University of Alberta

MATRIX METALLOPROTEINASE-2 IS PRESENT IN THE NUCLEUS

BY

JENNIFER ANNE KWAN 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pediatrics

Edmonton, Alberta, Canada

Fall 2002

University of Alberta

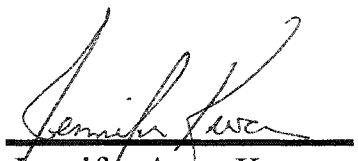
LIBRARY RELEASE FORM

NAME OF AUTHOR: **JENNIFER ANNE KWAN**

TITLE OF THESIS: **MATRIX METALLOPROTEINASE-2 IS PRESENT IN THE NUCLEUS**

DEGREE: **MASTER OF SCIENCE**

YEAR THIS DEGREE GRANTED: **2002**



Jennifer Anne Kwan
403 Rooney Crescent
Edmonton, Alberta
T6R 1C5

Date: June 18, 2002

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read and recommended to the Faculty of Graduate Studies and Research a thesis entitled **MATRIX METALLOPROTEINASE-2 IS PRESENT IN THE NUCLEUS** submitted by **Jennifer Anne Kwan** for acceptance in partial fulfillment of the requirements of the degree of Master of Science.

Richard Schulz
(Supervisor)

David E. Sims
(Committee Member)

Jason R.B. Dyck
(Committee Member)

John J. Greer
(Examiner)

Date: July 5, 2002

ABSTRACT

Matrix metalloproteinases (MMPs) are traditionally known for their role in remodeling the extracellular matrix. However recent investigations have demonstrated novel substrates for these enzymes and investigators have discovered that MMPs are localized intracellularly. This study was conducted to determine if MMP-2 is located in the nucleus of cardiac myocytes. Using both histochemical and biochemical techniques, MMP-2 was indeed found to be present in the nucleus, primarily in the latent form. In addition, the cleavage of poly (ADP-ribose) polymerase (PARP) by MMP-2 was investigated as a possible nuclear role for this protease. PARP is an enzyme involved in DNA strand repair. It is activated by oxidative stress and inactivated by proteolytic cleavage to an 85 kDa degradation product. This study shows that MMP-2 cleaves PARP *in vitro* to novel ~66 kDa and <45 kDa degradation products. This cleavage can be inhibited by MMP inhibitors. Although there were no detectable changes in the PARP degradation pattern in cardiac tissue from isolated perfused rat hearts subjected to two models of oxidative stress, both the ~66 kDa band and the <45 kDa band could be seen, consistent with *in vitro* degradation. This is the first evidence that MMP-2 is in the nucleus and that it can cleave PARP, forming a basis for a novel area of investigation.

ACKNOWLEDGMENTS

To my supervisor, Dr. Richard Schulz, for sharing his scientific experience with me, guiding me through this adventure and especially for letting me take my time with writing.

To my committee members, Dr. David E. Sims and Dr. Jason R.B. Dyck for all their advice and support in all matters. Thank you for letting me bother you both whenever I was a little unsure.

To Dr. John J. Greer for participating in my examination and for all his help with my figures.

To Drs. Costas J. Schulze, Wenjie Wang and Hernando Leon for providing me with the hearts and such sage advice.

To my parents for, well, everything. And to my brother for keeping me humble.

To everyone in the Schulz Lab for putting up with my messes, my silliness and my never-ending writing. And especially to Wenjie Wang for trying to let me defend before him.

To Margaret Horne, Dorota Wadowska and everyone at the University of Prince Edward Island for teaching me how to use the electron microscope and introducing me to the beauty of the other end of the country.

To Dr. Ming Chen for so kindly letting me use his electron microscopy facilities even though I left the province to learn how, and to Teresa Hopkins for sharing my EM trials and tribulations.

To Dr. Esmond Sanders, Dr. William M. Keyes and Eva Parker for teaching me about immunohistochemistry.

To Wilma L. Suarez-Pinzon for helping me start in the lab and for having faith in me as I continued, as well as for letting me in on all your little scientific secrets.

To Jason Iden for fixing all of my computer messes and putting up with the insanity.

And to all my wonderful friends who kept me sane in my personal life so that I could function academically, you know who you are.

Dedication

For my mom and dad, Shirley and Brewster,
for telling me that I should go get a Masters.

And for Costas for making sure I did.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

RESULTS

LIST OF FIGURES

RESULTS **PAGE**

ABBREVIATIONS

MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinases
$ONOO^-$	peroxynitrite
I/R	ischemia-reperfusion
PARP	poly (ADP-ribose) polymerase
BSA	bovine serum albumin
SDS	sodium dodecyl sulphate
IgG	immunoglobulin G
DMSO	dimethyl sulfoxide
Doxy	doxycycline
Phen	phenanthroline

CHAPTER I

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases best known for their actions in remodeling the extracellular matrix. Implicated in many pathological states, including ischemia-reperfusion injury, there is an increasing amount of evidence that these proteases have a much more complicated role than solely altering the extracellular matrix. With the use of MMP inhibitors on the horizon of disease therapy, it is important to fully understand the various roles of these enzymes and the possible effects their inhibition would present.

Recent studies demonstrate a number of non-matrix roles for MMPs including effects on cell growth and proliferation by cleavage or interactions with several growth factors (McCawley & Matrisian, 2001), vascular tone via cleavage of big endothelin (Fernandez-Patron *et al.,* 1999) and myocardial injury by cleavage of troponin I (Wang *et al., in press*). An increasing amount of recent evidence is also building which suggests an intracellular localization (Rouet-Benzineb *et al.,* 1999; Coker *et al.*, 1999) and novel roles for these "extracellular" enzymes (Fernandez-Patron *et al.,* 1999; Fernandez-Patron *et al.,* 2000). In this study, I will investigate the possibility that MMP-2 may be localized to the nucleus, as well as its possible role in cleaving a nuclear matrix protein, poly (ADP-ribose) polymerase (PARP).

A) MATRIX METALLOPROTEINASES

1) General Information

A family of more than 25 enzymes, matrix metalloproteinases are involved in both physiological processes, such as angiogenesis, wound healing and embyrogenesis and pathological states including cancer (Woessner, 1991), myocardial infarction, ischemia

Name	Number	Molecular Weight (kDa)	
		Latent	Active
Collagenases			
Collagenase 1	MMP-1	52	42
Collagenase 2	MMP-8	85	64
Collagenase 3	MMP-13	52	42
Collaganase 4	MMP-18	53	42
Gelatinases			
Gelatinase A	MMP-2	72	64
Gelatinase B	MMP-9	92	84
Stromelysins			
Stromelysin 1	MMP-3	57	45
Stromelysin 2	MMP-10	54	44
Stromelysin 3	MMP-11	64	46
Membrane-Type			
MT1-MMP	MMP-14	66	54
MT2-MMP	MMP-15	72	60
MT3-MMP	MMP-16	64	53
MT4-MMP	MMP-17	57	53

Table 1.1 Matrix Metalloproteinases

Among the over 25 currently identified matrix metalloproteinases, are these four major subfamilies. MMPs are grouped and named according to their primary substrate, or in the case of the membrane-type, their localization. Although, MMPs are generally characterized in either latent or active forms, recent evidence from Okamoto *et al.* (2001) indicates that the latent form of the enzyme, upon exposure to oxidants like peroxynitrite, can also be active without the loss of the propeptide.

and dilated cardiomyopathy (Cannon *et al.,* 1983; Sato *et al.,* 1983). All MMPs are synthesized as zymogens (proenzymes). Most are secreted that way (Nagase, 1997), however, there is a subtype, known as membrane type matrix metalloproteinases (MT-MMPs) that are membrane bound.

Activation of MMPs can occur by a conformational change caused by denaturing agents or by proteolytic removal of the inhibitory propeptide domain (Dollery *et al.,* 1995). This is known as the "cysteine switch" hypothesis, involving the dissociation of a cysteine residue from the zinc ion in the active site, allowing the enzyme to function. The first step is the disruption of this bond allowing the zinc ion to interact with a water molecule required for catalysis (Nagase, 1997). The resulting MMP intermediate can then be processed to the fully active form by further cleavage.

Another mechanism of activation has recently been shown by Okamoto *et al.* (2001). Peroxynitrite ($ONOO^-$), a reactive oxidant species, can activate MMPs in the presence of glutathione. Although the exact mechanism is not clear, it is thought that this results in the S-glutathiolation of the PRCGVPD sequence in the autoinhibitory domain. This highly conserved sequence is believed to be involved in maintaining the cysteine switch (Massova *et al.,* 1998). Thus the formation of disulfide S-oxide at this site will alter the ability of this domain to protect the active site.

In addition, a unique activation pathway for MMP-2 has been discovered. This cell surface activation involves both the membrane type metalloproteinase (MT-MMP) and an endogenous inhibitor, tissue inhibitor of metalloproteinase (TIMP-2). The N-terminal domain of TIMP-2 binds and inhibits MT-MMP allowing the C-terminal domain to act as a receptor for the 72 kDa form of MMP-2. An adjacent, uninhibited MT-MMP is then

required to cleave and activate the bound MMP-2. Following this initial cleavage, the residual propeptide is cleaved by another MMP-2 to produce the fully active enzyme (Strongin *et al.,* 1995).

Although, these enzymes are typically classified into "pro" and "active" forms, the latter being of approximately 8-10 kDa lower in molecular weight (Nagase *et al.,* 1990), these terms are often misleading. As demonstrated by Okamoto *et al.* (2001), it is possible to activate MMPs by S-glutathiolation, thus the "pro" form can be active without being cleaved to a lower molecule weight form. There may be a slight shift in the molecular weight, due to the addition of disulfide S-oxide, which would increase the molecular weight instead of decrease it. Therefore, the terms "pro" and "active" can only be used to identify the different sizes of an MMP with the understanding that both forms may be active enzymes.

Matrix metalloproteinase subfamilies are classified according to their primary substrate specificity, although many substrates are common to a number of MMPs. The gelatinases are of great interest in heart research due to their abundance in the cardiovascular system. This family consists of a 92 kDa zymogen known as MMP-9 and a lower molecular weight enzyme, MMP-2 (72 kDa). Both enzymes have a lower molecular weight "active" form; however MMP-2 is thought to be constitutive. As the name implies, the primary substrate for these MMPs is partially degraded collagen or gelatin.

2) MMP-2

This 72 kDa enzyme (64 kDa active form) is essential for cells to cross the basement membrane (Pauly *et al.,* 1994). Once activated, this enzyme has the ability to degrade

type IV and all non-fibrillar collagens of the basal lamina, as well as fibronectin, elastin and laminin (Denis & Verweij, 1997).

There has recently been a considerable amount of data demonstrating a diverse role for MMP-2 in various cellular functions. Sawicki *et al.* (1997) have illustrated that MMP-2 is involved in mediating platelet aggregation, while Fernandez-Patron *et al.* (1999) have proven that MMP-2 is involved in the regulation of vascular tone by cleaving both big endothelin-1 to "medium endothelin", which acts as a novel vasoconstrictor peptide. MMP-2 also degrades the endogenous vasodilator calcitonin gene-related peptide (Fernandez-Patron *et al.,* 2000), promoting vasoconstriction. Recent work done in our laboratory also demonstrates MMP-2 cleavage of an intracellular target, troponin I (Wang *et al., in press*), which is a regulatory element of the myocardial contractile proteins. This, to my knowledge, is the first study that shows an intracellular role for active MMP-2. Wang *et al.* (*in press*) show that MMP-2 could degrade troponin I but not troponin T or C when in complex, and both troponin I and troponin T individually. This work has opened up the possibility that MMP-2 has intracellular mechanisms in addition to its well accepted extracellular actions.

3) Inhibitors

MMPs have both natural and chemical inhibitors. The endogenous inhibitors of MMPs are known as tissue inhibitors of metalloproteinases (TIMPs). They are proteins that bind MMPs, both pro and active forms, in a 1:1 ratio (Creemers *et al.,* 2001; Gomez *et al.,* 1997), thereby inhibiting their proteolytic activity. Chemical inhibitors include tetracycline antibiotics (Nordstrom *et al.,* 1998), *ortho*-phenanthroline (Cheung *et al.,* 2000) and a growing number of new drugs such as PD 166793 (Ferrans, 2002), which

Inhibitor	MW (kDa)	Comments
TIMP-1	28.5	❖ inhibits active MMPs ❖ binds proMMP-9 ❖ inhibits apoptosis ❖ growth factor activity ❖ present in nucleus ❖ present in soluble form ❖ responsive to external stimuli
TIMP-2	21.0	❖ inhibits MMP-2 ❖ binds MT-MMP and proMMP-2 to activate the latter ❖ anti-angiogenic ❖ inhibits apoptosis ❖ present in soluble form ❖ constitutive ❖ growth factor activity
TIMP-3	21.0	❖ inhibits active MMPs ❖ insoluble (bound to ECM) ❖ promote detachment of transformed cells & accelerate morphological changes ❖ marker of differentiation ❖ anti-angiogenic ❖ induces apoptosis
TIMP-4	22.0	❖ inhibits active MMPs ❖ binds proMMP-2 ❖ abundant in heart and brain ❖ unknown chromosome location

Table 1.2 Tissue Inhibitors of Metalloproteinases (TIMPs)

Characteristics of the endogenous inhibitors of matrix metalloproteinases (MMPs). All four TIMPs bind the active site of the MMP enzyme via 12 highly conserved cysteine residues. In addition to their abilities to inhibit MMPs, TIMPs play an integral role in processes such as cell growth.

have been developed by pharmaceutical companies.

There are four currently identified TIMPs (1 through 4), all of which are able to bind MMPs. Inhibition occurs as the TIMPs interact non-covalently, via 12 conserved cysteine residues (Williamson *et al.,* 1990), with the zinc-binding site of the catalytic domain (Creemers *et al.,* 2001). TIMPs can also bind at additional sites on MMP-2 and MMP-9 (Woessner, 1994). MMPs and TIMPs are present in most tissues and body fluids (Gomez *et al.,* 1997), however, it is the balance between them that is essential for normal physiological functions. TIMP-1 is a soluble enzyme that is highly inducible (Gomez *et al.,* 1997). While it is similar to TIMP-2 in its ability to inhibit tumor growth, angiogenesis and apoptosis (Mannello & Gazzanelli, 2001), TIMP-1 can be found in the nucleus, whereas TIMP-2 is not (Zhao *et al.,* 1998). TIMP-2 is also a soluble protein however it tends to be constitutively expressed (Gomez *et al.,* 1997). When TIMP-2 can be modulated, it is altered in the opposite manner to TIMP-1 (Shapiro *et al.,* 1992). TIMP-3 is an insoluble protein that is found only in the extracellular matrix (Wick *et al.,* 1994). It is regulated in a cell cycle dependent manner (Wick *et al.,* 1994) acting as a marker for differentiation (Boudreau *et al.,* 1995). TIMP-4 is the most recent TIMP to be identified (Greene *et al.,* 1996). It is found primarily in the heart (Greene *et al.,* 1996) and has a role in inhibiting the cell surface activation of proMMP-2 (Hernandez-Barrantes *et al.,* 2001).

The basic principle behind chemical MMP inhibitors is the chelation of the zinc ion in the active site of the enzyme. One of the oldest known metalloproteinase inhibitors is *ortho*-phenanthroline, which can chelate the zinc ion without affecting the surrounding calcium concentration (Latt *et al.,* 1969). In addition, tetracycline antibiotics can inhibit

matrix metalloproteinases by chelation, in a manner independent of their antibiotic activity (Golub *et al.,* 1991). This includes the prevention of oxidative activation of the latent forms of MMPs (Weiss *et al.,* 1985). In fact, many studies are now looking at chemically modified tetracyclines for their ability to inhibit MMPs without antimicrobial properties, thus reducing the risk of bacterial resistance (Golub *et al.,* 1991). Compounds such as PD 166793 use carboxylic acid groups to chelate the zinc ion in MMPs, effectively inactivating these enzymes (Parker *et al.,* 1999). In addition, there are other drugs containing a hydroxamic group that chelate the zinc ion with high affinity (Parker *et al.,* 1999).

4) Localization

Since MMPs degrade the extracellular matrix, it is expected that these enzymes, once activated, would be found in the extracellular space. Tyagi *et al.* (1993) investigated proMMP-1, a collagenase, by indirect immunofluorescence microscopy and found it to be present within the endothelium and subendothelial space of the endocardium and in the interstitial spaces between cardiac muscle bundles. Although many studies have been done on various tissue types in various disease states, my thesis will primarily focus on the heart since the emerging studies that imply intracellular mechanisms for MMPs have been done primarily on this organ.

Looking at MMP-2 in isolated porcine left ventricular myocytes by immunofluorescence microscopy, Coker *et al.* (1999) found a strong MMP-2 signal both along the sarcolemmal surface of the myocyte and throughout the entire cell in a punctate fashion, as well as a strong perinuclear staining. Rouet-Benzineb *et al.* (1999) looked at samples from human left ventricle via confocal microscopy and found gelatinase activity

(both MMP-2 and MMP-9) mainly localized to the periphery of cardiomyocytes in normal hearts and associated with the sarcomeres in dilated cardiomyopathy hearts. MMP-9 also appeared in the T-tubule system. The striated staining pattern for MMP-2 in diseased hearts, similar to that of the sarcomere (Rouet-Benzineb *et al.,* 1999) corresponds to immunogold electron microscopy studies conducted in this laboratory (Wang *et al., in press*) that shows the presence of MMP-2 in the sarcomeres and mitochondria of sections obtained from isolated rat hearts. This study also showed the association of MMP-2 with purified myofilaments. Confocal microscopy depicted MMP-2 in rat heart sections in an intermittent and diffuse pattern that is both cytosolic and membrane-associated (Wang *et al., in press*). However, there has been little investigation into the nucleus as a possible site for MMP actions.

B) THE NUCLEAR MATRIX

The nuclear matrix plays an essential role in the function of the nucleus, in addition to its organization. It defines both the shape and structure while providing support for DNA replication, transcription and repair (Jackson & Cook, 1995). Composed mainly of proteins, the nuclear matrix also includes variable amounts of DNA and RNA (Martelli *et al.,* 1997). He *et al.* (1990) have deconstructed the matrix and found it to be composed of a nuclear lamina, an interior matrix of polymorphic fibers and masses that resemble nucleoli remnants. Although the exact composition of the nuclear matrix is not currently clear, it is known that proteolytic cleavage occurs in the nucleus. Martelli *et al.* (1997) speculate that cleavage of functional components may be necessary to inactivate enzymes that are no longer needed for apoptotic processes or to detach DNA from the matrix for

transcription. However, Mellgren (1991) notes that although proteolytic cleavage solubilizes the matrix, the fragments do not dissociate, which may account for the inability to detect histological changes (Arends *et al.,* 1990). Many types of proteases are present within the nucleus for a number of processes including apoptosis (Martelli *et al.,* 1997), regulation of the cell cycle (Gerogi *et al.,* 2002), signaling (Harada *et al.,* 1999), and nuclear matrix degradation (Owen & Campbell, 1995).

Recent work (Wang *et al., in press*) demonstrates that MMP-2 has the ability to cleave troponin I, both alone and in complex, and troponin T alone in cardiac myocytes much like Di Lisa *et al.* (1995) had shown the cleavage of troponin I and T by mu-calpain. Mellgrin (1991) illustrated the cleavage of the nuclear matrix by calpains, thus it is reasonable to investigate MMP-2 in this setting. Both μ-calpain and m-calpain are capable of cleaving several high molecular weight (>50 kDa) nuclear proteins, although Mellgren did not identify them. Yet, even though calpains have been shown to solubilize histone H1 kinase activity, indicating they have a role in the nucleus, Schollmeyer (1988) was unable to find m-calpain in the nucleus itself. On the other hand, Moraczewski *et al.* (1996) found μ-calpain in the nuclei of myoblasts and Fukuda *et al.* (1998) found that the nuclear fraction of the rat forebrain was rich in m-calpain.

While the components of the nuclear matrix are not well documented, they do include lamins (Lebel & Raymond, 1984), topoisomerase II (Kaufmann, 1989) and poly (ADP-ribose) polymerase (Kaufmann *et al.,* 1991), the last of which is the subject of this study. During apoptotic death, this enzyme, which is key in DNA repair, is degraded by a protease similar to interleukin-1β converting enzyme (Lazebnik *et al.,* 1994), which was later identified as caspase-3 (Tewari *et al.,* 1995). While Ito *et al.* (1996) have shown

that MMP-1, -2, -3 and -9 can degrade interleukin1ß, they did not investigate the possibility of whether MMPs could activate this cytokine. Nevertheless, since MMPs have been shown to interact with interleukin-1ß, it is possible that they could also act on PARP.

C) PARP

Also known as poly (ADP-ribose) synthetase and poly (ADP-ribose) transferase, poly (ADP-ribose) polymerase (PARP) is a 116 kDa enzyme that is inactivated to a 85 kDa by-product by caspase-3 (Tewari *et al.*, 1995). Activated to repair DNA strand breaks, PARP transfers ADP ribose moieties from NAD^+ to the DNA strand (Thiemermann *et al.*, 1997). The resulting product, nicotinamide, can then be restored to NAD^+ using ATP. While this process is essential for DNA repair, excessive activation of PARP can be very detrimental to the cell, depleting NAD^+ and ATP stores. This reduction slows glycolysis and mitochondrial respiration, effectively causing functional alterations and a necrosis-like death (Szabo & Dawson, 1998).

The expected time course of PARP activation and its degradation in relation to the activation of caspase-3 varies according to the method of inducing apoptosis. Rosenthal *et al.* (1997) show PARP activation after 2 days in human osteosarcoma cells undergoing slow (8-10 day), spontaneous cell death. Subsequent degradation of PARP occurs at 4-6 days. On the other hand, Bursztajn *et al.* (2000) show PARP activation after only 1.5 hr in human neuroblastoma cells exposed to staurosporine for 30 min to induce apoptosis. Although caspase-3 activation is seen immediately after exposure, PARP degradation is not seen until 1.5-5.5 hr. Still other studies on apoptosis in human prostate cancer cells

subject to paclitaxel (Au *et al.,* 1999) and mouse mammary epithelial cells stimulated with Se-methylselenocysteine (Unni *et al.,* 2001) show caspase-3 activation at 12-24 hr and PARP degradation after 24 hr to 72 hr. These studies do not prove that caspase-3 activation is necessary for PARP inactivation nor do they exclude the possibility of other mechanisms (ie. proteolytic enzymes) involved in this process. Although it is evident that there is a lag time between PARP activation and its subsequent degradation, there is little agreement as to how long.

In myocardial ischemia-reperfusion injury in rats after a regional, no-flow ischemia followed by reperfusion, PARP inhibitors have been shown to reduce infarct size and plasma creatine phosphokinase activity levels while improving both the histological profile and metabolic status of the reperfused myocardium (Zingarelli *et al.,* 1997). However, although PARP inhibitors are cytoprotective, they do not decrease DNA strand break formation (Szabo, 1996). One of the mechanisms implicated in the induction of strand breaks and subsequently poly (ADP-ribose) polymerase activation is oxidative stress, especially that mediated by $ONOO^-$.

D) PEROXYNITRITE

1) General Information

Generated by the near-diffusion rate combination of two oxygen free radicals, nitric oxide (NO) and superoxide anion (O_2^{-*}), $ONOO^-$ is now realized to be the toxic effector of NO. Although it is not a free radical itself, $ONOO^-$ has a plethora of actions including protein nitration (Moreno & Pryor, 1992), lipid peroxidation (Radi *et al.,* 1991),

carbohydrate oxidation (Moro *et al.,* 1995), thiol oxidation (Radi *et al.,* 1994) and DNA fragmentation (Lin *et al.,* 1997). The last of these causes PARP activation.

Stable at alkaline pH (pH 12), at lower pH (<8), such as that seen in a physiological milieu, $ONOO^-$ is protonated to peroxynitrous acid. This species is chemically unstable and rapidly dissociates to nitronium ion and a species much like the hydroxy radical, both of which are highly oxidant (Beckman *et al.,* 1994). Although it is not clear whether the effects of $ONOO^-$ are induced by the anion molecule itself or by one of these more reactive intermediate or products, it is clear that it contributes to oxidative stress injury.

2) PARP Activation

Although little is known about the mechanism by which $ONOO^-$ causes DNA strand breakage and how these strand breaks subsequently activate PARP, many studies have shown an increase in PARP activity upon exposure of both cells and tissue to $ONOO^-$ (Gilad *et al.,* 1997; Szabo *et al.,* 1996; Ishida *et al.,* 1996). Indeed most studies look at the effect of PARP inhibitors on oxidative injury. The general theory is that $ONOO^-$ causes DNA strand breaks, which in turn activates PARP and depletes NAD^+ and ATP, causing cell death (Szabo & Dawson, 1998). This mechanism has been implicated in both ischemia-reperfusion injury and inflammation, as well as other pathologies where oxidant injury plays a role.

3) MMP Activation

Peroxynitrite has also recently been shown to enhance the release and activity of MMP-2 (Wang *et al.,* 2002). In this study, infusion of authentic $ONOO^-$ into isolated rat hearts elicited a rapid and significant increase in MMP-2 activity in the coronary effluent prior to the onset of the decline in mechanical function of the hearts. As previously

described in the MMP section, Okamoto *et al.* (2001) have shown that the MMP activation by $ONOO^-$ occurs via a glutathione dependent S-glutathiolation of the protein by disulfide S-oxide formation. During the acute reperfusion phase following ischemia, the time course of $ONOO^-$ formation and MMP activation and release is similar (Yasmin *et al.,* 1997; Cheung *et al.,* 2000). Thus, a working hypothesis is that an increase in intracellular MMP-2 activity caused by the exposure to the oxidative stress mediated by $ONOO^-$, results in an increase in PARP degradation and resultant decline in cardiac mechanical function.

E) HYPOTHESIS

MMP-2 is present within the nucleus and has a possible biological role that involves cleaving PARP.

F) STUDY OBJECTIVES

In this study, I propose that MMP-2 is indeed present within the nucleus and contributes to oxidative stress injury by cleaving PARP. This proposed mechanism occurs independently of the depletion of energy stores but rather causes injury due to a decreased ability of PARP to repair DNA strand breaks. Thus I will determine whether MMP-2 can be localized to the nucleus, and whether it is biochemically active there. The susceptibility of PARP to cleavage by MMP-2 both *in vitro* and in *ex vivo* heart homogenates will also be investigated. To demonstrate that PARP degradation can be attributed to MMP activity I will repeat these studies in the presence of MMP inhibitors.

REFERENCES

Arends MJ, Morris RG & Wyllie AH. Apoptosis: the role of endonuclease. *American Journal of Pathology* 1990; 136:593-608

Au JL, Kumar RR, Li D & Wientjes MG. Kinetics of hallmark biochemical changes in paclitaxel-induced apoptosis. *AAPS PharmSci* 1999; 1:E8

Beckman JS, Chen J, Ischiropoulos H & Crow JP. Oxidative chemistry of peroxynitrite. *Methods in Enzymology* 1994; 233:229-240

Boudreau N, Sympson CJ, Werb Z & Bissell MJ. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* 1995; 267:891-893

Bursztajn S, Feng JJ, Berman SA & Nanda A. Poly (ADP-ribose) polymerase induction is an early signal of apoptosis in human neuroblastoma. *Brain Research: Molecular Brain Research* 2000; 76:363-376

Cannon RO, Butany JW, McManus BM, Speir E, Kravitz AB & Bolli R. Early degradation of collagen after acute myocardial infarction in the rat. *American Journal of Cardiology* 1983; 52:390-395

Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski M & Schulz R. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000; 101:1833-1839

Coker ML, Dorscher MA, Thomas CV, Galis ZS & Spinale FG. Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations. *American Journal of Physiology (Heart and Circulatory Physiology)* 1999; 277:H777-H787

Creemers EEJM, Cleutjens JPM, Smits JFM & Daemen MJAP. Matrix metalloproteinase inhibition after myocardial infarction. *Circulation Research* 2001; 89:201-210

Denis LJ & Verweij J. Matrix metalloproteinase inhibitors: present achievements and future prospects. *Investigational New Drugs* 1997; 15:175-185

Di Lisa F, De Tullio R, Salamino F, Barbato R, Melloni E, Siliprandi M, Schiaffino S & Pontremoli S. Specific degradation of troponin T and I by mu-calpain and its modulation by substrate phosphorylation. *Biochemistry Journal* 1995; 308 (pt 1):57-61

Fernandez-Patron C, Radomski MW & Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circulation Research* 1999; 85:906-911

Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW & Davidge ST. Vascular matrix metalloproteinase-2 dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. *Circulation Research* 2000; 87:670-676

Ferrans VJ. New Insights into the world of matrix metalloproteinases. *Circulation* 2002; 105:405-407

Fukuda S, Harada K, Kunimatsu M, Sakabe T & Yosida K. Postischemic reperfusion induces alpha-fodrin proteolysis by m-calpain in the synaptosome and nucleus in rat brain. *Journal of Neurochemistry* 1998; 70:2526-2532

Georgi AB, Stukenberg PT & Kirschner MW. Timing of events in mitosis. *Current Biology* 2002; 12:105-114

Gilad E, Zingarelli B, Salzman AL, Szabo C. Protection by inhibition of poly (ADP-ribose) synthetase against oxidant injury in cardiac myoblasts *in vitro*. *Journal of Molecular and Cellular Cardiology* 1997; 29:2585-2597

Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA & Rifkin BR. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. *Critical Reviews in Oral Biology and Medicine* 1991; 2:297-321

Gomez DE, Alonso DF, Yoshiji H & Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *European Journal of Cell Biology* 1997; 74:111-122

Greene J, Wang M, Liu YE, Raymond LA, Rosen C & Shi YE. Molecular cloning and characterization of human tissue inhibitor of metalloproteinases-4. *Journal of Biological Chemistry* 1996; 271:30375-30380

Harada S, Smith RM & Jarett L. Mechanisms of nuclear translocation of insulin. *Cell Biochemistry and Biophysics* 1999; 31:307-319

He D, Nickerson JA & Penman S. Core filaments of the nuclear matrix. *Journal of Cell Biology* 1990; 110:569-580

Hernandez-Barrantes S, Shimura Y, Soloway PD, Sang QA & Fridman R. Differential roles of TIMP-4 and TIMP-2 in proMMP-2 activation by MT1-MMP. *Biochemistry and Biophysics Research Communications* 2001; 281:126-130

Ishida H, Ichimori K, Hirota Y, Fukahori M Nakazawa H. Peroxynitrite induced cardiac myocyte injury. *Free Radical Biology and Medicine* 1996; 20:343-350

Ito A, Mukaiyama A, Itoh Y, Nagase H, Thorgersen IB, Endhild JJ, Sasaguri Y & Mori Y. Degradation of interleukin 1β by matrix metalloproteinases. *Journal of Biological Chemistry* 1996; 271:14657-14660

Jackson DA & Cook PR. The structural basis of nuclear function. *International Review of Cytology* 1995; 162A:125-149

Kaufmann SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Research* 1989; 49:5870-5878

Kaufmann SH, Brunet G, Talbot B, Lamarr D, Dumas C, Shaper JH & Poirier G. Association of poly (ADP-ribose) polymerase with the nuclear matrix: the role of intermolecular disulfide bond formation, RNA retention and cell type. *Experimental Cell Research* 1991; 192:524-535

Latt SA, Homquist B & Vallee BL. Thermolysin: a zinc metalloenzyme. *Biochemistry and Biophysics Research Communications* 1969; 37:333-339

Lazebnik YA, Kaufmann SH, Desnoyers A, Poirier GG & Earnshaw WC. Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994; 371:346-347

Lebel S & Raymond Y. Lamin B from rat liver nuclear exists both as a lamina protein and as an intrinsic membrane protein. *Journal of Biological Chemistry* 1984; 259:2693-2696

Lin KT, Xue JY, Sun FF & Wong PYK. Reactive oxygen species participate in peroxynitrite-induced apoptosis in HL-60 cells. *Biochemical and Biophysical Research Communications* 1997; 230:115-119

Mannello F & Gazzanelli G. Tissue inhibitors of metalloproteinases and programmed cell death: conundrums, controversies and potential implications. *Apoptosis* 2001; 6:479-482

Martelli AM, Bareggi R, Bortul R, Grill V, Narducci P & Zweyer M. The nuclear matrix and apoptosis. *Histochemical Cell Biology* 1997; 108:1-10

Massova I, Kotra L, Fridman R & Mobashery S. Matrix metalloproteinases: structures, evolution, and diversification. *FASEB Journal* 1998; 12:1075-1095

McCawley LJ & Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! *Current Opinions in Cell Biology* 2001; 13:534-540

Mellgren RL. Proteolysis of nuclear proteins by μ-calpain and m-calpain. *Journal of Biological Chemistry* 1991; 266:13920-13924

Moreno JJ & Pryor WA. Inactivation of α1-protease inhibitor by peroxynitrite. *Chemical Research in Toxicology* 1992; 5:425-431

Moraczewski J, Piekarska E, Bonavaud S, Wosinske K, Chazaud B & Barlovatz-Meimon G. Differential intracellular distribution and activities of mu- and m-calpains during the differentiation of human myogenic cells in culture. *Comptes Rendus de l'Académie des Sciences III* 1996; 319:681-686

Moro MA, Darley-Usmar VM, Lizasoain I, Su Y, Knowles RG, Radomski MW & Moncada S. The formation of nitric oxide donors from peroxynitrite. *British Journal of Pharmacology* 1995; 116:1999-2004

Nagase H, Enghild JJ, Suzuki K & Salvesen G. Stepwise activation of mechanisms of the precursor of matrix metalloproteinase-3 (stromelysin) by proteinases and (4-aminophenyl) mecuric acetate. *Biochemistry* 1990; 29:5783-5789

Nagase H. Activation mechanisms of matrix metalloproteinases. *Biological Chemistry* 1997; 378:151-160

Nordstrom D, Lindy O, Lauhio A, Sorsa T, Santavirta S & Konttinen YT. Anti-collagenolytic mechanism of action of doxycycline treatment in rheumatoid arthritis. *Rheumatoid International* 1998; 17:175-180

Okamoto T, Akaike T, Sawa T, Miyamoto Y, van der Vilet A & Maeda H. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *Journal of Biological Chemistry* 2001; 276:29596-29602

Owen CA & Campbell EJ. Neutrophil proteinases and matrix degradation. The cell biology of pericellular proteolysis. *Seminars in Cell Biology* 1995; 6:367-376

Parker M, Lunney E, Ortwine DF, Pavlovsky AG, Humblet C & Brouillette CG. Analysis of the binding of hydroxamic acid and carboxylic acid inhibitors to the stromelysin-1 (matrix metalloproteinase-3) catalytic domain by isothermal titration calorimetry. *Biochemistry* 1999; 38:13592-13601

Pauly RR, Passanti A, Bilato C, Cheng L, Papadopolous N, Gluzband YA, Smith L, Weinstein C, Lakatta EG & Crow MT. Migration of cultured vascular smooth muscle cells thorough a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. *Circulation Research* 1994; 75:41-54

Radi R, Beckman JS, Bush KM & Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Archives of Biochemistry and Biophysics* 1991; 288:481-487

Radi R, Rodriguez M, Castro L & Telleri R. Inhibition of mitochondrial electron transport by peroxynitrite. *Archives of Biochemistry and Biophysics* 1994; 308:89-95

Rosenthal DS, Ding R, Simbulan-Rosenthal CM, Vaillancourt JP, Nicholson DW & Smulson M. Intact cell evidence for the early synthesis, and subsequent late apopain-mediated suppression, of poly (ADP-ribose) during apoptosis. *Experimental Cell Research* 1997; 232:313-321

Rouet-Benzineb P, Buhler JM, Dreyfus P, Delcourt A, Dorent R, Perennec J, Crozatier B, Harf A & Lafuma C. Altered balance between matrix gelatinases (MMP-2 and MMP-9) and their tissue inhibitors in human dilated cardiomyopathy: potential role of MMP-9 in myosin heavy chain degradation. *European Journal of Heart Failure* 1999; 1:337-352

Sato S, Ashraf M, Millard RW, Fujiwara H & Schwartz A. Connective tissue changes in early ischemia of porcine myocardium: an ultrastructural study. *Journal of Molecular and Cellular Cardiology* 1983; 15: 261-275

Sawicki G, Salas E, Murat J, Miszta-Lane H & Radomski MW. Release of gelatinase A during platelet activation mediates aggregation. *Nature* 1997; 386:616-619

Shapiro SD, Kobayashi DK & Welgus HG. Identification of TIMP-2 in human alveolar macrophages. *Journal of Biological Chemistry* 1992; 267:13890-13894

Schollmeyer JE. Calpain II involvement in mitosis. *Science* 1988; 240:911-913

Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA & Goldberg GI. Mechanism of cell surface activation of 72 kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. *Journal of Biological Chemistry* 1995; 270:5331-5338

Szabo C. DNA strand breakage and activation of poly-ADP ribosyl-transferase: a cytotoxic pathway triggered by peroxynitrite. *Free Radical Biology and Medicine* 1996; 21:855-869

Szabo C & Dawson VL. Role of poly (ADP-ribose) synthetase in inflammation and ischemia-reperfusion. *Trends in Pharmacological Science* 1998; 19:287-298

Szabo C, Zingarelli B, O'Connor M & Salzman AL. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite. *Proceedings of the National Academy of Sciences* 1996; 93:1753-1758

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS & Dixit VM. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell* 1995; 81:801-809

Thiemermann C, Bowes J, Myint FP & Vane JR. Inhibition of the activity of poly (ADP-ribose) synthetase reduces ischemia-reperfusion injury in the heart and skeletal muscle. *Proceedings of the National Academy of Science* 1997; 94:679-683

Tyagi SC, Ratajska A & Weber KT. Myocardial matrix metalloproteinase(s): localization and activation. *Molecular and Cellular Biochemistry* 1993; 126:49-59

Unni E, Singh U, Ganther HE & Sinha R. Se-methylselenocysteine activates caspase-3 in mouse mammary epithelial tumor cells in vitro. *Biofactors* 2001; 14:169-177

Wang W, Sawicki G & Schulz R. Peroxynitrite induced myocardial injury is mediated through matrix metalloproteinase-2. *Cardiovascular Research* 2002; 53:165-174

Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JRB, Sawicki G & Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia-reperfusion injury. *Circulation* 2002; *in press*

Weiss SJ, Peppin G, Ortiz X, Ragsdale C & Test ST. Oxidative autoactivation of latent collagenase by human neutrophils. *Science* 1985; 227:747-749

Wick M, Burger C, Brusselbach S, Lucibello FC & Muller R. A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation and senescence. *Journal of Biological Chemistry* 1994; 269:18953-18960

Williamson RA, Marston FAO, Angal S, Koklitis P, Panico M, Morris HR, Carne AF, Smith BJ, Harris TJR & Freedman RB. Disulfide bond assignment in human tissue inhibitor of metalloproteinases (TIMP). *Biochemistry Journal* 1990; 268:267-274

Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB Journal* 1991; 5:2145-2154

Woessner JF Jr. The family of matrix metalloproteinases. *Annals of the New York Academy of Science* 1994; 732:11-21

Yasmin W, Strynadka KD & Schulz R. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovascular Research* 1997; 33:422-432

Zhao WQ, Li H, Yamashita K, Guo XK, Hoshino T, Yoshida S, Shinya T & Hayakawa T. Cell cycle-associated accumulation of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the nuclei of human gingival fibroblasts. *Journal of Cell Science* 1998; 111:1147-1153

Zingarelli B, Cuzzocrea S, Zsengeller Z, Salzman AL & Szabo C. Protection against myocardial ischemia and reperfusion injury by 3-aminobenzimide, an inhibitor of poly (ADP-ribose) synthetase. *Cardiovascular Research* 1997; 36:205-215

CHAPTER II

MATERIALS AND METHODS

A) MATERIALS

1) Standards and Postive Controls

Culture media from HT-1080 cells, a human fibrosarcoma cell line (American Type Culture Collection, Rockville, MD), was used as a standard for gelatinase detection for both zymography and MMP-2 western blotting. Cells were maintained in Eagle's minimum essential medium with 10% fetal calf serum at 37°C in a humidified incubator with 5% CO_2. After washing 3 times in serum-free media, the HT 1080 cells were incubated in serum-free medium with phorbol ester 12-o-tetradecanoyl-phorbol-13-acetate (0.1 μM) for 24 hr at 37°C. The cells were then centrifuged (1500 g at for 10 min room temperature) and the supernatant was collected, aliquoted and stored at -80°C.

Purified MMP-2 and MMP-9 were purchased from Oncogene (Boston, MA). Molecular weight markers for western blotting (myosin, β-galactosidase, bovine serum albumin, ovalbumin) and silver staining (myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin) were purchased from Bio-Rad (Hercules, CA). In the case of the *ex vivo* PARP western blots (Figure 3.14 and Figure 3.15) purified bovine PARP (0.1 μg/ lane, Biomol Research Laboratories Inc, Plymouth Meeting, PA) was used as a standard. Purified bovine collagen (6 μg/ lane, a gift from Dr. Paul Scott, Department of Biochemistry, University of Alberta) was used as a standard to verify the proteolytic activity of MMP-2 for the *in vitro* studies while purified caspase-3 (30 ng/lane, Calbiochem, San Diego, CA), which is known to degrade PARP (Tewari *et al.*, 1995), was used as a positive control. A rat heart homogenate sample from the peroxynitrite study was used as a positive control for the western blots testing the purity of the nuclear extracts.

2) Antibodies

A polyclonal, peptide generated MMP-2 antibody (a gift from Dr. Mieczyslaw Wozniak, Department of Clinical Chemistry, Medical University, Warsaw, Poland) was produced by injecting rabbits with a nine amino acid long synthetic peptide based on a domain from human MMP-2 (NGKEYNSCT 221-230, Sawicki *et al.*, 1998). The fragment was conjugated to m-maleimidobenzoyl-N-hydroxysuccinimide prior to injection.

A second rabbit-derived polyclonal MMP-2 antibody, generated against the catalytic domain of *Escherichia coli*-expressed active rat MMP-2, was purchased from Chemicon International Inc. (Temecula, CA) to verify histological results. With both antibodies, rabbit IgG (Sigma-Aldrich Canada, Oakville, ON) was used as negative control. In both instances, a goat anti-rabbit horseradish peroxidase secondary antibody (Transduction Laboratories, Mississauga, ON) was used.

Poly (ADP-ribose) polymerase (PARP) western blots were performed with a mouse monoclonal antibody C2-10 against rat PARP (a gift from Dr. Guy Poirier, Laval University, QC) and goat-anti mouse secondary antibody (Transduction Laboratories). The *ex vivo* PARP western blots were run with a polyclonal rabbit anti-PARP antibody (Calbiochem) and the goat anti-rabbit secondary antibody described above.

To assess the purity of the nuclear extracts, antibodies to muscle carnitine palmitoyltransferase (M-CPT-1, a gift from Dr. Gebre Woldegiorgis, Department of Biochemistry and Molecular Biology, University of Barcelona, Spain), a mitochondrial enzyme; soluble guanylate cyclase (Calbiochem), a cytoplasmic enzyme; and the sodium/potassium ATPase (Chemicon International Inc.), a membrane bound protein,

were all used to eliminate the possibility of contamination by these fractions. A goat anti-rabbit secondary antibody (Transduction Laboratories) was used for both the M-CPT-1 and soluble guanylate cyclase blots while a rabbit anti-chicken secondary antibody (Chemicon International) was used for the sodium-potassium ATPase antibody.

3) Peroxynitrite Preparation

Peroxynitrite was freshly prepared by simultaneously discharging two syringes connected to a common line and filled with ice-cold 2M $NaNO_2$ (solution A) and a mixture of 11.1 M nitric acid and 8.2 M H_2O_2 (solution B) into a rapidly stirring excess of ice-cold 4.2 M NaOH (Yasmin *et al.*, 1997). This solution has a pH ~12, at this pH $ONOO^-$ is stable for the purpose of the experiments. Decomposed $ONOO^-$ was prepared by discharging the syringes into an empty beaker. The low pH due to the nitric acid results in rapid decomposition of $ONOO^-$. The equivalent volume of NaOH solution was then added 5 min after solutions A and B were thoroughly mixed such that the decomposed $ONOO^-$ has the same pH as the authentic $ONOO^-$ solution. Excess H_2O_2 was removed by filtering the final solution through $Mn(IV)O_2$ and the concentration of $ONOO^-$ was determined via UV spectroscopy (λ_{max} = 302 nm, ε = 1670 M^{-1} x cm).

B) ANIMAL MODEL

Adult male Sprague-Dawley rats (250-350 g, n = 55) were used for these studies. The animals were maintained in the animal care facilities of the Health Science Laboratory Animal Services at the University of Alberta, and were provided with normal rat chow and water. This investigation complies with the Guide to the Care and Use of

Experimental Animals published (revised 1993) by the Canadian Council on Animal Care.

C) HEART PERFUSIONS

Rats were euthanized with sodium pentobarbital (360 mg/kg; Euthanyl^{TM}, Bimedia MTC, Cambridge, ON). The heart was rapidly excised by transecting the diaphragm and opening the chest by making an incision along the sternum. After cutting the pericardium, the heart was removed and rinsed in ice-cold Krebs-Henseleit buffer [118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 1.25 mM $CaCl_2$ (or for some experiments 3 mM $CaCl_2$ plus 0.5 mM EDTA)]. The hearts were perfused in Langendorff mode as described below.

1) Heart Perfusions for Histological Studies

Hearts taken "directly from rat" were cannulated via the aorta and retrogradedly perfused at 37°C for 10 minutes with a low calcium (1.25 mM), oxygenated (95% oxygen: 5% carbon dioxide) Krebs-Henseleit buffer at a constant pressure of 80 mm Hg in order to rinse them free of blood before embedding for immuohistochemical studies using electron (n = 7) microscopy.

2) Peroxynitrite Study

Hearts were excised as above and perfused according to Wang *et al.* (2002) in Langendorff mode at 37°C via the aorta at a constant flow of 10 ml/min using a peristaltic pump (Buchler Instruments Inc., Fort Lee, NJ) with the high calcium Krebs-Henseleit buffer (3 mM $CaCl_2$ plus 0.5 mM EDTA).

An incision was made in the left atrium so that a water filled latex balloon connected to a pressure transducer (model 60-3002, Harvard Apparatus, Holliston, MA) could be inserted into the left ventricle. The volume was adjusted to an end diastolic pressure of 8-12 mmHg. A second transducer was also placed close to the heart in the aortic infusion line in order to measure coronary perfusion pressure, which, along with left ventricular pressure and heart rate were recorded on the MP100 system (Biopac Systems Inc., Santa Barbara, CA). The temperature of the heart was maintained at 37°C by placing a water-jacketed glass chamber around it. Drug solutions were infused through a side-port proximal to the aortic cannula by a Gilson mini pump (Minipuls 3, Villiers Le Bel, France) at a constant rate of 0.1 ml/min.

The hearts were allowed to equilibrate for 20 min before the infusion of U46619 (1-100 nM, Sigma), a thromboxane mimetic, to increase the coronary perfusion pressure from 40 to 100-150 mmHg. 15 min later, previously decomposed $ONOO^-$ or $ONOO^-$ (80 μM) was infused into the hearts for 15 min prior to a 15 min washout period. After the perfusion period, hearts were freeze-clamped with tongs cooled to liquid nitrogen temperature, followed by immersion in liquid nitrogen and stored at -80°C.

Parallel groups of hearts (n = 6 per group) were infused simultaneously with $ONOO^-$ and either PD-166793 (2 μM, Parke-Davis, Ann Arbor, MI), an MMP inhibitor, or the antioxidant glutathione (GSH, 300 μM, Sigma) and U46619.

3) Ischemia-Reperfusion Study

Hearts were cannulated for perfusion in Langendorff mode using the high calcium Krebs-Henseleit buffer, at a constant pressure of 60 mm Hg. Several groups of hearts (n = 6 per group) were perfused. The control group was aerobically perfused for 75 min.

After a 25 min stabilization period, a second set of hearts were subjected to global, no-flow ischemia for 20 min (at 37°C) followed by 30 min of reperfusion. In additional experiments, doxycycline (100 μM) or phenanthroline (100 μM, both from Sigma) were infused into the hearts for 10 min before and 10 min after the ischemic period. The hearts were subsequently freeze-clamped, cooled in liquid nitrogen and stored at -80°C.

D) ELECTRON MICROSCOPY

1) Tissue Embedding

Following a 10 min perfusion in Langendorff mode, each heart was perfusion fixed with 0.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (60 mL, pH 7.3, room temperature), administered by a syringe attached to a side port proximal to the aortic cannula, while the heart was immersed in the same solution. The ventricles were chopped into ~1 mm cubes and then immersed for a further 90 min in fresh 0.5% glutaraldehyde. After fixation, the pieces were dehydrated in a series of increasing ethanol concentrations (50%, 2 x 70% and 2 x 95% for 10 min each, 2 x 100% for 15 min) and placed directly into acrylic resin (LR White Medium), which was changed twice and then left on a rotater overnight. The next day, pieces were placed in gelatin capsules and polymerized in LR White with an accelerator for 10 min at 4°C.

Thick sections (0.5 μm) were cut at room temperature on an ultramicrotome (Reichert-Jung Ultracut E) for light microscopy and stained with toluidine blue. These sections were used for orientation in order to select optimal samples for ultramicrotomy for electron microscopy.

Thin sections (90 nm) were then cut and placed on 300 mesh nickel grids for immunostaining and subsequent heavy metal staining with uranyl acetate and lead stain for visualization with a transmission electron microscope (Hitachi H-600 or H-7000, accelerating voltage 75 kV). Black and white pictures were taken with the microscope itself and the negatives were developed and converted to photographs (Kodak Polychromatic III paper).

2) Immunostaining

Once on grids, the sections were blocked in 0.5 M glycine (30 min) at room temperature, to eliminate any free aldehyde moieties, and then with 1% bovine albumin serum (BSA, Sigma) in PBS (pH 7.4) for 10 min to prevent non-specific binding. Sections were incubated with either of the MMP-2 antibodies (1:500, in the case of the peptide-generated antibody or 1:25 for the Chemicon antibody) for 18 hr at 4°C. Negative controls were prepared by either incubating with rabbit IgG or by omitting the primary antibody. After washing with 1% BSA solution, the sections were labeled with protein A-colloidal gold, (20 nm, CedarLane Laboratories Ltd., Hornby, ON) diluted 1:50 in PBS for 1 hr at room temperature, washed in PBS and then double distilled water and left to dry for heavy metal staining.

In order to visualize the tissue, sections were stained with a saturated uranyl acetate solution for 30 min at room temperature in the dark, rinsed in double-distilled water and dried before staining with lead salts (0.2 M lead nitrate, 0.2 M lead acetate, 0.2 M lead citrate, 0.05 M sodium citrate, 0.12 M NaOH) (Sato, 1968).

All materials used for electron microscopy were obtained from Marivac Ltd. (Halifax, NS) unless otherwise specified.

E) NUCLEAR EXTRACTS

Various protocols for isolating nuclei were attempted on rat hearts (Widnell *et al.*, 1967; Maggio *et al.*, 1963; Hogeboom *et al.*, 1952). However, they failed to produce nuclei of sufficient yield or purity, thus human heart and rat liver nuclear extracts (prepared according to Gorski *et al.*, 1986) were purchased from Geneka Biotechnology Inc. (Montreal, QC).

F) PARP SEQUENCE ANALYSIS

The primary amino acid sequence for bovine PARP was inputted into the LALIGN peptide comparison program (www.ch.embnet.org/software/LALIGN_form.html) along with 22 MMP-2 cleavage recognition sequences (8 amino acids each). The program compares the homology of each recognition sequence with that of PARP and reports the percent homology within a certain number of amino acids. Sites that had a homology greater then 60% for all 8 amino acids were considered, with those showing greater than 50% for 7 of 8 amino acids also recorded.

G) PARP CLEAVAGE ASSAY

Purified bovine poly (ADP-ribose) polymerase (PARP) was purchased from Biomol Research Laboratories Inc. for use in *in vitro* studies. Preliminary studies determined that 0.25 μg/lane of PARP prepared as a dilution in Tris buffer (50 mM Tris, 5 mM $CaCl_2$, 159 mM NaCl) [20 μl of PARP solution plus 4 μl of 6x loading buffer (30% glycerol, 3% β-mercaptoethanol, 6% SDS, 10 mg/100 ml bromophenol blue, 0.13 M Tris, pH 8)] in a 10 lane SDS-PAGE gel was ideal for PARP visualization. This concentration was

incubated with increasing concentrations of purified human MMP-2 (1, 5, 10 or 30 ng in 0.5 M Tris buffer, total volume 20 µl; Oncogene) at 37°C for either 20 min or 2 hr. Purified bovine type IV collagen (6 µg/ lane) was used as a standard to verify the proteolytic activity of MMP-2. Purified caspase-3 (30 ng/lane), which is known to degrade PARP (Tewari *et al.*, 1995), was used as a positive control for these studies.

Parallel assays were performed with MMP inhibitors to show that PARP cleavage indeed occurred as a result of MMP-2 activity. Human TIMP-2 (0.5 µM, Chemicon International, Inc.), an endogenous inhibitor of MMPs, and doxycycline (100 µM, Sigma) were added to the *in vitro* tubes and incubated as above (for either 20 min or 2 hr at 37°C).

Following the incubation, 6x loading buffer was added to each sample at a ratio of 1:5 (v:v) to stop the reaction. After vortexing, the samples were loaded onto an 8% acrylamide gel for electrophoresis followed by either silver staining or western blotting for PARP.

H) TISSUE HOMOGENATES

Frozen hearts were crushed at liquid nitrogen temperature using a mortar and pestle before sonication for 15 sec in ice-cold homogenization buffer (50 mM Tris-HCl pH 7.4, 3.1 mM sucrose, 1 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 2 µg/ml aprotinin, 0.1% Triton X-100). The homogenate was then centrifuged at 10000 g for 10 min at 4°C, and the supernatant was collected and stored at -80°C.

I) PROTEIN ASSAY

The amount of protein in homogenates and extracts was measured using a bicinchoninic acid (BCA) assay. This assay is dependent on quantification of the color obtained from a BCA-copper mixture reacting with the protein in the sample. Protein standard (BSA) of varying concentrations (1.5-10 μg/10 μl) was loaded in triplicate into a 96 well plate. The samples were then diluted (1:20 for homogenates, 1:5 for nuclear extracts) and loaded in triplicate into wells of the same 96 well plate. 200 μl of the BCA-copper sulfate mixture (50:1) was added to each well and the plate was incubated for 30 min at 37°C. The plate was then read on a UVmax Kinetic Microplate Reader at a wavelength of 560 nm and analyzed using the Softmax program.

J) GELATIN ZYMOGRAPHY

1) Protocol

Gelatin zymography was used to measure MMP activity via a previously described protocol (Cheung *et al.*, 2000). Briefly, non-reduced samples were loaded onto an 8% polyacrylamide gel co-polymerized with 2 mg/ml gelatin. After a 60-90 min electrophoresis period (150V, 4°C), gels were washed 3 x 20 min in Triton X-100 (2.5% v:v, Sigma) to remove the sodium dodecyl sulphate (SDS, Caledon Laboratories Inc., Georgetown, ON) and 3 x 20 min in incubation buffer (50 mM Tris-HCl pH 7.6, 5 mM $CaCl_2$, 150 mM NaCl, 0.05% NaN_3) before incubation in this buffer for 18-24 hr at 37°C. The next day, the gels were stained at room temperature (2% Coomassie Brilliant Blue G, 25% methanol, 10% acetic acid) for 2 hr, and then destained for 3 hr (2% methanol, 4% acetic acid) prior to photographing (Fisherbiotech Photodocumentation Camera with

Polaroid ISO $80/20^0$ Black & White Instant Pack Film). Pictures were scanned (HP 6100 scanner, Hewlett-Packard) for densitometric analysis using Quantity One software (Bio-Rad). In this program, the intensity of each band is measured after subtracting the background noise. The obtained arbitrary units of each lane were then expressed as a percent of the control.

In some instances, MMP inhibitors (100 μM doxycycline or 100 μM phenanthroline) were included in the incubation buffer during the overnight incubation period to verify that the gelatinolytic activity was indeed due to MMPs.

2) Possible Activation of MMPs by PARP

To investigate the possibility of PARP activating the gelatinases found in the nuclear extracts, the human heart nuclear extract was incubated at 37°C for either 20 min or 2 hr in the presence of various concentrations of purified bovine PARP. 0, 0.1, 0.3, 1 or 3 μg of PARP was added to 10 μg of human heart nuclear extract *in vitro*. After incubation, the samples were then run on zymograms as described above. In addition, parallel samples that were not incubated, but included both PARP and the heart nuclear extract, were analyzed by zymography.

K) WESTERN BLOTS

1) MMP-2

MMP-2 was detected by western blotting using a semi-dry transfer protocol (Wang *et al., in press*). 20 μg of protein was loaded onto an 8% polyacrylamide SDS-PAGE gel and run at 150 V for 60-90 min at room temperature before semi-dry transfer onto a polyvinylidene difluoride membrane (PVDF, Bio-rad). Membranes were blocked

overnight at 4°C in a 5% milk (in PBS, pH 7.6) solution prior to a 2 hr incubation with the polyclonal MMP-2 antibody (1:5000, Chemicon) at room temperature. Blots were washed 4 x 5 min in TTBS buffer (0.01 M Tris pH 7.6, 0.1% Tween, 0.1 M NaCl), and incubated with a goat-anti-rabbit secondary antibody (1:5000, Transduction Laboratories) for 1 hr at room temperature. The membrane was washed again before visualizing the bands by chemiluminscent detection using an ECL Plus kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). The membranes were exposed to X-ray film (Kodak Biomax MR-1 Film, Sigma) for autoradiography. The developed films were scanned and analyzed similar to the zymogram photos.

2) Purity of Nuclear Extracts

Western blots were performed to verify the purity of the purchased nuclear extracts in the same manner as stated above. Antibodies to the mitochondrial enzyme, M-CPT-1 (1:3000); cytoplasmic enzyme soluble guanylate cyclase (1:3000); and the sodium/potassium ATPase (1:2000), a plasma membrane bound protein, were used to eliminate the possibility of contamination by these fractions. The goat anti-rabbit secondary antibody (1:5000) was used for both the M-CPT-1 and soluble guanylate cyclase blots and a rabbit anti-chicken secondary antibody (1:250,000) was used for the sodium-potassium ATPase blot.

3) PARP

Western blots for PARP and its degradation products were performed following the protocol of Alexandre *et al.* (2000). Samples loaded onto an 8% polyacrylamide gel were electrophoresed for 60-90 min (150 V) at room temperature before being wet-transferred onto nitrocellulose membranes (Bio-Rad) for 2 hr at 100 V. The membranes

were then blocked for 1 hr at room temperature in PBSMT (5% milk, 0.1% Tween 20 in PBS) and incubated overnight at room temperature with a monoclonal mouse anti-PARP C2-10 antibody (1:10000) or a polyclonal rabbit anti-PARP antibody (1:1000, Calbiochem). The membranes were then washed 3 x 5 min in PBSMT buffer and placed in goat-anti-mouse (1:5000) or goat anti-rabbit (1:5000) secondary antibody for 30 min at room temperature. After washing, the membranes were placed in PBS for 30 min before detection with a chemiluminscent ECL kit (Amersham Pharmacia Biotech UK Ltd.).

L) SILVER STAINING

Silver staining of the samples from the *in vitro* PARP degradation assay were performed by loading the samples onto an 8% SDS-PAGE for electrophoresis under reducing conditions (60-90 min, 150 V). All reactions took place at room temperature and, unless otherwise specified, solutions were made with ddH_2O. Subsequent to electrophoresis, gels were washed for 1 hr in each of the following solutions: 50% methanol and 10% acetic acid, 5% methanol and 7% acetic acid, and 10% glutaraldehyde before briefly washing in double-distilled water and being left overnight at in the same. The next morning, the gels were placed in a 5 μg/L dithiothreitol solution for 1 hr, washed and then placed in 0.1% silver nitrate for 1 hr. The gels were developed with the addition of a developing solution (3% w:v Na_2CO_3 and 1 μl/ml formaldehyde). When the observed bands reached the desired intensity, the reaction was stopped with 7% acetic acid. These gels were also photographed for analysis as described above.

M) STATISTICS

The functional data for the *ex vivo* experiments were subjected to statistical analysis using either a paired Student's t-test or one or two-way repeated measures ANOVA with Neuman Keuls as a post-hoc test, as appropriate. The densitometric analyses of the zymogram, silver stained SDS-PAGE and western blot bands were analyzed by one-way ANOVA also with a Neuman Keuls post-hoc test. The data are expressed as mean ± standard error of the mean and statistical significance was considered to occur if the p-value was less than 0.05.

REFERENCES

Alexandre S, Rast C, Nguyen-Ba G, Poirier GG & Vasseur P. PARP degradation in apoptotic Syrian hamster embryo (SHE) cells compared to HL60 cell line. *Biochimie* 2000; 82:1115-1122

Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW & Schulz R. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000; 101:1833-1839

Gorski K, Carneiro M & Schibler U. Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* 1986; 47:767-776

Hogeboom GH, Schneider WC & Streibich MJ. Cytochemical studies; on isolation and biochemical properties of liver cell nuclei. *Journal of Biological Chemistry* 1952; 196: 111-121

Maggio R, Siekevitz P & Palade GE. Studies in isolated nuclei. I. Isolation and chemical characterization of a nuclear fraction from guinea pig liver. *Journal of Cell Biology* 1963; 18:267-291

Polak JM & Van Noorden S. *Introduction to Immunocytochemistry* 2nd ed. Bios Scientific Publishers: New York, USA. 1997

Sato, T. A modified method of lead staining of thin sections. *Journal of Electron Microscopy* 1968; 17:158-159

Sawicki G, Sanders EJ, Salas E, Wozniak M, Rodrigo J & Radomski MW. Localization and translocation of MMP-2 during aggregation of human platelets. *Thrombosis & Haemostasis* 1998; 80:836-839

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS & Dixit VM. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhabitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell* 1995; 81:801-809

Wang W, Sawicki G & Schulz R. Peroxynitrite induced myocardial injury is mediated through matrix metalloproteinase-2. *Cardiovascular Research* 2002; 53:165-174

Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JRB, Sawicki G & Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 2002; *in press*

Widnell CC, Hamilton TH & Tata JR. The isolation of enzymatically active nuclei from the rat heart and uterus. *Journal of Cell Biology* 1967; 32:766-770

Yasmin W, Strynadka KD & Schulz R. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovascular Research* 1997; 33:422-432

CHAPTER III

RESULTS

A) LOCALIZATION OF MMP-2

1) Ultrastructural Localization of MMP-2

Immunogold electron microscopy of rat left ventricles allowed for the determination of the subcellular localization of MMP-2 within cardiac myocytes. Probing with both MMP-2 antibodies revealed staining along the myofibrils and in mitochondria (data not shown), as previously detected. In addition, there was a significant presence of MMP-2 in the nuclei (Figure 3.1 upper and middle). The distribution of MMP-2 in the nucleus was relatively homogenous with a degree of association with the condensed chromatin. There was no intracellular staining of the sections when probed with an unrelated IgG (Figure 3.1 lower). MMP-2 was also found intracellularly within endothelial cells, including the nucleus (data not shown).

2) Purity of Nuclear Extracts

Various attempts to isolate nuclei from rat hearts were explored using several different protocols. However, I was unable to produce a fraction with a yield sufficient for further analysis. As well, the purity of the fraction I obtained was questionable. As a result, both human heart and rat liver nuclear extracts were purchased from Geneka Biotechnology Inc. To verify the purity of these extracts, the samples were tested for the presence of sodium/potassium ATPase (Figure 3.2 A) as a marker for possible membrane contamination, soluble guanylate cyclase (Figure 3.2 B) as a marker for cytosolic proteins and muscle carnityl palmerase transferase-1 (Figure 3.2 C) to exclude mitochondrial contamination. There was little or no contamination with any of these proteins in either of the commercially available nuclear extracts.

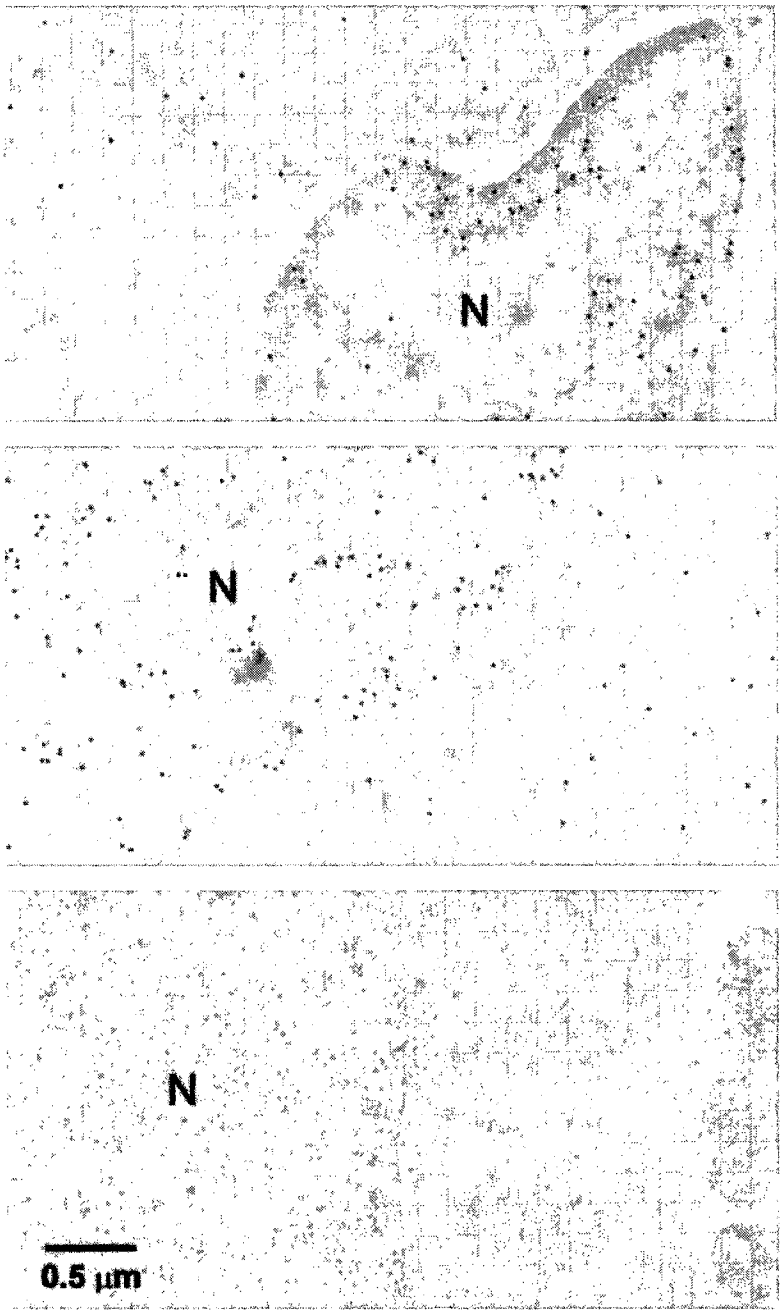


Figure 3.1 Nuclear Localization of MMP-2 in Cardiac Myocytes

Immunogold staining of rat cardiac myocytes from sectioned hearts for transmission electron microscopy. Sections probed with MMP-2 antibody (**upper** and **middle**) followed by protein A-colloidal gold (20 nm particles) show MMP-2 staining in the nucleus (**N**). The negative control (IgG) sections (**lower**) show no background staining. All micrographs were taken at the same magnification (n = 7 hearts with MMP-2 and control IgG stained in each). *Hearts perfused by Dr. Costas J. Schulze.*

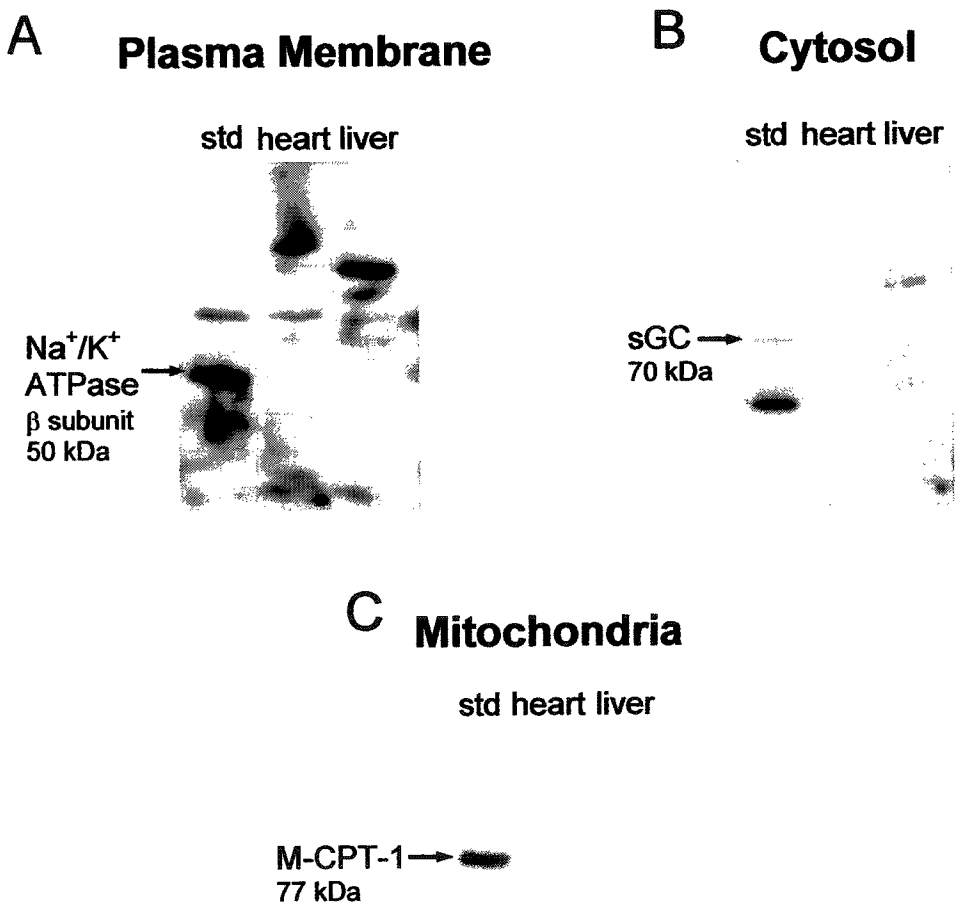


Figure 3.2 Western Blots Demonstrating Purity of Nuclear Extracts

In order to assess the purity of the purchased nuclear extracts, western blots for several enzymes specific to other subcellular fractions were run. In the left lane of each gel 40 μg of rat heart homogenate was loaded as a standard (std). The middle lane is the human heart nuclear extract (10 μg) and the right lane is the rat liver nuclear extract (10 μg). There are no noticeable bands for either sodium/potassium ATPase (Na^+/K^+ ATPase, **A**), soluble guanylate cyclase (sGC, **B**) or muscle carnitine palmitoyltransferase (M-CPT-1, **C**) in either of the nuclear extracts suggesting that there is no contamination with either the membrane, cytosolic or mitochondrial fractions, respectively. (n = 3)

3) Biochemical Assays for MMP-2 in the Nucleus

Nuclear extracts from both human heart and rat liver were assayed for gelatinase activity and MMP-2 expression by gelatin zymography (Figure 3.3 A) and western blotting (Figure 3.3 B), respectively. Both the rat liver and human heart nuclear extracts displayed gelatinase activity for MMP-9 and MMP-2. Although both extracts predominantly showed MMP-2 in the 72 kDa form, a lesser amount of active MMP-2 (64 kDa) could also be detected. In addition, the human heart nuclear extract showed a higher molecular weight band of gelatinolytic activity (~130 kDa) that was not apparent in either the HT 1080 standard or the rat liver nuclear extract.

Western blots of each nuclear extract indicated the presence of active MMP-2 (Figure 3.3 B). However, in accordance with the zymography results there was a greater amount of MMP-2 present in the human heart nuclear extract than in the rat liver nuclear extract. Thus MMP-2 is present in both the 72 kDa and 64 kDa forms in nuclei from both rat liver and human heart. However the 72 kDa form is predominant.

4) Inhibition of Nuclear Gelatinase Activity

In order to attribute the gelatinolytic activity of the nuclear extracts to MMPs, additional zymograms were incubated in the presence of MMP inhibitors (Figure 3.4 to Figure 3.6). After electrophoresis, the zymogram gels were washed and then incubated with the specified inhibitor. In both the human heart nuclear extract (Figure 3.4) and the rat liver nuclear extract (Figure 3.6), both 100 μM doxycycline (Doxy) and 100 μM phenanthroline (Phen) inhibited the nuclear extract gelatinase activities corresponding to MMP-2, MMP-9 and the ~130 kDa band. The less potent MMP inhibitor, doxycycline, caused only a partial inhibition that was not statistically significant upon densitometric

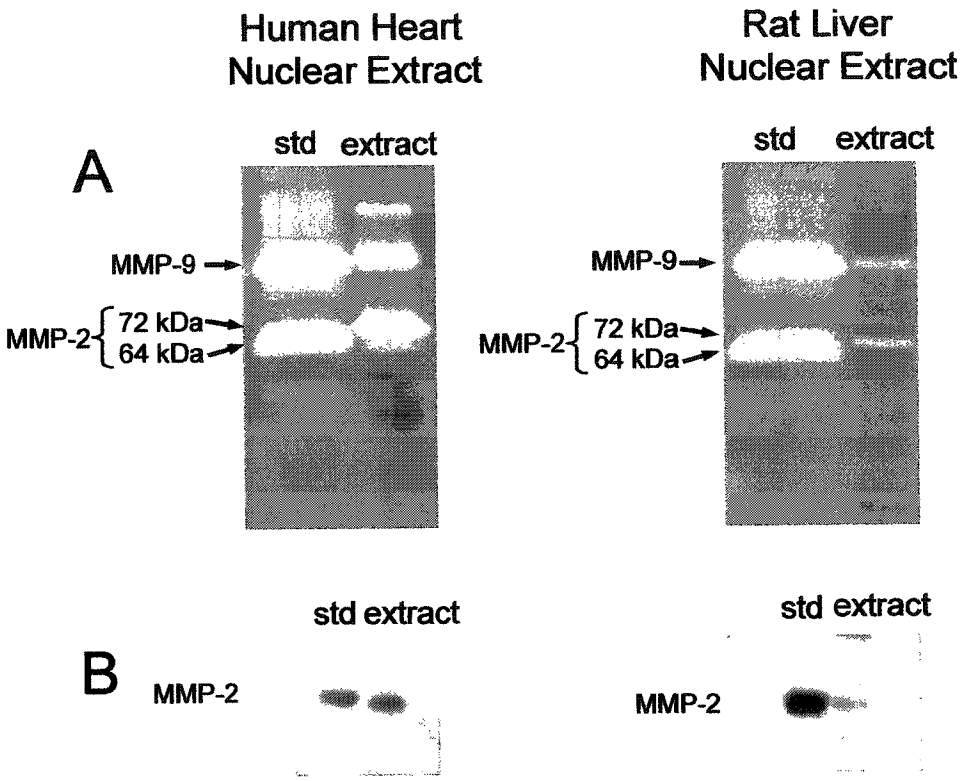


Figure 3.3 Gelatinase Activity and MMP-2 Expression in Nuclear Extracts

A) Gelatin zymograms for human heart (left) and rat liver (right) nuclear extracts (10 μg/lane). std indicates culture medium from HT 1080 cells, which possesses both MMP-9 and MMP-2 activities. While it is evident that both the nuclear extracts contain both MMP-9 and 72 kDa MMP-2 there is less activity present in the rat liver nuclear extract. A lesser amount of 64 kDa MMP-2 activity is evident in the human heart nuclear extract. As well, there is an unknown band that appears ~130 kDa in the human heart nuclear extract. (n = 5) **B)** After loading 10 μg of protein into each lane, both extracts were examined for the presence of 64 kDa MMP-2 by western blot. Similar to the zymography results, MMP-2 is present in both nuclear extracts, however, there is less in the rat liver. (n = 4)

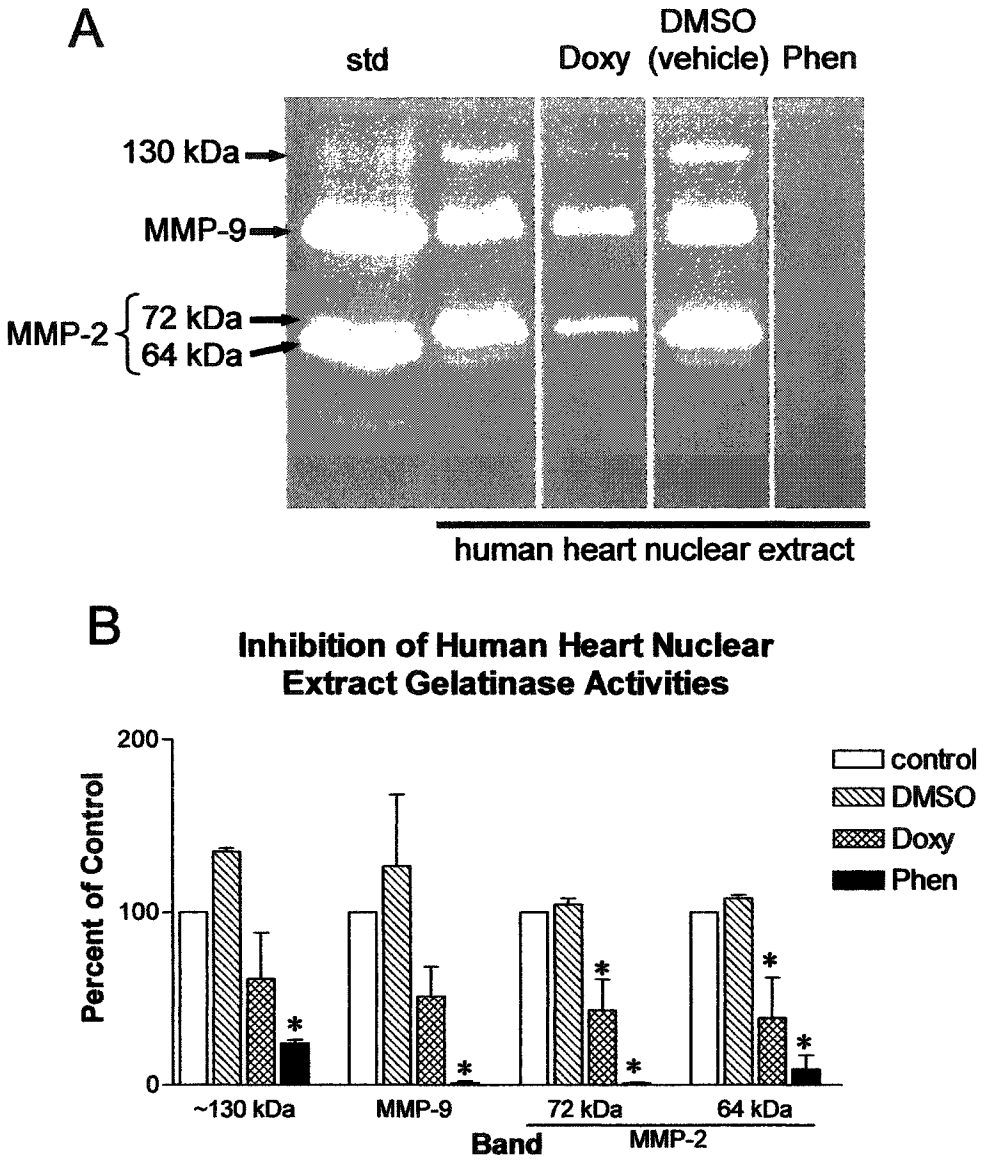


Figure 3.4 Inhibition of Gelatinase Activity in Human Heart Nuclear Extracts

A) Zymogram depicting the inhibition of human heart nuclear extract gelatinase activity by various MMP inhibitors. std is culture medium from HT 1080 cells, which shows both MMP-2 and MMP-9 activity. The second lane from the left is the human heart nuclear extract alone. Tetracycline antibiotic, doxycycline (Doxy, 100 μM) caused partial inhibition of all gelatinolytic activity, while phenanthroline (Phen, 100 μM) caused a complete inhibition. The vehicle for phenanthroline (DMSO, <0.05%) did not affect gelatinase activity. **B)** Summary densitometric analysis of the changes in gelatinase activities ($n = 4$, $^*p < 0.05$ compared to control).

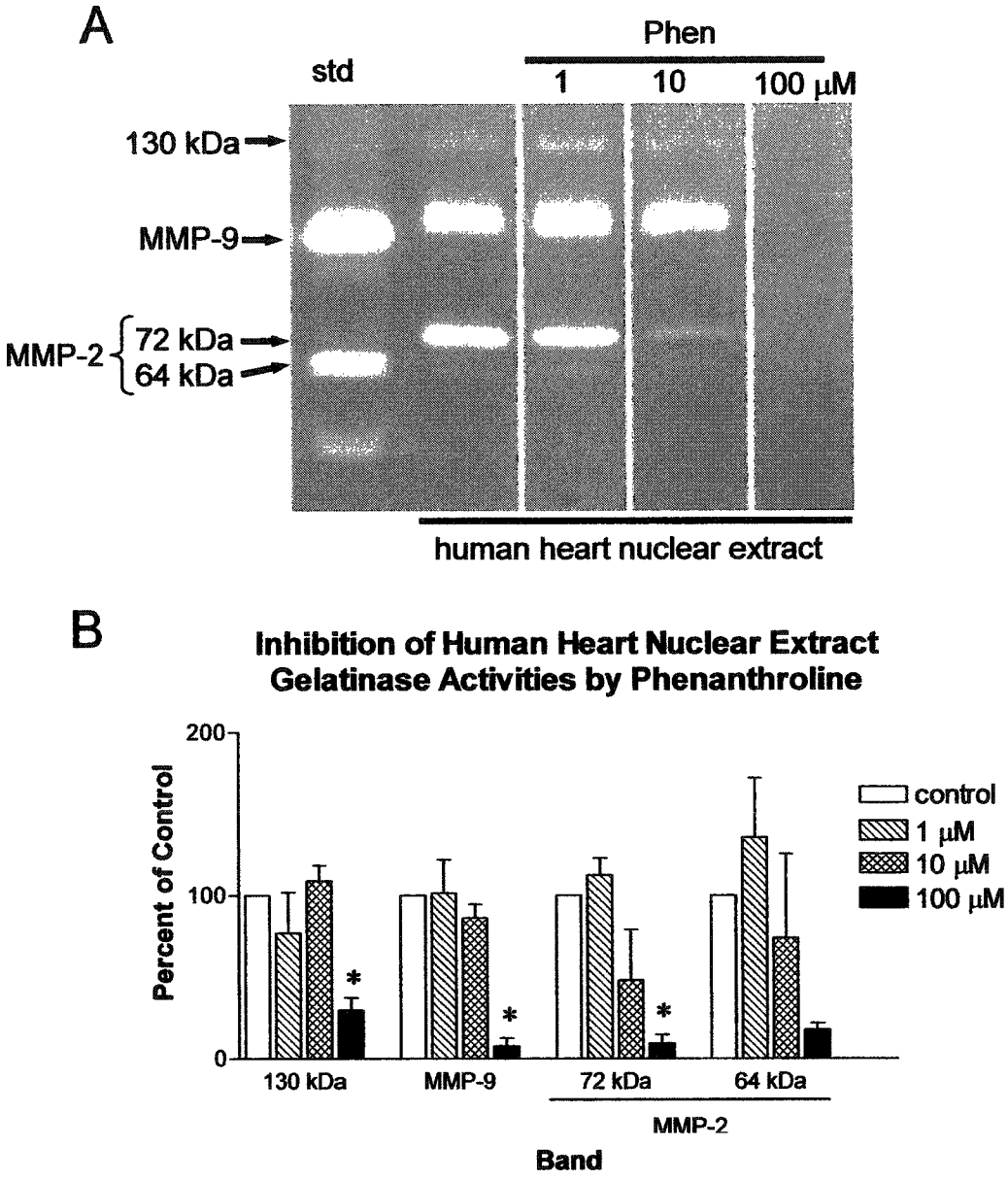


Figure 3.5 Concentration Dependent Inhibition of Gelatinase Activities by Phenanthroline

Phenanthroline (Phen) inhibits the gelatinase activity of human heart nuclear extracts in a concentration-dependent manner. **A)** Zymogram of the inhibition. Both MMP-2 and MMP-9 activity is seen in std, culture medium from HT 1080 cells. The second lane is the human heart nuclear extract alone. The following lanes are samples that have been subjected to increasing concentrations of phenanthroline, a gelatinase inhibitor. **B)** Summary densitometric analysis of the changes in gelatinase activities ($n = 3$, $^*p < 0.05$ compared to control).

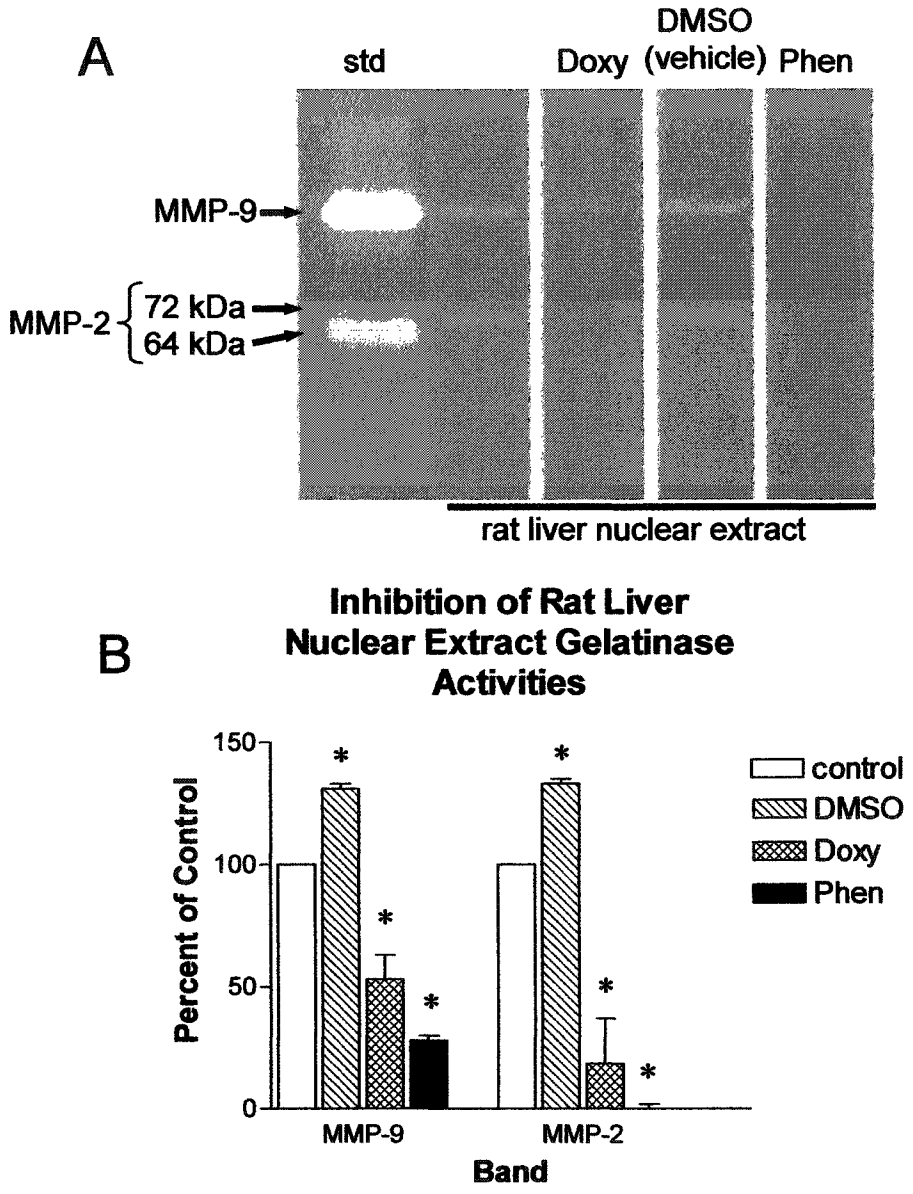


Figure 3.6 Inhibition of Gelatinase Activity in Rat Liver Nuclear Extracts

A) Zymogram depicting the inhibition of rat liver nuclear extract gelatinase activity by various MMP inhibitors. std is culture medium from HT 1080 cells expressing both MMP-2 and MMP-9 activity. The second lane from the left is the rat liver nuclear extract alone. The third lane from the left was a sample that was incubated in the presence of the tetracycline antibiotic, doxycycline (Doxy, 100 μM) that caused partial inhibition of gelatinolytic activity. Phenanthroline (Phen, 100 μM) abolished gelatinolytic activity, while the vehicle for phenanthroline (DMSO, <0.05%) did not affect gelatinase activity. **B)** Summary densitometric analysis of these bands ($n = 4$, $^*p < 0.05$ compared to control).

analysis of the higher molecular weight bands of the human heart nuclear extract. The vehicle for phenanthroline (DMSO, <0.05%) did not have any inhibitory effect, although it did enhance the activities of the rat liver nuclear extracts. The inhibitory effect of phenanthroline was concentration dependent (Figure 3.5) with 1 μM being subthreshold and 100 μM showing significant inhibition of all gelatinolytic activities. The zymograms for the rat liver extracts reflected a similar pattern, however, there was less basal activity as seen previously (Figure 3.6). Therefore, the gelatinase activities seen in the nuclear extracts can be attributed to MMPs.

A) PARP DEGRADATION

1) PARP Sequence Analysis

Various 8 amino acid MMP-2 cleavage recognition sequences were compared to the primary protein sequence of bovine PARP to examine possible cleavage sites. Among the sites found, there were three with a homology of > 60% for all 8 amino acids that were identified (Figure 3.7). Sites centered around amino acid 308 and 385 had a 62.5% homology to two different MMP-2 cleavage sequences. The site at amino acid 500 showed a 75% homology to the GPKGSRGA MMP-2 cleavage sequence. This site also showed high homology to 11 other MMP-2 cleavage recognition sequences (2 with 62.5% and 9 with 57.1% homology for 7 amino acids).

2) *In Vitro* PARP Degradation

One of the potential nuclear targets of MMP-2 is poly (ADP-ribose) polymerase (PARP). This enzyme, which is essential in DNA repair, resides in the nucleus and is subjected to proteolytic cleavage as a means to terminate its actions. It is conventionally

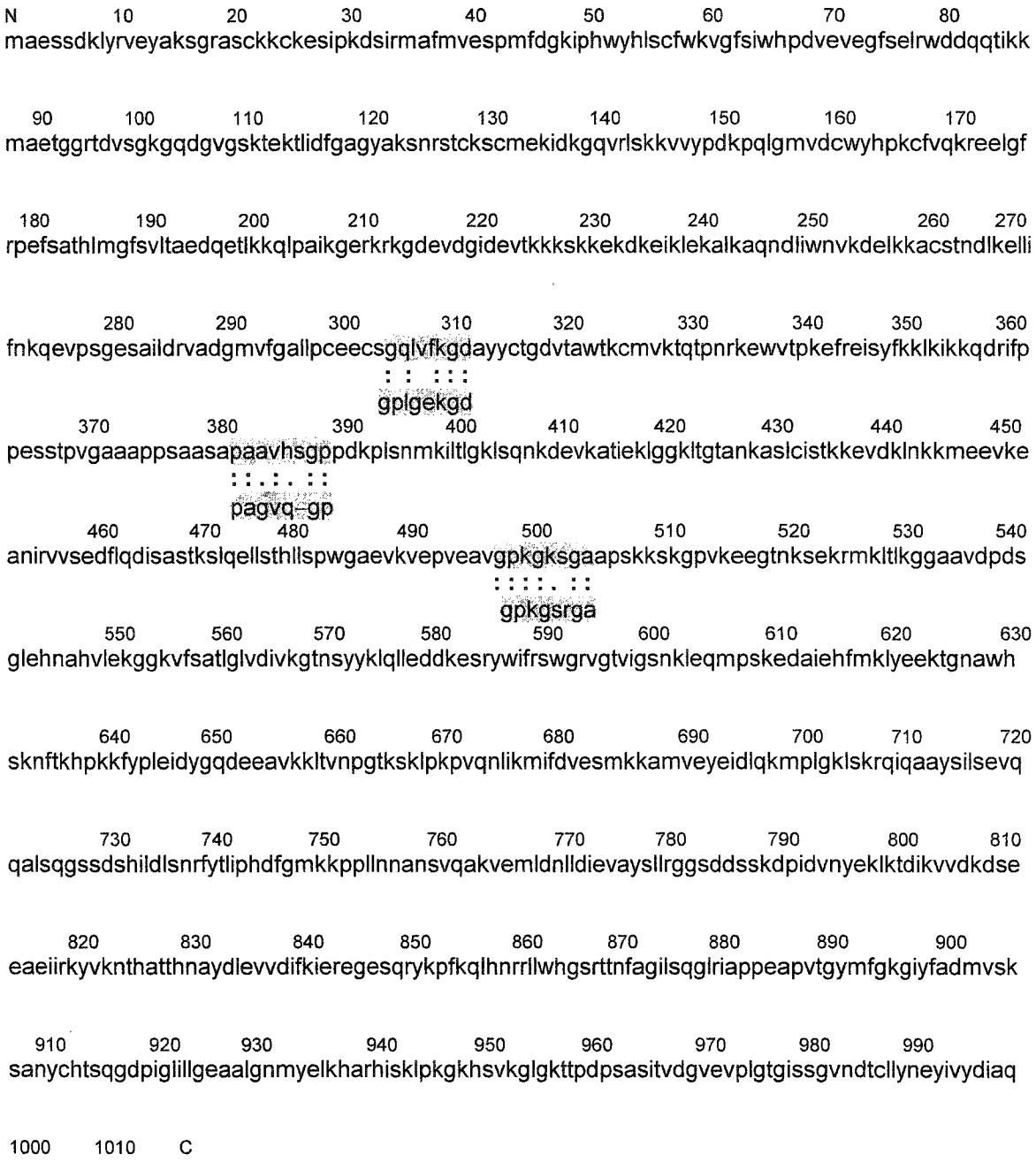


Figure 3.7 Possible MMP-2 Cleavage Sites in the Bovine PARP Sequence

Bovine PARP sequence sites with greater than 60% homology to various 8 amino acid MMP-2 cleavage recognition sequences. Comparative sequencing produces two sites at ~310 and ~380 with 62.5% homology and one site at ~500 with 75% homology. The site at ~500 is also homologous to two further 8 amino acid MMP-2 cleavage recognition sequences with 62.5% homology. Two dots denote the same amino acid whereas one dot denotes a similar amino acid.

known to be degraded to a 85 kDa product by caspase-3 (Tewari *et al.,* 1995). Following a 20 min (Figure 3.10) or 2 hr (Figure 3.8) incubation of various concentrations of human recombinant MMP-2 with bovine PARP, silver stain analysis revealed a concentration-dependent loss of the 116 kDa PARP band (Figure 3.9 A and 3.11 A). However, degradation by MMP-2 did not yield the expected 85 kDa degradation product, but rather a ~66 kDa band (Figure 3.8 A and Figure 3.9 A). However, this band failed to appear with only a 20 min incubation period (Figure 3.10 A and Figure 3.11 A). As well, there appears to be a contaminating band at <45 kDa in all lanes containing PARP. Caspase-3 was incubated with PARP as a positive control (Figure 3.8 B, lane 1), which depicts the expected 85 kDa degradation band. To eliminate the possibility of the ~66 kDa band, seen in Figure 3.8 A, being MMP-2 itself, MMP-2 was run alone under the same conditions and there were no visible bands (Figure 3.8 B, lane 2).

Western blot analysis of the 2 hr incubation also demonstrated a concentration-dependent loss of the 116 kDa PARP band and the appearance of a degradation product of <48 kDa (Figure 3.8 C). After a 20 min incubation with MMP-2, there was no visible loss of the 116 kDa band, however, the <48 kDa band does appear at this time (Figure 3.10 B and Figure 3.11 B). The 66 kDa band seen in the silver stain was not detected by western blot, possibly due to the absence of a compatible epitope. While a band of ~40 kDa did also appear in the silver stained gel, this was apparently a contaminant in the PARP standard (Figure 3.8 A).

3) Pharmacological Inhibition of PARP Degradation by MMP-2

MMP inhibitors were added to the *in vitro* PARP degradation assay to demonstrate that PARP degradation was indeed due to MMP-2 activity. Both silver stain analysis and

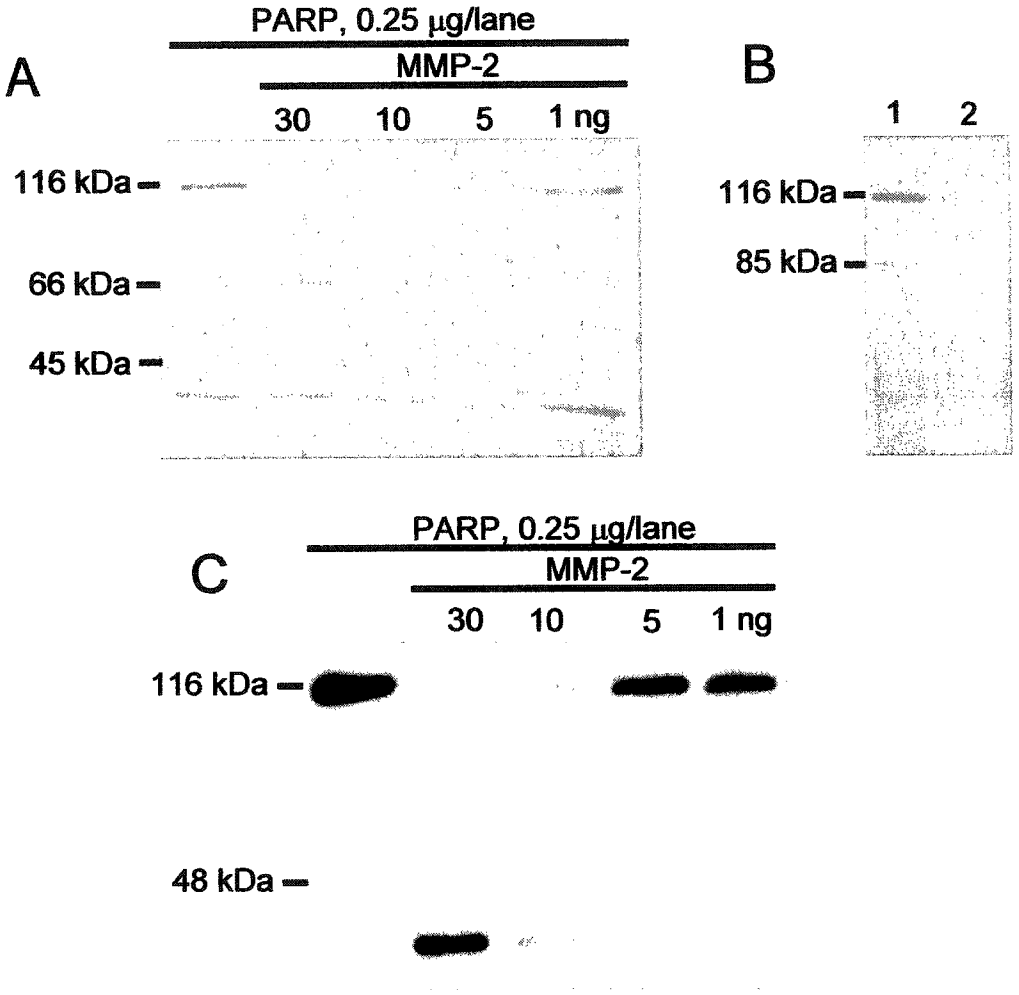


Figure 3.8 ***In Vitro*** **Degradation of PARP by MMP-2 (2 hr incubation)**

Concentration dependent degradation of PARP after 2 hr *in vitro* incubation with MMP-2. **A)** Silver stained SDS-PAGE depicting this degradation. The left lane is the commercial preparation of PARP alone, which shows bands at both 116 kDa (PARP) and an unknown band of <45 kDa. Increasing amounts of MMP-2 cause a loss of the 116 kDa band and the appearance of a band at 66 kDa. **B)** Lane 1: 0.25 µg of PARP was incubated with caspase-3 (30 ng) for 2 hr as a positive control. Caspase-3 degrades PARP to the expected 85 kDa degradation product. Lane 2: MMP-2 (30 ng) was incubated and run alone to eliminate the possibility of the 66 kDa band, seen in A, being MMP-2 itself instead of a PARP degradation product. **C)** Western blot of the *in vitro* degradation using a polyclonal PARP antibody. The antibody detects the band at 116 kDa (PARP). Addition of MMP-2 shows a concentration dependent loss of the 116 kDa band. However, the 66 kDa degradation band is not detected. Instead a band at <48 kDa appears in a concentration dependent manner. (n = 5)

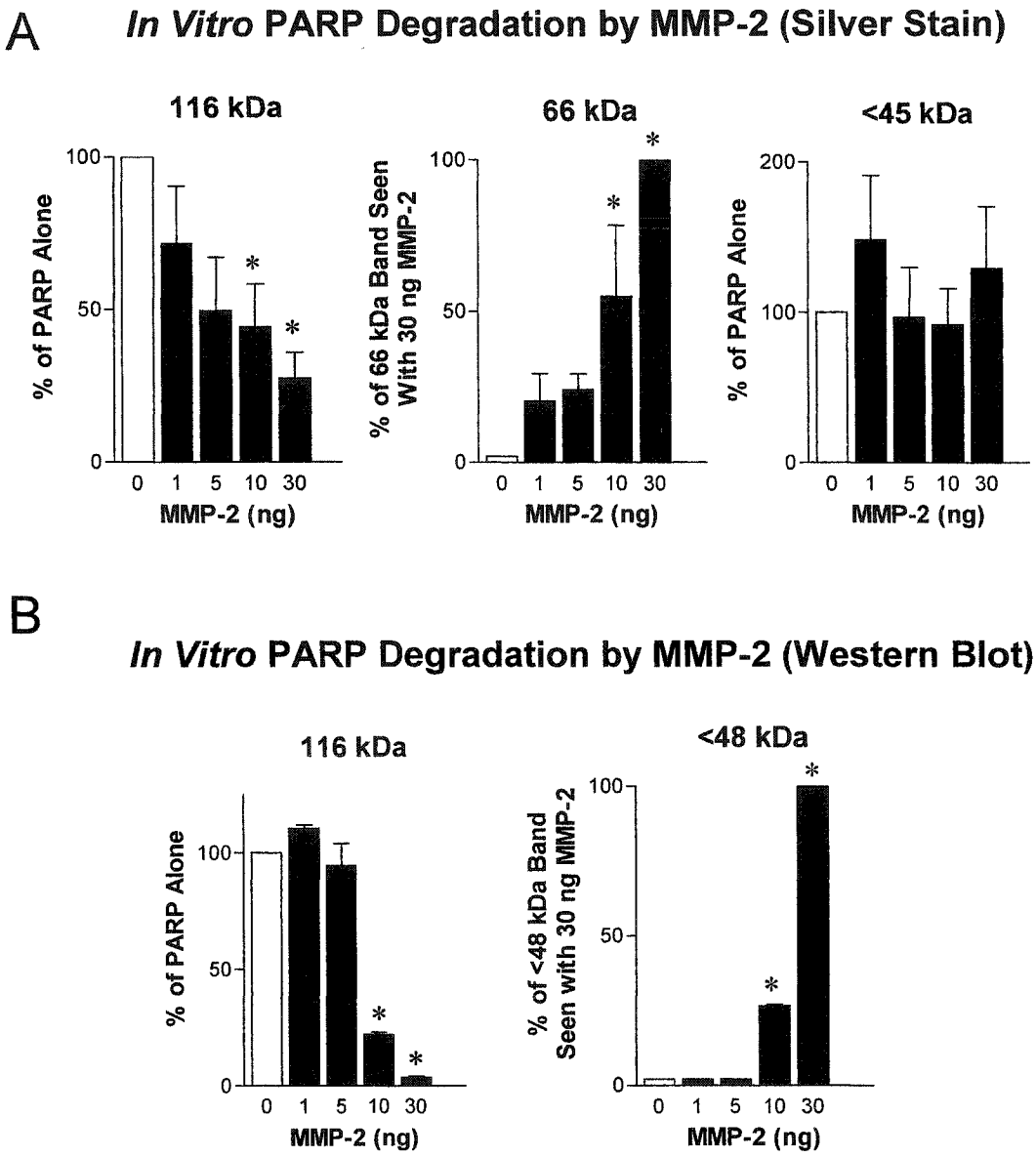


Figure 3.9 **Densitometric Analysis of *In Vitro* PARP Degradation (2 hr incubation)**

Summary densitometric analysis of PARP and its degradation products as depicted in Figure 3.8. **A**) Analysis of silver stained SDS-PAGE gels (n = 4) reveals a concentration-dependent decrease in the 116 kDa PARP band with a corresponding increase in a 66 kDa degradation product. **B**) Densitometric analysis of western blots for PARP (n = 5) also demonstrate a concentration-dependent decrease in the 116 kDa PARP band. However, there is a corresponding appearance of a band at <48 kDa. (*$p < 0.05$ compared to PARP alone).

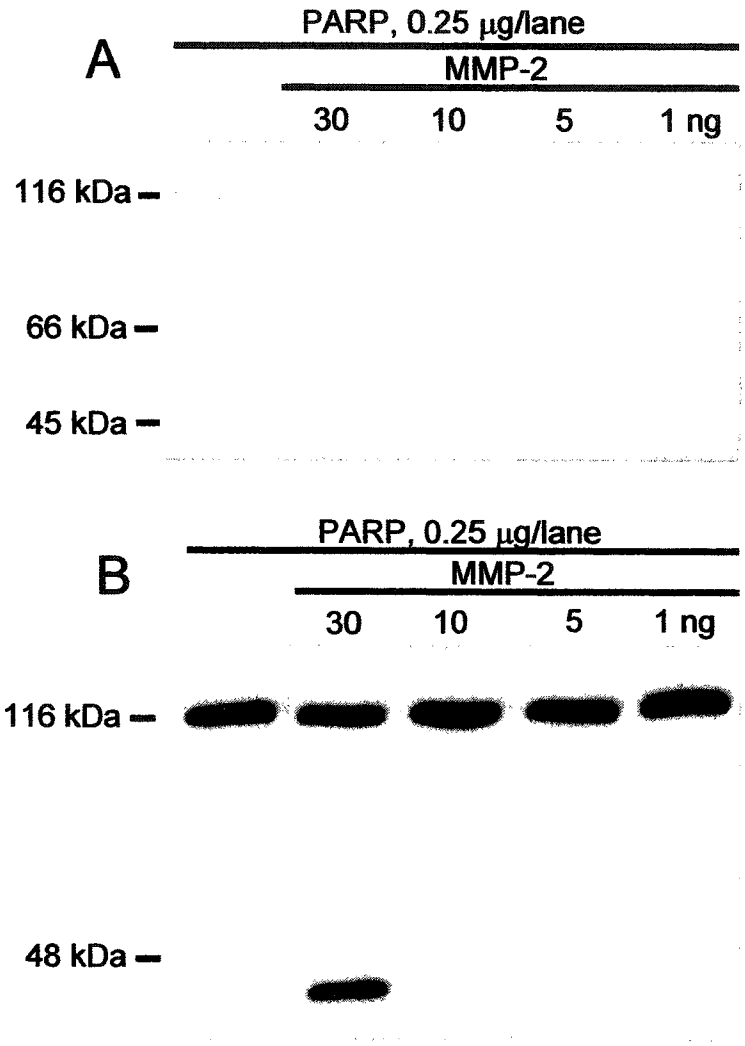


Figure 3.10 ***In Vitro*** **Degradation of PARP by MMP-2 (20 min incubation)**

Concentration dependent degradation of PARP after 20 min *in vitro* incubation with MMP-2. **A)** Silver stained SDS-PAGE depicting this degradation. The left lane is the commercial preparation of PARP alone, which shows the 116 kDa PARP band. Increasing amounts of MMP-2 cause a loss of this band. **B)** Western blot of the *in vitro* degradation using a monoclonal PARP antibody. The antibody detects the band at 116 kDa (PARP). Addition of MMP-2 shows a concentration dependent appearance of a band at <48 kDa. (n = 5)

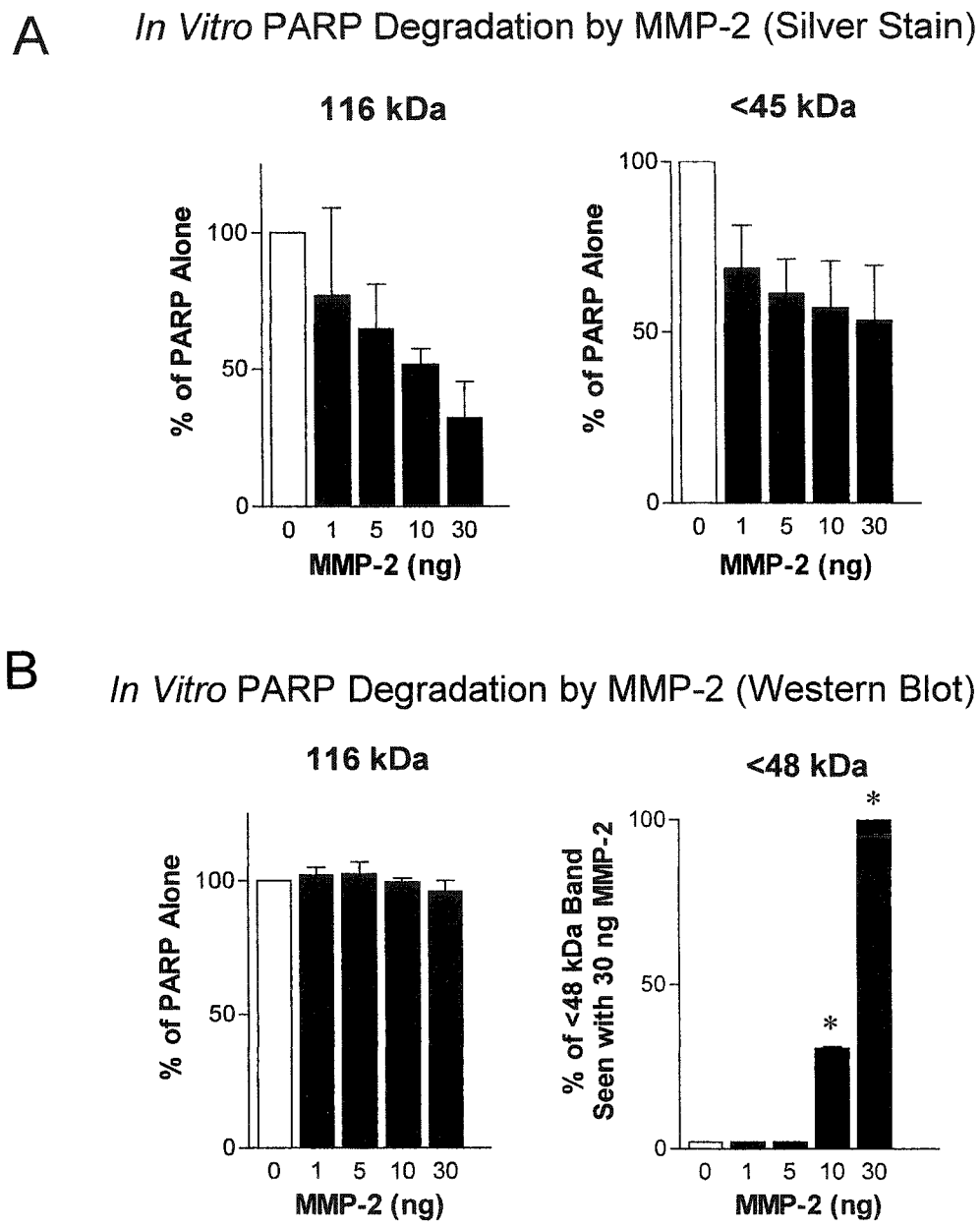


Figure 3.11 Densitometric Analysis of *In Vitro* PARP Degradation by MMP-2 (20 min incubation)

Summary densitometric analysis of representative data shown in Figure 3.10. Although the decrease in the 116 kDa PARP band is evident in the silver stained SDS-PAGE gels (**A**), it is not apparent by western blotting for PARP (**B**). However, the western blots reveal the accumulation of a <48 kDa degradation band with increasing concentrations of MMP-2 ($n = 5$, $^*p < 0.05$ compared to PARP alone).

western blot revealed that TIMP-2 (0.5 μM) and doxycycline (100 μM) could both inhibit PARP degradation by MMP-2 after both 2 hr (Figure 3.12) and 20 min of incubation (data not shown). Densitometric analysis of these bands shows a statistically significant loss of the 116 kDa PARP band in the presence of 30 ng MMP-2 in the western blots (Figure 13.3 B) but not by silver staining (Figure 3.13 A), although qualitatively, the effect was similar. However, both the silver stained gels (Figure 3.13 A) and the western blots (Figure 3.13 B) show that the MMP inhibitors can attenuate the appearance of the 66 kDa and < 48 kDa degradation products, respectively. Interestingly, a faint band between 48 and 80 kDa can be seen in the western blot when TIMP-2 was used as an inhibitor (Figure 3.12 B), the identity of which is unknown.

The suggestion was made that perhaps PARP could activate MMP-2 in the nucleus so that varying concentrations of PARP (0, 0.1, 0.3, 1 or 3 μg) were incubated (0, 20 or 120 min at 37°C) with the human heart nuclear extract and then analyzed by zymography to assay for gelatinase activity. However, there was no noticeable change in the activities of MMP-9 or MMP-2 at either 72 kDa or 64 kDa (data not shown).

4) *Ex Vivo* Studies for PARP Degradation

Western blots for PARP were run on ventricular homogenates from isolated perfused rat hearts in two models of oxidative stress, which are known to activate myocardial MMP-2. Hearts were either subjected to a 15 minute infusion of 80 μM $ONOO^-$ as a model of exogenous oxidative stress, or subjected to 20 minutes of global, no-flow ischemia followed by 30 minutes of reperfusion as a model of endogenous oxidative stress. Both models of oxidative stress injury showed significant loss of cardiac mechanical function (Tables 3.1 and 3.2). Importantly, there was already notable PARP

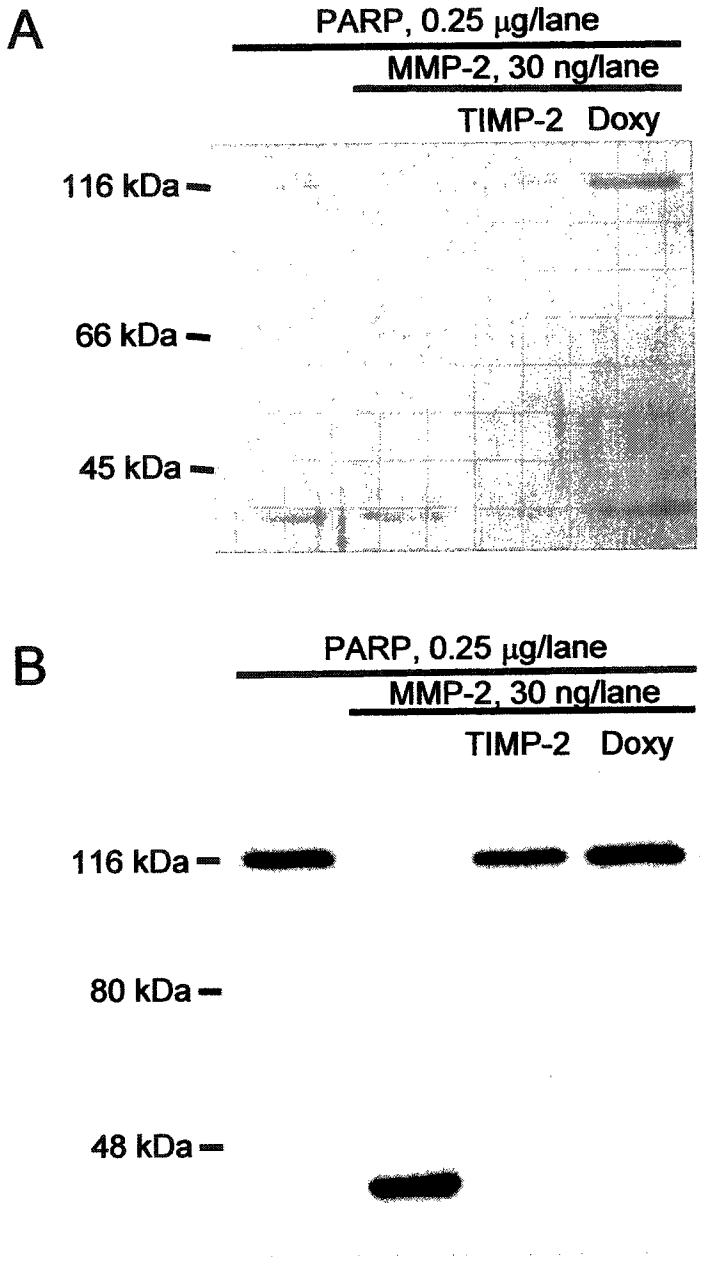


Figure 3.12 Inhibition of *In Vitro* Degradation of PARP by MMP-2 (2 hr incubation)

A) Silver staining analysis of a SDS-PAGE gel reveals bands at 116 kDa and <45 kDa for PARP alone (left lane). The addition of MMP-2 shows a loss of the 116 kDa band with the appearance of a 66 kDa degradation product. Both 0.5 µM TIMP-2 and 100 µM doxycycline (Doxy) inhibit this degradation. **B)** A PARP western blot of the same conditions reveals a 116 kDa band for PARP alone, while the addition of MMP-2 shows a complete loss of the 116 kDa band and the appearance of a band at <48 kDa. (n = 3)

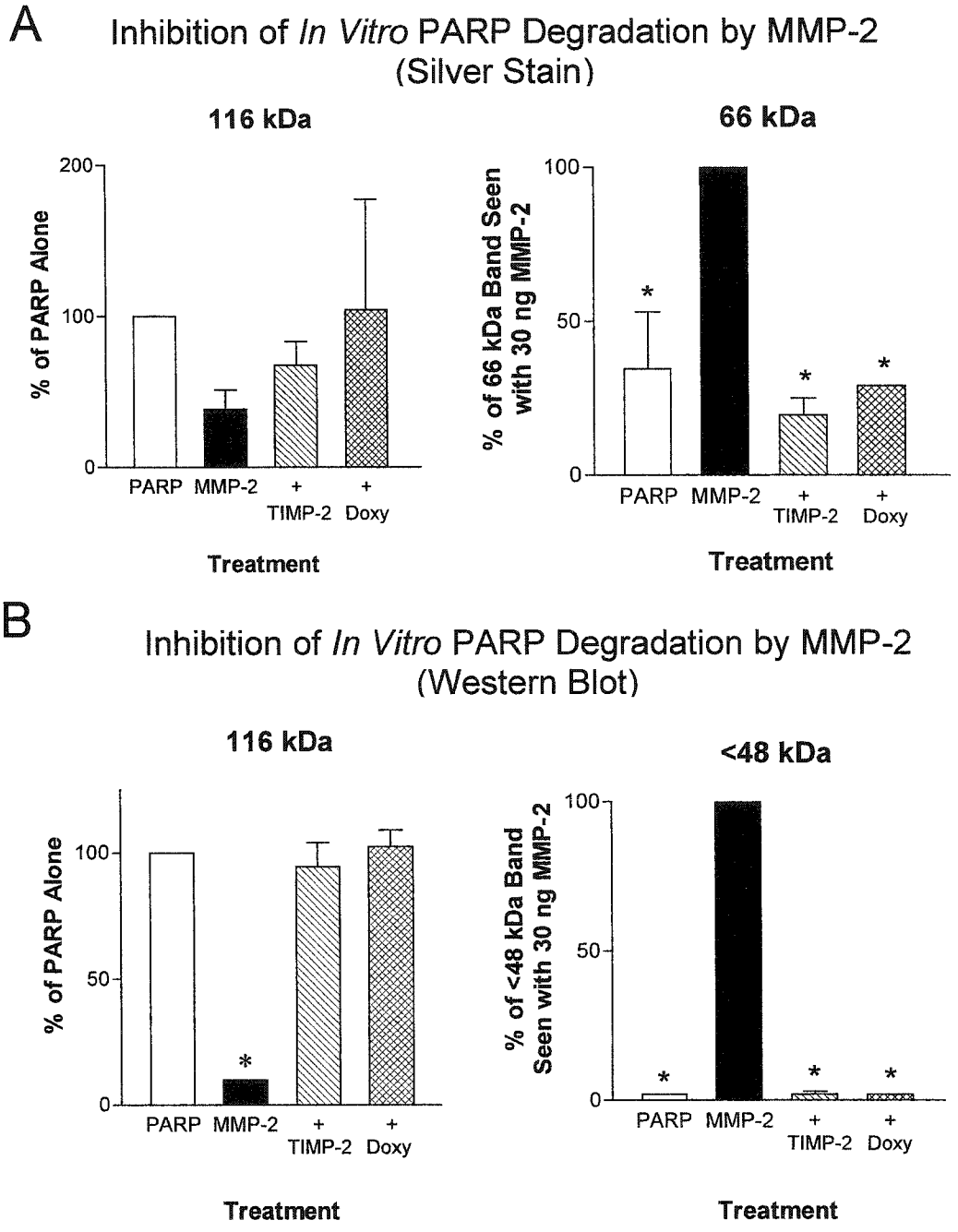


Figure 3.13 Densitometric Analysis of Inhibition *In Vitro* PARP Degradation (2 hr incubation)

A) Densitometric analysis of silver stained SDS-PAGE gels depicted in Figure 3.12, showing the protective effect of the MMP inihibtors in preventing the loss of the 116 kDa PARP band and the appearance of the 66 kDa degradation product. Analysis of western blots (**B**) also shows the protection from 116 kDa PARP degradation, as well as preventing the accumulation of the <48 kDa degradation product. *$p < 0.05$ compared to PARP alone, *$p < 0.05$ compared to MMP-2

	HR (beats/min)		LVDP (mmHg)		HR x LVDP	
	baseline	15 min	baseline	15 min	baseline	15 min
dec. $ONOO^-$	331 ± 12	306 ± 38	54 ± 12	50 ± 10	18230 ± 4234	14810 ± 3536
$ONOO^-$	311 ± 7	265 ± 31	57 ± 5	29 ± 9*	17470 ± 1629	8339 ± 3281*
$ONOO^-$ + PD166793	281 ± 24	257 ± 32	58 ± 3	62 ± 6	16590 ± 1933	15680 ± 1919
$ONOO^-$ + GSH	291 ± 12	279 ± 14	53 ± 6	55 ± 10	15510 ± 2011	15550 ± 2991

*$p < 0.05$ compared to baseline
n = 6 hearts per group

Table 3.1 Functional Data for Peroxynitrite Infused Hearts

Heart rate (HR) and left ventricular developed pressure (LVDP) both before (baseline) and after 15 minutes of infusion with either decomposed peroxynitrite (dec. $ONOO^-$) or peroxynitrite ($ONOO^-$, 80 μM). Other hearts were co-infused with the MMP inhibitor PD 166793 (2 μM) or glutathione (GSH, 300 μM), an antioxidant. There was a significant decrease in LVDP and HR x LVDP upon peroxynitrite infusion that was prevented by PD 166793 or GSH.

	HR (beats/min)	LVDP (mmHg)	HR x LVDP
Aerobic	286 ± 17	80 ± 9	22970 ± 3179
I/R	302 ± 19	25 ± 10*	6708 ± 2511*
I/R + Doxy	294 ± 12	62 ± 9†	18510 ± 3043†
I/R + Phen	257 ± 9	78 ± 7†	20030 ± 1502†

*$p < 0.01$ compared to aerobic
†$p < 0.01$ compared to I/R
n = 6 hearts per group

Table 3.2 Functional Data for Ischemia-Reperfused Hearts

Heart rate (HR) and left ventricular developed pressure (LVDP) for hearts subjected to a 75-minute aerobic perfusion in Langendorff mode or those subjected to 20 minutes of ischemia followed by a 30 minute reperfusion period (I/R). Additional hearts were infused with doxycycline (Doxy, 100 μM) or phenanthroline (Phen, 100 μM), two MMP inhibitors, for both ten minutes before and after ischemia. Both doxycycline and phenanthroline attenuated the decrease in function caused by ischemia-reperfusion injury.

degradation in the respective control hearts (Figures 3.14 and Figure 3.15). The co-infusion of $ONOO^-$ with 2 μM PD-166793, a MMP inhibitor, or with the antioxidant glutathione (50 μM) to the hearts improved the mechanical function (Table 3.1) but did not alter the PARP degradation pattern (Figure 3.14). Nor did the MMP inhibitors, doxycycline (100 μM) or phenanthroline (100 μM), alter the PARP degradation pattern of the ischemia-reperfused hearts (Figure 3.15), despite improving their function (Table 3.2). There was no change in the gelatinase activity of any of these hearts compared to the controls (data not shown).

In all of the experimental hearts, from both the peroxynitrite study (Figure 3.14) and the ischemia-reperfusion study (Figure 3.15), the 116 kDa band was apparent, as was the expected 85 kDa degradation band. Importantly, both a band of approximately 66 kDa and another one of about 40 kDa were apparent in the western blots under all conditions.

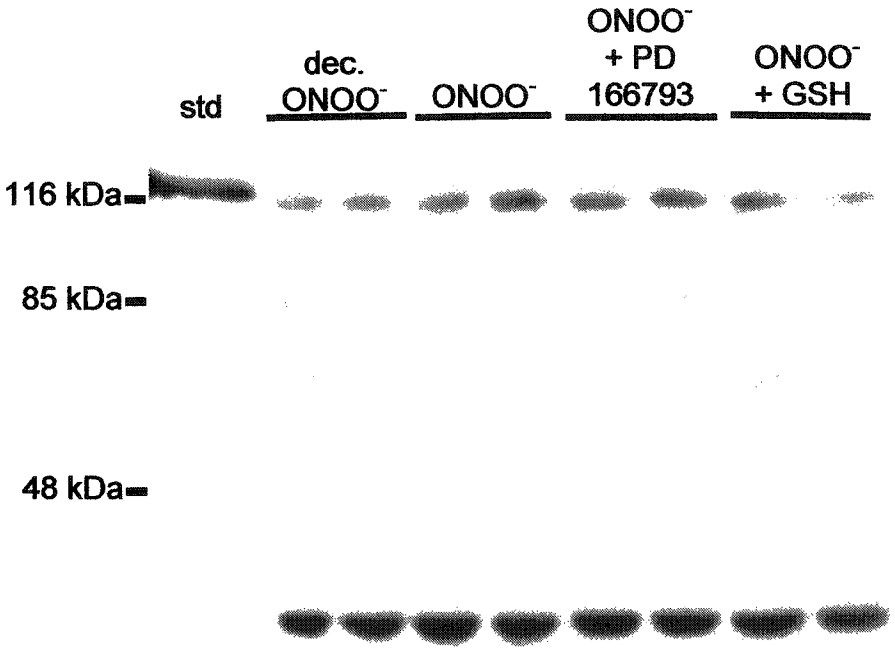


Figure 3.14 Western Blot Analysis of PARP Degradation in Isolated Perfused Rat Hearts Infused With Peroxynitrite

Isolated perfused rat hearts (two per group shown, n=4 total) were homogenized and extracts were prepared before SDS-PAGE and immunoblotting for PARP. std denotes PARP from whole cell extracts of human HL60 leukemia cells. 20 μg of each extract was loaded into each lane. There was no visible change in the PARP degradation pattern whether the hearts were aerobically perfused or infused for 15 min either with decomposed peroxynitrite (dec. $ONOO^-$) or 80 μM peroxynitrite ($ONOO^-$) alone or co-infused with either a MMP inhibitor, PD 166793 (2 μM) or the antioxidant glutathione (300 μM). However, it is important to note that the degradation pattern includes bands at ~66 kDa and <48 kDa. *Hearts perfused by Dr. Wenjie Wang.*

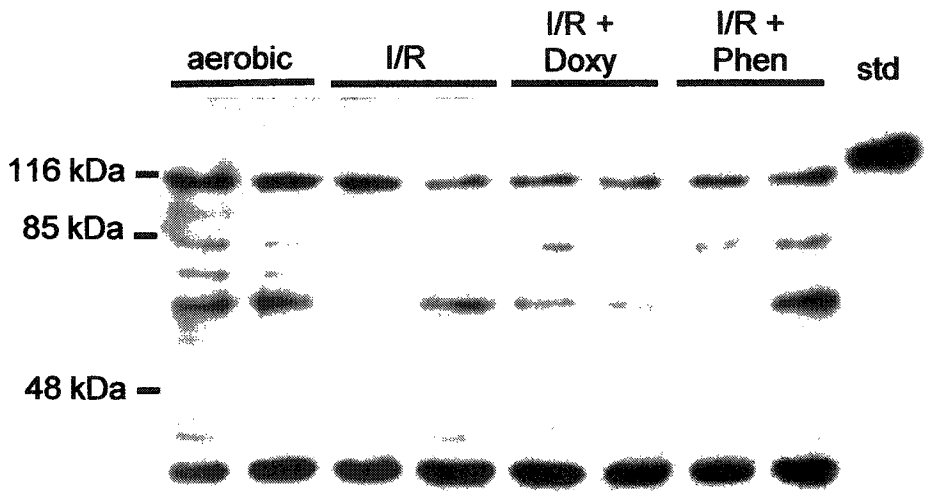


Figure 3.15 Western Blot Analysis of PARP Degradation in Isolated Perfused Rat Hearts Subjected to Ischemia-Reperfusion Injury

Homogenates (20 μg/lane) from isolated perfused rat hearts (two per group shown, n=4 total) were run on a SDS-PAGE and immunoblotted to determine the PARP degradation pattern. std indicates PARP from whole cell extracts of human HL60 leukemia cells. After 20 minutes ischemia and 30 minutes of reperfusion (I/R), the PARP degradation pattern did not change in comparison to the aerobically perfused hearts. The addition of the MMP inhibitors doxycycline (Doxy, 100 μM) or phenanthroline (Phen, 100 μM) did not alter this pattern. However, there is evidence for degradation bands at ~66 kDa and <48 kDa. *Hearts perfused by Dr. Wenjie Wang.*

CHAPTER IV

DISCUSSION

A) The Presence of MMP-2 in the Nucleus

Although MMPs are traditionally known for their ability to act on the extracellular matrix, there is increasing evidence for more diversified targets of these proteases. Indeed, novel MMP-2 targets include the vasoactive peptides big endothelin-1 (Fernandez-Patron *et al.*, 1999) and calcitonin gene-related peptide (Fernandez-Patron *et al.,* 2000), as well as troponin I (Wang *et al., in press*). This last protein, a regulatory protein of the contractile actin-myosin ATPase, is an intracellular target associated with the sarcomeres whose cleavage may contribute to myocardial contractile dysfunction following ischemia-reperfusion. These novel targets identify novel roles for MMPs and suggest possible intracellular actions, which warrant further investigation into other potential biological functions and locations of MMP-2.

Although there have been many studies which identify MMPs in different tissue types in the body, over the past decade investigators have begun to delve deeper into the localization of these enzymes. Indeed the intracellular localization of MMP-2 and -9 (Coker *et al.*, 1999; Rouet-Benzineb *et al.*, 1999) has already been observed in both isolated cardiac myocytes and human left ventricular tissue. In accordance with the confocal studies by Rouet-Benzineb *et al.* (1999), our lab has recently shown that, in isolated rat hearts after ischemia-reperfusion, MMP-2 can be seen within the sarcomeres of cardiac myocytes, closely associated with the myofilaments (Wang *et al., in press*), as well as in mitochondria. These results, which include biochemical evidence showing the co-localization of MMP-2 with troponin I, were shown by both confocal immunofluorescence and immunogold electron microscopy. Thus, a growing amount of evidence is developing that reveals the localization of MMP-2 within the cell.

In this study I was able to identify that MMP-2 is associated with cardiac myofibrils (sarcomeres) and within mitochondria in accordance with Wang *et al.* (*in press*). In addition, I found that MMP-2 is also present in the nucleus, which became the focus of this thesis. I investigated this further due to the presence of the nuclear matrix, which may represent a site of action for MMP-2.

The nuclear matrix is a proteinacious structure that functions in a similar manner to the extracellular matrix, acting as an anchor for many molecules, much as the extracellular matrix anchors cells (Martelli *et al.,* 1997). Indeed nuclear matrix degradation is integral in DNA fragmentation (Kaufmann *et al.,* 1993), DNA conformation and unwinding (Ruh *et al.,* 1996) and chromatin condensation (Oberhammer *et al.,* 1994). Thus there may be a possible role for MMPs in reorganizing this matrix.

In this study, both immunogold electron microscopy of rat heart ventricles (Figure 3.1) and biochemical assays on nuclear extracts from both human heart and rat livers showed evidence of MMP-2 within the nucleus (Figure 3.3). Although both nuclear extracts had gelatinase activities corresponding to both MMP-2 and its related gelatinase MMP-9, both were predominantly found at molecular weights corresponding to the zymogen forms (proMMP-2 and –9). However, active MMP-2 (64 kDa) could also be seen in the human heart nuclear extract zymograms and in both nuclear extracts by western blotting (Figure 3.3). In order to verify that this gelatinase activity is indeed due to the MMPs, parallel zymograms were incubated in the presence of MMP inhibitors. Both doxycycline, a tetracycline antibiotic shown to inhibit MMPs independent of its antibiotic activity (Golub *et al.,* 1991), and phenanthroline, a zinc chelator (Latt *et al.,*

1969), reduced the gelatinase activity in both nuclear extracts (Figures 3.4 to 3.6). The greater potency of phenanthroline as an MMP inhibitor in comparison to doxycycline was as expected from previous studies (Cheung *et al.,* 2000).

The ~130 kDa band of gelatinase activity that was seen in the human heart nuclear extract but not in the standard or rat liver nuclear extract is most likely the 135 kDa neutrophil derived gelatinase (Kjeldsen *et al.,* 1993). This gelatinase was found to be a complex of the 92 kDa MMP-9 and an associated 25 kDa protein called NGAL (neutrophil gelatinase-associated lipocalin) and could possibly appear if there was significant neutrophil adherence when the heart extract was prepared.

Although we have been able to show that MMP-2 is indeed present in the nucleus, primarily in the proMMP-2 form, it is not known how the enzyme arrives there. Personal communications from Dr. Patrick Anglard (INSERM, Illkirch, France) indicates that they have found a nuclear isoform of stromelysin-3 (MMP-14) by studying its promoter. By looking at an inducible upstream promoter, Anglard's group has found that it controls the expression of beta-stomelysin-3, a novel isoform of this enzyme (Luo *et al.,* 2002). This isoform is directly translated into an active enzyme that is located in the nucleus. The protein resembles a N-terminal truncated form lacking ~20% of the catalytic site; however, the catalytic activity remains. Although Luo *et al.* (2002) have not demonstrated any role for this novel isoform, they suspect that it may be involved in the activity of promoter genes associated with the cell cycle or in apoptosis.

In addition, Luo *et al.* (2002) investigated several other MMPs for the presence of methionine in the N-terminal part of the catalytic domain (the start site of translation for this novel human beta-stromelysin isoform) and found it in the same position or nearby in

10 other human MMPs, however they did not investigate either MMP-2 or MMP-9. As well, there are seven other MMPs that have be identified to have more than one transcript (Sternlicht & Werb, 2001; Velasco *et al.,* 1999; Pei et al., 2000; Velasco *et al.,* 1999), six of these containing the methionine residue in the N-terminal part of the catalytic domain (Luo *et al.,* 2002). Therefore it is highly possible that MMP-2 found in the nucleus may be a novel isoform, however, this may involve a change in the molecular weight, which was not seen in this study and may be too small to be resolved within the SDS-PAGE. Yet both of the antibodies used recognize the catalytic domain or the hinge region of MMP-2, thus they should be able to detect an N-terminal truncated isoform of this enzyme.

B) PARP DEGRADATION BY MMP-2

One of the possible targets for MMP-2 in the nucleus is PARP. Although PARP is one of the primary enzymes involved in DNA strand repair (Szabo & Dawson, 1998), its presence in the nuclear matrix (Martelli *et al.,* 1997) makes it an attractive target in this study. As well, Lazebnik *et al.* (1994) have shown that PARP is degraded by a protease much like the interleukin-1β converting enzyme. This enzyme was later found to be caspase-3 (Tewari *et al.,* 1995), which cleaves PARP effectively in order to cause its inactivation. MMPs have also been found to cleave interleukin-1β (Ito *et al.,* 1996). Although MMPs do not activate interleukin-1β, they do degrade this molecule. Thus it may be possible that MMP-2 could be involved in the degradation of PARP.

In vitro investigation showed that MMP-2 can indeed degrade PARP in a concentration dependent manner (Figures 3.8 to 3.11). As well, in the presence of the

MMP inhibitors TIMP-2 or doxycycline, this degradation was markedly reduced (Figure 3.12 and Figure 3.13). Of interest is the appearance of a 66 kDa degradation band in the silver stained SDS-PAGE, which does not appear in the PARP western blot. A simple explanation for this may be that the epitope that the antibody is directed to does not recognize the 66 kDa fragment. In addition, there is a band at ~40 kDa, which is detected by the PARP antibody, yet in the silver stained gel it appears even in the absence of MMP-2. It may therefore be that this band is indeed a degradation fragment but that it is masked by a similar size contaminant which is observed in the silver stain.

Investigation of the PARP protein sequence (Figure 3.7) reveals three sites with a high homology to known MMP-2 cleavage recognition sequences. The site at amino acid 500 showed greater than 50% homology to at least 11 recognition sequences within seven amino acids, thus it is highly likely, although not conclusive, that MMP-2 cleaves at this site. On average, each amino acid within a peptide is about 100 Da. Hydrophobicity and secondary folding will influence the distance a polypeptide will run on a SDS-PAGE gel. Thus, given an approximate range of 10 kDa, it is possible that one of these cleavage sites could result in a band of 66 kDa.

Since both PARP and MMP-2 can be activated by $ONOO^-$, we have looked for PARP cleavage by MMP-2 in two *ex vivo* models of oxidative stress known to occur through the generation of $ONOO^-$. PARP is activated by DNA strand breakage caused by $ONOO^-$ (Gilad *et al.*, 1997; Szabo *et al.*, 1996, Ishida *et al.*, 1996). Okamoto *et al.* (2001) and Wang *et al.* (2002) have shown that $ONOO^-$ can activate MMPs in human neutrophils and in isolated perfused rat hearts, respectively. Therefore, it is possible that MMP-2 in the nucleus could act upon PARP via a mechanism initiated by $ONOO^-$.

Western blots for PARP were therefore run on heart homogenates prepared from isolated, perfused rat hearts that were subjected to $ONOO^-$ infusion (Figure 3.14). This was done with a concentration (80 μM) known to enhance the release of MMP-2 prior to an irreversible loss of cardiac mechanical function (Wang *et al.*, 2002). However, there was no change in the PARP degradation pattern in these hearts in comparison to those infused with decomposed $ONOO^-$ as a control. As well, the co-infusion of PD 166793, a MMP inhibitor, or the $ONOO^-$ scavenger glutathione, treatments that prevented $ONOO^-$-induced injury (Table 3.1), did not alter the degradation pattern. This is despite the notion that one might expect an attenuated degradation of PARP under these circumstances. However, the oxidative stress caused by the application of exogenous $ONOO^-$ via the coronary circulation may be smaller than that experienced by the heart in a pathological setting. We therefore looked at an ischemia-reperfusion injury model that is known to involve both endogenous generation of $ONOO^-$ (Yasmin *et al.*, 1997) and involves the activation of MMP-2 (Cheung *et al.*, 2000) in its pathogenesis.

After a 20 minute ischemic period followed by 30 minutes of reperfusion to simulate myocardial stunning injury, PARP western blots from these hearts showed no changes in comparison to the aerobic controls (Figure 3.15). The infusion of the MMP inhibitors doxycycline or phenanthroline into the hearts did not affect this degradation although their function was improved (Table 3.2). Cheung *et al.* (2000) have shown, in isolated rat hearts, an increased release of MMP-2 within the first few minutes of reperfusion after ischemia, followed by diminished tissue MMP activity. Recent evidence shows that ischemic-reperfused hearts also lose TIMP-4 and as a result, the myocardium shows a net positive proteolytic activity by *in situ* zymography (Schulze *et al., in review*).

Importantly, in both the homogenates from the $ONOO^-$ study and the ischemia-reperfusion study, western blots showed bands at ~66 kDa and at <48 kDa. These bands may coincide with the degradation bands in the *in vitro* assay as PARP is cleaved by MMP-2.

The relationship between PARP activation and its degradation is still uncertain. Although PARP inactivation may be due to mechanisms other than degradation, none have been shown. In fact, investigators tend to use the terms interchangeably. Benchoua *et al.* (2002) explain that degradation of PARP effectively inactivates the enzyme by disabling its ability to repair DNA strand breaks and to synthesize ADP-ribose polymers. However, although little evidence exists to suggest otherwise, it is possible that the cleavage of PARP, by MMP-2 or by caspases, simply results in its degradation after it has been inactivated by other means.

While there is little evidence that PARP degradation by MMP-2 is involved in oxidative stress injury, it may be beneficial to investigate other types of cellular injury or nuclear processes further. While evidence for MMPs in intracellular compartments is just now being investigated, both Zhao *et al.* (1998) and Ritter *et al.* (1999) have found a role for TIMP-1 in the nucleus. While Ritter *et al.* (1999) have shown that TIMP-1 translocates to the nucleus after binding to the surface of human MCF-7 breast carcinoma cells, Zhao *et al.* (1998) suggest that it may pass into the nucleus via an active transport mechanism against a concentration gradient (Melchior & Gerace, 1995). Both studies speculate that TIMP-1 present in the nucleus is involved in the cell cycle and in cell growth, and both discuss the possibility of the inhibition of a nuclear MMP. While Zhao *et al.* (1998) were unable to find TIMP-2 or TIMP-3 in the nucleus they did investigate

HeLa cells and detected a stromelysin-like MMP activity in the nuclear fraction. Perhaps this activity is the same as that of the new stromelysin-3 isoform observed by Luo *et al.* (2002). To date there have been no investigations into TIMP-4 in the nucleus.

In this study we have identified MMP-2 in the nucleus, primarily in the 72 kDa form. As well, we have shown that MMP-2 is capable of cleaving PARP *in vitro* to novel degradation products at ~66 kDa and <45 kDa. This cleavage would have similar effects as that of caspase-3 in inactivating the enzyme, thus playing a protective role when PARP is overactivated, or a detrimental one by inactivating PARP, thus preventing it from acting on DNA repair. Investigations of isolated perfused hearts have shown that although there is no change in the PARP degradation pattern after two types of oxidative stress injury, both the ~66 kDa band and the <45 kDa degradation bands are visible.

Since I have shown here that MMP-2 is able to cleave PARP *in vitro*, but there were no evident changes in PARP cleavage in hearts subjected to oxidative stress, in the absence or presence of MMP inhibitors, it is therefore possible that MMP-2 plays a minor role in the cleavage of PARP. Whether this possible action of MMP-2 is protective in that MMP-2 degrades PARP alongside caspase-3 in a pathological setting of energy depletion, or that MMP-2 causes further damage by degrading the PARP necessary for DNA repair is yet unclear. Perhaps the role of MMP-2 would become more apparent in a more severe model of injury such as a longer ischemic time, which would then induce apoptosis. However, although we have not found an express role for MMP-2 in the nucleus, we have opened up an important and novel area of research as a result of its detection there. As well, these novel findings could have an impact on the potential use of MMP inhibitors clinically, as they may affect certain nuclear processes.

C) LIMITATIONS

No scientific study is without limitations. Given the objectives of this study I have used a number of diverse protocols to address my hypothesis. The extensive use of antibodies for both the histochemical studies and the western blotting relies heavily on the specificity of the antibodies. In the immunogold electron microscopy, unrelated IgG was used as a negative control. As another negative control, several sections were also stained with the primary antibody omitted. Both negative controls showed little to no staining. Western blots for MMP-2 were verified by the zymograms, an enzymatic assay. However there is some question as to the specificity of the polyclonal PARP antibody used for the heart homogenate western blots. Multiple dilutions and different amounts of protein loading were tested. The recommended 1:1000 dilution of the antibody indeed was the ideal concentration, yet the multitude of bands that appear on the blots raises questions about its specificity. Other blots attempted with a monoclonal C2-10 PARP antibody (Oncogene) failed to detect any PARP in the samples at all (data not shown).

With any embedding technique, there is a brief moment of ischemia as the tissue is transferred from the animal or crystalloid perfusion solution to the fixation media. In the electron microscopy study we have attempted to minimize this period by switching directly to fixative while the heart is still being perfused with oxygenated physiological buffer on the working heart apparatus.

In examing the interaction between MMP-2 and PARP the differences between the *in vitro* setting and those *in vivo* are considerable. The *in vitro* setting has the advantage of an isolated system with minimal complicating factors, however it lacks any biological components of the cell, which might contribute to these enzymes physiological and

pathological roles. For example, *in vivo* MMPs exist in both the latent and active form normally bound to TIMPs, of which there are four isoforms. *In vitro* I have only added active MMP-2 with only TIMP-2. Also, in this study the samples were incubated for 20 min or 2 hr before analysis by a SDS-PAGE. According to the study done by Wang *et al.* (2002), in which MMP-2 was released within the first few minutes of $ONOO^-$ infusion, the 20 min incubation time of MMP-2 with PARP is a reasonable one. It is also may bereasonable to expect that a 2 hr incubation is fairly long. However, Fernandez-Patron *et al.* (2000) found that MMP-2 is capable of cleaving calcitonin gene-related peptide in a time dependent manner with cleavage peaking after 3 hr incubation, MMP-2 was also able to cleave big-endothelin-1 to a novel vasoactive peptide after 6 hr (Fernandez-Patron *et al.*, 1999). As well, Rouet-Benzineb *et al.* (1999) showed myosin heavy chain degradation after a 24 hr incubation with MMP-2.

The disadvantage to both zymography and silver staining is the uncertainty in the purity of any given band. However, the use of MMP inhibitors in zymography verified that the gelatinolytic activities were at least due to matrix metalloproteinases. An example can be seen by comparing the silver stain to the PARP western blot (Figure 3.8), the appearance of a <45 kDa band in all PARP lanes in the silver stain probably masks the <48 kDa PARP degradation band that is seen on the western blot. Likewise, zymography cannot differentiate whether the 72 kDa band is latent MMP-2 alone or whether it has been activated by oxidants such as $ONOO^-$, without proteolytic cleavage. As well, zymograms cannot be used as a direct measurement of net proteolytic activity *in situ* as the protocol involves the activation of the latent enzymes.

Unfortunately, the synthetic MMP inhibitors that are currently available are not only selective for MMP-2. They all have the common ability to chelate zinc and thus may inhibit other zinc containing enzymes. Barring any contaminants, the MMP inhibitors used in the *in vitro* assays can be considered specific for MMPs as PARP does not contain a metal ion. However, PARP activity was not measured.

The isolated heart model allows us to mimic many physiological conditions while controlling for a large number of variables. Although it is useful for pharmacological, biochemical and functional studies of the heart and its perfusate, this model lacks the components of whole blood. Thus the data generated from these studies is independent of the possible effects of erythrocytes, leukocytes, platelets, plasma and other circulating factors present in the blood. In addition, the isolated perfusion of the heart acts as an oxidative stress itself, thus even the control hearts may have undergone a slight injury. This injury could be evaluated by examining the histological changes between aerobically perfused hearts and those taken directly from the rat. The jump from a specific *in vitro* assay and all of the additional complicating factors of the intracellular milieu and other components of cells or tissue is a large one. Thus although MMP-2 cleaves PARP *in vitro*, it may do so in a minor role. Further studies are needed to determine the physiological and pathological significance of this finding.

D) FUTURE RESEARCH

These investigations lay the foundation for a whole new area of study. As the amount of research into possible intracellular roles for MMPs increases, the investigation into possible nuclear roles for these enzymes becomes increasingly more viable. Further

experiments to be performed would include the characterization of the ~66 kDa and <45 kDa PARP degradation bands that resulted from the *in vitro* experiments. Although we did attempt to analyze these bands by mass spectrometry, there was not enough protein for detection. To verify that the degradation bands that appear in the heart homogenate western blots coincide with those identified in the *in vitro* assay, it would be necessary to sequence these bands too. In addition, it would be beneficial to co-localize MMP-2 and PARP within the nucleus by either immunogold electron microscopy or fluorescence microscopy.

In addition to these experiments, this research will be further enhanced by exploring various *in vivo* and *ex vivo* models. In this study we looked at two models of oxidative stress injury based on the ability of $ONOO^-$ to activate both MMPs and PARP via different mechanisms. However, one of the major pathologies involving the nucleus, apoptosis, was not addressed in this study. Indeed, apoptosis occurs after a longer period of ischemia (Stadler *et al.,* 2001) then a model of stunning that we used. Moreover, MMPs are known to play an integral role in cancer and tumor cell invasion (Goldberg & Eisen, 1991), which may implicate a role for these enzymes in altering the cell cycle from within the nucleus. Indeed both Zhao *et al.* (1998) and Ritter *et al.* (1999) speculate that TIMP-1 plays a role in the cell cycle and cell growth.

As well, there are many other proteins within the nuclear matrix that are subject to degradation. One of the possible MMP targets may be nuclear lamins. Much like PARP, these proteins are cleaved by interleukin-1β enzyme-like proteases (Lazebnik *et al.,* 1995) during apoptosis and by different calcium-dependent nuclear matrix proteases for

activity (Madsen *et al.,* 1992). Therefore there are several pathways that MMP-2 could be involved in and many proteins that may be degraded by MMP-2.

Future investigations should also include the related gelatinase MMP-9. While the histochemical assays I performed as a prelude to this work could not detect MMP-9 in the intracellular space (data not shown), zymograms depict gelatinase activity that corresponds to MMP-9. This disparity may be accounted for as the MMP-9 antibody that was used detects the active (84 kDa) form of MMP-9 while the gelatinolytic activity on the zymograms appears to correspond to the latent (92 kDa) form. Indeed, Rouet-Benzineb *et al.* (1999) found MMP-9 present in the sarcomeres of human cardiac myocytes, much like MMP-2. Although there have not been any intracellular targets identified for MMP-9 yet, it is possible that they are similar to those of MMP-2.

There are vast possibilities of the potential roles of MMPs in the nucleus. Since proteases are involved in signaling (Harada *et al.,* 1999), the cell cycle (Gerogi *et al.,* 2002), nuclear matrix degradation (Owen & Campbell, 1995) and apoptosis (Martelli *et al.*, 1997), MMPs maybe involved in any of these. Thus the presence of MMP-2 in the nucleus constitutes an extensive and exciting new area of research.

REFERENCES

Benchoua A, Couriaud C, Guegan C, Tartier L, Couvert P, Friocourt G, Chelly J, Menissier-De Murcia J & Onteniente B. Active caspase-8 translocates into the nucleus of apoptotic cells to inactivate poly(ADP-ribose) polymerase-2. *Journal of Biological Chemistry* 2002; *in press*

Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW & Schulz R. Matrix metalloproteinases-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000; 101:1833-1839

Coker ML, Doscher MA, Thomas CV, Galis ZS & Spinale FG. Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations. *American Journal of Physiology (Heart and Circulatory Physiology)* 1999; 277:H777-H787

Fernandez Patron C, Radomski MW & Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. *Circulation Research* 1999; 85:906-911

Fernandez-Patron C, Stewart KG, Zhang Y, Koivunen E, Radomski MW & Davidge ST. Vascular matrix metalloproteinase-2 dependent cleavage of calcitonin gene-related peptide promotes vasoconstriction. *Circulation Research* 2000; 87:670-676

Georgi AB, Stukenberg PT & Kirschner MW. Timing of events in mitosis. *Current Biology* 2002; 12:105-114

Gilad E, Zingarelli B, Salzman AL, Szabo C. Protection by inhibition of poly (ADP-ribose) synthetase against oxidant injury in cardiac myoblasts *in vitro*. *Journal of Molecular and Cellular Cardiology* 1997; 29:2585-2597

Goldberg GI & Eisen AZ. Extracellular matrix metalloproteinases in tumor invasion and metastasis. *Cancer Treatment Research* 1991; 53:421-440

Golub LM, Ramamurty NS, McNamara TF, Greenwald RA & Rifkin BR. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. *Critical Reviews in Oral Biology and Medicine* 1991; 2:297-321

Harada S, Smith RM & Jarett L. Mechanisms of nuclear translocation of insulin. *Cell Biochemistry and Biophysics* 1999; 31:307-319

Ischida H, Ichimori K, Hirota Y, Fukahori M & Nakazawa H. Peroxynitrite induced cardiac myocyte injury. *Free Radical Biology and Medicine* 1996; 20:343-350

Ito A, Mukaiyama A, Itoh Y, Nagase H, Thorgersen IB, Enghild JJ, Sasaguri Y & Mori Y. Degradation of interleukin 1β by matrix metalloproteinases. *Journal of Biological Chemistry* 1996; 271:14657-14660

Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE & Poirier GG. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy induced apoptosis. *Cancer Research* 1993; 53:3976-3985

Kjeldsen L, Johnsen AH, Sengelov H & Borregaard N. Isolation and primary structure of NGAL,a novel protein associated with human neutrophil gelatinase. *Journal of Biological Chemistry* 1993; 268:10425-10432

Latt SA, Homquist B & Vallee BL. Thermolysin: a zinc metalloenzyme. *Biochemistry and Biophysics Research Communications* 1969; 37:333-339

Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG & Earnshaw WC. Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994; 371:346-347

Lazebnik YA, Takahashi A, Moir RD, Goldman RD, Poirier GG, Kaufmann SH & Earnshaw WC. Studies of the lamin proteinases reveal multiple parallel biochemical pathways during apoptotic execution. *Proceedings of the National Academy of Science USA* 1995; 92:9042-9046

Luo D, Mari B, Stoll I & Anglard P. Alternative splicing and promoter usage generates an intracellular stromelysin-3 isoform directly translated as an active matrix metalloproteinases. *Journal of Biological Chemistry* 2002; *in press*

Madsen KR, Fiskum G & Clawson GA. Regulation of nuclear scaffold protease activity by calcium. *Experimental Cell Research* 1992; 187:343-345

Martelli AM, Bareggi R, Bortul R, Grill V, Narducci P & Zweyer M. The nuclear matrix and apoptosis. *Histochemical Cell Biology* 1997; 108:1-10

Malchior F & Gerace L. Mechanisms of nuclear protein import. *Current Opinions in Cell Biology* 1995; 7:310-318

Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R & Pavelka M. Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B, without enhanced activation of cdc2 kinase. *Journal of Cell Biology* 1994; 126:827-837

Okamoto T, Akaike T, Sawa T, Miyamoto Y, van der Vilet A & Maeda H. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *Journal of Biological Chemistry* 2001; 276:29596-29602

Owen CA & Campbell EJ. Neutrophil proteinases and matrix degradation. The cell biology of pericellular proteolysis. *Seminars in Cell Biology* 1995; 6:367-376

Pei D, Kang T & Qi, H. Cysteine array matrix metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. *Journal of Biological Chemistry* 2000; 275:33988-33997

Ritter LM, Garfield SH & Thorgeirsson UP. Tissue inhibitor of metalloproteinases-1 (TIMP-1) binds to the cell surface and translocates to the nucleus of human MCF-7 breast carcinoma cells. *Biochemical and Biophysical Research Communications* 1999; 257:494-499

Rouet-Benzineb P, Buhler JM, Dreyfus P. Delcourt A, Dorent R, Perennec J, Crozatier B, Harf A & Lafuma C. Altered balance between matrix gelatinases (MMP-2 and MMP-9) and their tissue inhibitors in human dilated cardiomyopathy: potential role of MMP-9 in myosin-heavy chain degradation. *European Journal of Heart Failure* 1999; 1:337-352

Ruh MF, Dunn R 2nd & Ruh TS. Interrelationships between nuclear structure and ligand activated intracellular receptors. *Critical Reviews in Eukaryotic Gene Expression* 1996; 6:271-283

Schulze CJ, Wang W, Suarez-Pinzon WL, Sawicki G & Schulz R. Imbalance between tissue inhibitor of metalloproteinases-4 and matrix metalloproteinases during acute myocardial ischemia-reperfusion injury. *in review*

Stadler B, Phillips J, Toyoda Y, Federman M, Levitsky S & McCully JD. Adenosine-enhanced ischemic preconditioning modulates necrosis and apoptosis: effects of stunning and ischemia reperfusion. *Annals of Thoracic Surgery* 2001; 72:555-563

Sternlicht MD & Werb Z. How matrix metalloproteinases regulate cell behavior. *Annual Reviews in Cellular Developmental Biology* 2001; 17:463-516

Szabo C & Dawson VL. Role of poly (ADP-ribose) synthetase in inflammation and ischemia-reperfusion. *Trends in Pharmacological Science* 1998; 19:287-298

Szabo C, Zingarelli B, O'Connor M & Salzman AL. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity in macrophages and smooth muscle cells exposed to peroxynitrite. *Proceedings of the National Academy of Science* 1996; 93:1753-1758

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS & Dixit VM. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhabitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell* 1995; 81:801-809

Tyagi SC, Ratajska A & Weber KT. Myocardial matrix metalloproteinase(s): localization and activation. *Molecular and Cellular Biochemistry* 1993; 126:49-59

Velasco G, Cal S, Merlos-Suarez A, Fernando AA, Alvarez S, Nakano A, Arribas J & Lopez-Otin C. Human MT6-matrix metalloproteinase: identification, progelatinase A activation, and expression in brain tumors. *Cancer Research* 2000; 60:877-82

Velasco G, Pendas AM, Fueyo A, Knauper V, Murphy G & Lopez-Otin C. Cloning and characterization of human MMP-23, a new matrix metalloproteinase predominantly expressed in reproductive tissues and lacking conserved domains in other family members. *Journal of Biological Chemistry* 1999; 274:4570-4576

Wang W, Sawicki G & Schulz R. Peroxynitrite induced myocardial injury is mediated through matrix metalloproteinase-2. *Cardiovascular Research* 2002; 53:165-174

Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JRB, Sawicki G & Schulz R. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia-reperfusion injury. *Circulation* 2002; *in press*

Yasmin W, Strynadka K & Schulz R. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovascular Research* 1997; 33:422-432

Zhang X, Sakamoto T, Hata Y, Kubota T, Hisatomi T, Murata T, Ishibashi T & Inomata H. Expression of matrix metalloproteinases and their inhibitors in experimental retinal ischemia-reperfusion injury in rats. *Experimental Eye Research* 2002; 74:577-584

Zhao WQ, Li H, Yamashita K, Guo XK, Hoshino T, Yoshida S, Shinya T & Hayakawa T. Cell cycle-associated accumulation of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the nuclei of human gingival fibroblasts. *Journal of Cell Science* 1998; 111:1147-1152