 3 and 9 increased, XIAP levels decreased in the same tissues. This indicates that as needed, XIAP breaks into its fragments in order to combat against apoptosis by binding to specific caspases and preventing cell damage. This is a delicate balance, as seen in most biological systems, determining a cell's fate. Research for a better understanding of theses pathways could give us mechanisms by which we could perhaps increase the initiation of antiapoptotic proteins to prevent cell destruction, or possibly block their activity and allow unwanted cells to die.

World wide there is a continual growth of cardiovascular disease, and it is apparent that there will also be an increase in CBP surgeries. With more patients undergoing CBP it stands to reason that the percentage of cardiorenal syndrome will further increase as well. A greater understanding of the mechanism of action causing cardiorenal syndrome would greatly improve the outcomes of patients undergoing CBP without the added burden of acute renal dysfunction, or worse, renal failure. To improve cardiac patients' overall outcome from such events, these studies as well as further investigations, may provide treatments preventing the harmful effects caused by cardiorenal syndrome.

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VITA

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Thesis: RENAL INJURY, AS INDICATED BY APOPTOSIS IN THE KIDNEY, RESULTS FROM THE PRODUCTION AND RELEASE OF CYTOKINES DUE TO CARDIAC ISCHEMIA

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Education: Graduated from Adams City High School, Commerce City, Colorado in June 1970; received Associates in Arts from Tulsa Community College, Tulsa, Oklahoma, in May 1994, with a Major in Psychology and a Minor Sociology; received Bachelor of Arts from Langston University, Tulsa, Oklahoma in May 1996 with a Major in Psychology and a Minor in Sociology; completed the requirements for Doctor of Osteopathic Medicine from Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in May, 2005; and completed the requirements for Biomedical Science Doctorate of Philosophy from Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in May, 2005.

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Undergraduate: Call Rape Awareness Survey at Tulsa Community College, Tulsa, Oklahoma—designed and coordinated a survey conducted by Tulsa's local Call Rape organization during the time frame of January – May 1996; Alumnae Survey at University Center of Tulsa, Tulsa, Oklahoma—developed a survey to assess achievements and successes of the alumnae of the University Center of Tulsa during the time frame of August – December 1995; Oklahoma Teenage Pregnancy Survey at Langston University, Tulsa, Oklahoma—designed a survey that required interviewing professionals who worked directly with Oklahoma teenage pregnant girls during the time of January – May 1995.

Doctoral: *Renal injury, as indicated by apoptosis in the kidney, results from the production and release of cytokines due to cardiac ischemia* at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma during the time frame of August 2001 – April 2005.