Acetaminophen-mediated cardioprotection via inhibition of the mitochondrial permeability transition pore-induced apoptotic pathway

by

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ABSTRACT OF THE DISSERTATION

Acetaminophen-mediated cardioprotection via inhibition of the mitochondrial permeability transition pore-induced apoptotic pathway

by NORELL MELISSA HADZIMICHALIS

Dissertation Director:

Gary F. Merrill, Ph.D.

Historically, acetaminophen has been employed as a safe and effective analgesic and antipyretic agent. However, our laboratory has recently reported that acetaminophen also confers functional cardioprotection following cardiac insult, including ischemia/reperfusion, hypoxia/reoxygenation, and exogenous peroxynitrite and hydrogen peroxide administration. In the current study, we examined the mechanism of acetaminophen-mediated cardioprotection following ischemia/reperfusion injury. Langendorff-perfused guinea pig hearts were exposed to acute treatment with acetaminophen (0.35 mM) or vehicle (Krebs-Henseleit buffer) beginning at 15 minutes of a 30-minute baseline stabilization period. Low-flow global myocardial ischemia was subsequently induced for 30 minutes followed by 60 minutes of reperfusion. Upon completion of reperfusion,

hearts were homogenized and separated into cytosolic and mitochondrial fractions. Mitochondrial swelling and mitochondrial cytochrome *c* release were assessed and found to be significantly and completely reduced following reperfusion in acetaminophen-treated hearts when compared to vehicle. In a separate group of hearts, ventricular myocytes were isolated and subjected to fluorescence-activated cell sorting. Acetaminophen-treated hearts showed a significant decrease in late stage apoptotic myocytes when compared to vehicle-treated hearts following injury (58±1% vs. 81±5%, respectively). These data, together with electron micrograph analysis, suggest that acetaminophen mediates cardioprotection, in part, by inhibiting the mitochondrial permeability transition pore and subsequent apoptotic pathway.

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I. INTRODUCTION

1. Background

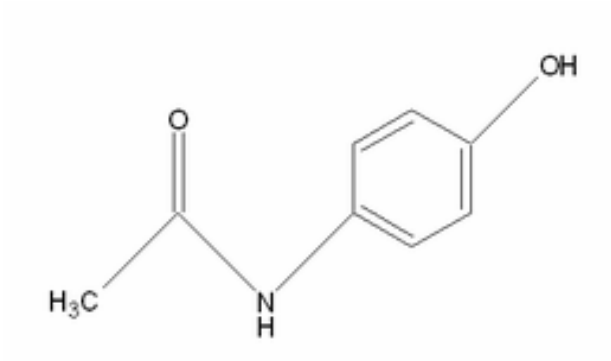
1.1 A brief history of acetaminophen

Historically, acetaminophen (paracetamol, APAP; Figure 1) has been employed as an analgesic and antipyretic agent. Today it remains a key ingredient in many popular over-the-counter medications including Tylenol, Anacin, and Datril. Acetaminophen was originally synthesized by the reduction of p-nitrophenol to p-aminophenol with tin and glacial acetic acid followed by acetylation (Prescott, 2001). It was first used clinically by von Mering (1893), however, despite potent antipyretic and analgesic properties it was determined that the side effects were too great to recommend use. Additional studies by Hinsberg and Treupel (1894) further detailed the antipyretic properties of acetaminophen. They determined that a 500 mg oral dose of acetaminophen was as effective in reducing fever as 700 mg phenacetin or 1 g antipyrine, medically accepted drugs for fever reduction in the late 19th century. Despite promising preliminary studies on the antipyretic and analgesic properties of acetaminophen, other drugs such as aspirin, phenacetin, acetanilide, and antipyrine remained more popular until the mid 20th century (Prescott, 2001).

In 1948, Brodie and Axelrod (1948a; 1948b) discovered acetaminophen to be the major metabolite of acetanilide and phenacetin in man. This finding renewed interest in the drug and provoked promotion of acetaminophen as a “Triogesic” in combination with aspirin and caffeine in the United States in 1950.

Acetaminophen became available as a non-prescription drug in 1955 and was subsequently marketed in the United Kingdom in 1956. Prolific investigation found acetaminophen to be as effective as aspirin in reducing fever and pain caused by radiant heat, cancer, dental surgery, or arthritis. Studies spanning the following two decades confirmed the safety of this drug claiming that, unlike other popular analgesic agents of the time, acetaminophen did not produce gastrointestinal toxicity. Today acetaminophen remains one of the leading over-the-counter drugs used for reducing both fever and pain (Prescott, 2001).

A



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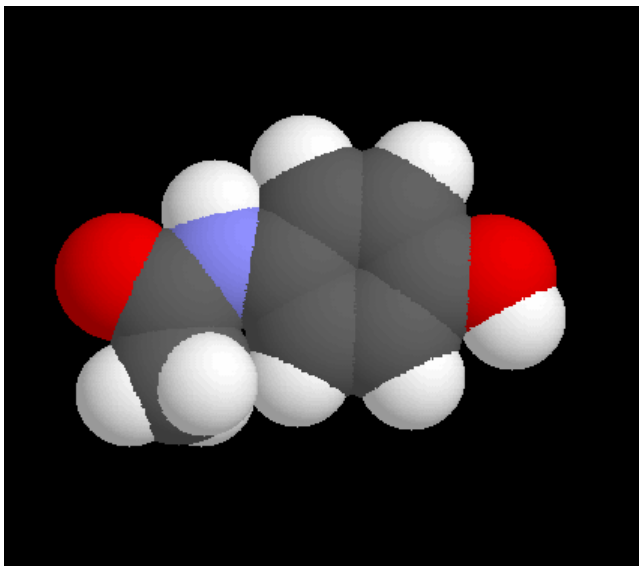


Figure 1. (A) *Chemical structure and (B) space-filling model of acetaminophen (paracetamol, APAP).* The benzene ring core is substituted by one hydroxyl group, which distinguishes this compound as a phenol.

1.2 Acetaminophen as an analgesic antipyretic agent

Since isolation of the constitutively expressed cyclooxygenase (COX) enzyme in 1976 (Hemler and Lands) and discovery of its inducible COX-2 isoform in 1991 (Xie *et al.*), much investigative effort has focused on their roles in both basic research and clinical environments. Non-steroidal anti-inflammatory drugs (NSAIDs), commonly used to treat inflammation, joint pain, headache, and fever, have been shown to produce gastrointestinal toxicity when used chronically (Vane and Botting, 1997). The basis for these adverse effects was thought to be related to COX-1 inhibition, while the positive antipyretic, analgesic, and anti-inflammatory effects are thought to be associated with COX-2 inhibition (Masferrer et al., 1994; Seibert and Masferrer, 1994; Luo et al., 2005) However, shortly after their commercial introduction, COX-2-specific inhibitors were reported to produce unfavorable cardiovascular side effects and resulted in voluntary manufacturer withdrawal of the compounds (Cotter and Wooltorton, 2005; Luo et al., 2005; Salzberg and Weir, 2007).

Despite widespread use as both an analgesic and antipyretic agent, the mechanism of acetaminophen's action in this regard is not fully clear. Unlike NSAIDs with similar effects in reducing pain and fever, acetaminophen lacks anti-inflammatory capabilities. Studies suggest that acetaminophen acts to inhibit central prostaglandin synthesis by competing with arachidonic acid for the active site on the COX enzyme (Botting, 2000). However, the exact nature of COX enzyme inhibition is controversial. While some investigators report that

acetaminophen attenuates prostaglandin synthesis by inhibiting a novel COX enzyme, COX-3, other investigators claim that COX-3 is merely a COX-1 splice variant (Botting, 2000; Kis et al., 2005).

1.3 Acetaminophen as a cardioprotective agent

In addition to its role as an analgesic and antipyretic agent, acetaminophen has been reported to exhibit cardioprotective efficacy when administered during ischemia/reperfusion, hypoxia/reoxygenation, or exogenous peroxynitrite and hydrogen peroxide administration. We have found both chronic and acute acetaminophen treatment to be cardioprotective following ischemia/reperfusion in the isolated perfused guinea pig myocardium (Merrill *et al*., 2001; Merrill and Goldberg, 2001; Golfetti *et al* ., 2002; Golfetti *et al*., 2003). Additional studies from our laboratory have demonstrated that acute acetaminophen treatment also provides protection in a canine model of myocardial infarction (Merrill *et al*., 2004).

Using isolated and perfused guinea pig hearts, we have established that both chronic and acute acetaminophen administration preserve the myocardium structurally and functionally (Merrill *et al*., 2001; Merrill and Goldberg, 2001; Golfetti *et al*., 2002; Golfetti *et al*., 2003). In acute studies, acetaminophen-treated hearts (0.35 mM) exhibited greater preservation of mechanical function (i.e. left ventricular developed pressure, LVDP), myofibrillar ultrastructure, and significant attenuation of reactive oxygen species when compared to vehicle-

treated hearts following 20 minutes of low-flow global myocardial ischemia and 40 minutes of reperfusion (Merrill and Goldberg, 2001). Additional work from Golfetti *et al*. (2002) showed that creatine kinase activity (an indicator of tissue damage) was also significantly reduced during reperfusion in acetaminophen-treated hearts.

In chronic studies, guinea pigs were given acetaminophen-treated drinking water (0.35 mM) *ad libitum* for 10 days. Hearts were subsequently extracted and subjected to ischemia/reperfusion as described above. Golfetti *et al*. (2003) established that hearts chronically treated with acetaminophen experienced similar protection to those in the acute studies. For example, acetaminophen-treated hearts demonstrated significantly greater retention of LVDP, attenuation of reactive oxygen species, and preserved myofibrillar ultrastructure when compared to vehicle-treated hearts. Taken together, these studies suggest that the mechanical, structural, and biochemical cardioprotective efficacy of acetaminophen during ischemia/reperfusion extends from an acute to a chronic treatment environment.

Canine studies corroborate these findings and further demonstrate the cardioprotective efficacy of acetaminophen following ischemia/reperfusion. Merrill *et al*. (2004) examined vehicle- and acetaminophen-treated (total dose, 30 mg/kg iv) dogs exposed to 60 minutes left anterior descending coronary artery occlusion followed by 180 minutes of reperfusion. At the completion of the experiment, hearts were simultaneously stained with Evan's blue dye and triphenyltetrazolium chloride to visualize viable tissue outside and inside the area

at risk, respectively. Necrotic tissue remained colorless. When compared to vehicle-treated hearts, acetaminophen-treated hearts were found to have significantly more viable tissue, a greater ability of coronary venous effluent to attenuate peroxynitrite, and visibly preserved myofibrillar ultrastructure. These results demonstrate the translative capacity of *ex vivo* studies to the *in vivo* arena.

More recent reports from Zhu *et al*. (2006) further support these data. In these studies rats were treated daily with 5 mg/kg intraperitoneal injections of acetaminophen beginning 7 days prior to surgery (permanent left coronary artery ligation) and extending until 2 days following the surgery. Results showed that acetaminophen-treated rats experienced a significant reduction in mortality rate following myocardial infarction when compared to vehicle-treated rats. In addition, electrocardiograms of treated rats 10 days post-treatment showed noticeable reductions in ST elevation (an indicator of electrical damage following myocardial infarction) when compared to vehicle. Triphenyltetrazolium chloride staining was also used to show a significant reduction in left ventricular infarct size in acetaminophen- versus vehicle-treated rats.

Based on the results of these animal studies, we conclude that acetaminophen has a cardioprotective role during ischemia/reperfusion. It is currently believed that the mechanism of action may involve antioxidant properties of this drug conveyed by its phenolic structure (Figure 1). Additional work is required in order to further delineate the pathway for this observed protection.

Rork *et al*. (2004) have extended this work with acetaminophen to investigate its effects in the setting of hypoxia/reoxygenation. In these studies, our laboratory exposed isolated perfused guinea pig hearts to 6 minutes of hypoxia followed by 36 minutes of reoxygenation and examined hemodynamic, metabolic, mechanical, ultrastructural, and biochemical indices of function. We found that acetaminophen-treated hearts retained significantly greater mechanical function, preserved myofibrillar ultrastructure, and a significantly greater ability to neutralize peroxynitrite-dependent chemiluminescence at all recorded time periods. In addition, creatine kinase activity was significantly decreased during both hypoxia and reoxygenation in acetaminophen- versus vehicle-treated hearts. Thus, we concluded that the cardioprotective efficacy of acetaminophen (0.35 mM) could be extended from an ischemia/reperfusion environment to also include myocardial protection from hypoxia/reoxygenation injury.

In addition to serving as a cardioprotective agent following ischemia/reperfusion and hypoxia/reoxygenation, acetaminophen has been shown to have protective effects in other cardiovascular injuries including arrhythmogenesis and atherosclerosis. Work from Merrill and Goldberg (2001) suggests that acetaminophen has the potential to attenuate sodium pentobarbital induced ventricular arrhythmias *ex vivo*. Acetaminophen-treated guinea pig hearts analyzed for ventricular salvos (VS) and ventricular premature beats (VPB) for 90 minutes post sodium pentobarbital administration were found to be significantly less arrhythmic when compared to vehicle. Results from this study

encouraged more recent *in vivo* investigation. Merrill *et al*. (2007) examined the effects of therapeutic acetaminophen treatment on either oubain- or myocardial infarction-induced ventricular arrhythmias in dogs. Results revealed that acetaminophen-treated dogs experienced a significant decrease in percent ectopy when compared to vehicle-treated dogs.

Atherosclerosis, a cardiovascular disease characterized by myeloperoxidase-induced LDL oxidation and the development of vascular atherosclerotic plaques, has also been shown recently to be a target of acetaminophen administration. Nachtigal *et al*. (2005) investigated the role of acetaminophen in the progression of aortic atherosclerosis. After 22 weeks of acetaminophen treatment (1.3 mg/mouse/day), the average numbers of aortic plaques, aneurysms, and periaortic vascular channels were significantly reduced when compared with vehicle-treated apolipoprotein E-deficient mice. In addition, the number of periaortic inflammatory infiltrates, in the presence of acetaminophen, was significantly lowered. No significant differences were noted between groups in either average food intake or average weight gain. These data suggest that long-term treatment with acetaminophen might be effective in reducing vascular disease. Additional evidence from hypercholesterolemic rabbits (Rogers *et al*., 1999) support the idea of an anti-atherosclerotic role for acetaminophen via a reduction of vascular fatty streaks. More recently, Chou and Greenspan (2002) have provided conclusive evidence associating acetaminophen treatment with a reduction in atherosclerotic plaques. These

studies show that 0.25 mM concentrations of acetaminophen and lower attenuate myeloperoxidase induced LDL metabolism.

For a number of years, mechanistic evidence of acetaminophen-mediated cardioprotection has been notably lacking. However, recently Rork *et al*. (2006) have shown that acetaminophen attenuates peroxynitrite-activated matrix metalloproteinase-2-mediated troponin I cleavage via direct inhibition of peroxynitrite in guinea pig hearts. While this work is promising, additional mechanistic data are essential to further characterize the role of acetaminophen in providing myocardial protection in cases of ischemia/reperfusion and hypoxia/reoxygenation injury. Table 1 summarizes the more recent discoveries concerning acetaminophen treatment during cardiovascular injury.

Table 1. Current studies on the effect of acetaminophen during various cardiovascular injuries.

			- acute treatment, 0.3-0.6mM	hydroxyl radicals and peroxynitrite). APAP also provides functional and structural protection.
Merrill and Goldberg	2001	Basic Res Cardiol.	- Langendorff perfused hearts - 20 mins. I / 40 mins. R - acute treatment, 0.35mM	APAP is cardioprotective against I/R via antioxidant mechanisms (inhibition of hydroxyl radicals and peroxynitrite). APAP attenuates ventricular arrhythmias.
Merrill	2002	AJP Heart	- Langendorff perfused hearts - 20 mins. I / 40 mins. R - APAP given at onset of I - acute treatment, 0.35mM	APAP is cardioprotective against I/R via antioxidant mechanisms (inhibition of hydroxyl radicals, peroxynitrite, and protein oxidation).
Golfetti et al.	2002	Exp Biol Med	- Langendorff perfused hearts - 20 mins. I / 40 mins. R - APAP given at onset of R - acute treatment, 0.35mM	APAP is cardioprotective against I/R via antioxidant mechanisms (inhibition of peroxynitrite) when administered at the onset of reperfusion. APAP also attenuates CK production.
Chou and Greenspan	2002	Biochim Biophys Acta.	- culture media - acute treatment, 0.025-0.25mM	APAP is protective against atherosclerosis via inhibition of myeloperoxidase-hydrogen peroxide-nitrate mediated modification of LDL.
Golfetti et al.	2003	Exp Biol Med	- Langendorff perfused hearts - 20 mins. I / 40 mins. R - APAP given at onset of R - chronic treatment, 0.35mM	APAP is cardioprotective against I/R when administered chronically. Cardioprotection is measured as functional and structural improvement, and both CK and peroxynitrite inhibition.
Merrill et al.	2004	AJP Heart	- MI - left coronary artery ligation - acute treatment, 30mg/kg iv	APAP significantly reduces infarct size.
Rork et al.	2004	Exp Biol Med	- Langendorff perfused hearts - 6 mins. H, 36 mins. ReO - acute treatment, 0.35mM	APAP is cardioprotective against H/ReO via antioxidant mechanisms (inhibition of peroxynitrite). APAP also provides significant functional and structural protection under this injury.
Rork et al.	2006	J Mol Cell Cardiol.	- Langendorff perfused hearts - exogenous peroxynitrite administration - acute treatment, 0.35mM	APAP is cardioprotective via attenuation of peroxynitrite-activated matrixmetalloproteinase-2 mediated cleavage of troponin I.
Zhu et al.	2006	AJP Heart	- MI - permanent left coronary artery ligation - chronic treatment 5mg/kg/day	Chronic APAP treatment significantly decreases mortality rate and infarct size. In addition, catalase and superoxide dismutase activities were increased in the treated-group.
Merrill et al.	2007	Exp Biol Med	- MI and oubain-induced disturbances - acute treatment, 30mg/kg iv	Therapeutic administration of APAP results in significant attenuation of percent ectopy and all ventricular ectopic beats (except ventricular salvos).

I, ischemia; R, reperfusion; APAP, acetaminophen; CK, creatine kinase; LDL, low density lipoproteins; MI, myocardial infarction; H, hypoxia; ReO, reoxygenation (Merrill and Goldberg, 2001; Chou and Greenspan, 2002; Golfetti et al., 2002; Merrill, 2002; Golfetti et al., 2003; Rork et al., 2004; Rork et al., 2006; Zhu et al., 2006; Hadzimichalis et al., 2007).

1.4 Cytochrome *c*, apoptotic cell death, and myocardial ischemia/reperfusion

Post-ischemia reperfusion, although vital for the survival of the myocardium, results in substantial myocardial damage including cell death (Sutherland and Hearse, 2000). While the relative contributions of necrosis and apoptosis to overall tissue injury is a topic of much debate, both pathways have been clearly implicated following ischemia/reperfusion. Much recent attention has focused on elucidating the cell death pathways active during cardiovascular injury in an attempt to explore pathway-related therapeutics.

Apoptosis has been implicated in various cardiovascular diseases including ischemia/reperfusion injury, myocardial infarction, atherosclerosis, and end-stage heart failure (MacLellan and Schneider, 1997; Zidar et al., 2007). Morphologically, apoptosis is characterized by chromatin condensation, cytoskeletal alterations, membrane blebbing, nuclear fragmentation, and cytoplasmic condensation. Ultimately, dying cells form apoptotic bodies which are subsequently engulfed by other cells via phagocytosis. Unlike necrosis, apoptosis involves an active, energy-dependent cell death, decrease in cell volume, minimal inflammation, and preservation of membrane integrity (MacLellan and Schneider, 1997; Haunstetter and Izumo, 1998). Consequently, gross tissue injury is avoided. In addition, it has been shown that reperfusion versus ischemia alone leads to a greater increase specifically in apoptotic myocytes (Gottlieb et al., 1994; Logue et al., 2005).

Mechanistically, myocardial cells undergoing apoptosis follow one of two major death pathways, both leading to aspartate-specific cysteine protease (caspase) activation. The extrinsic cell death pathway begins extracellularly in response to apoptotic signals. Ligands initiate apoptosis by binding to their associated cell surface receptor. Adaptor proteins such as TRADD and FADD, and procaspase-8 are recruited to the complex and procaspase-8 is cleaved and subsequently activated (Zhu *et al*., 2006). Caspase-8 then activates downstream effector procaspases (i.e. procaspase-3) which ultimately carry out characteristic end-stage steps of apoptosis such as DNA fragmentation (Foo *et al*., 2005). Studies in Langendorff-perfused murine hearts (Jeremias *et al*., 2000) implicate this pathway in ischemia/reperfusion-induced cell death. Western blot analysis revealed enhanced levels of the Fas death receptor ligand CD95 in the extracellular fluid, particularly during the reperfusion phase of ischemic injury. In addition, loss of function mutations in the Fas death receptor result in a reduction of apoptotic cell death following myocardial ischemia/reperfusion (Jeremias *et al*., 2000; Krijnen *et al*., 2002).

Alternatively, the intrinsic (i.e. mitochondrial) cell death pathway is initiated in response to conditions occurring within the cell and also plays a prominent role in apoptosis following ischemia/reperfusion injury. Following stimuli such as oxidative stress, various proteins, including cytochrome *c*, are released from the mitochondrial intermembrane space into the cytosol. Cytochrome *c* then binds to the apoptosis protease activating factor-1 (Apaf-1) adapter molecule forming the apoptosome complex, which subsequently recruits and activates procaspase-9.

Caspase-9 then cleaves and activates downstream executioner/effector caspases such as caspase-3 to carry out apoptotic cell death (Green and Kroemer, 2004; 2005). Borutaite *et al*. (2003) have implicated the intrinsic cell death pathway as contributing to myocardial cell death during ischemia. They demonstrated that following prolonged ischemia, significant amounts of cytochrome *c* were present in the cytosol in addition to an increase in caspase-3 activation. More recently Vanden Hoek *et al*. (2003) have shown that the burst of damaging oxidants released during reperfusion initiates the intrinsic pathways of apoptotic cell death. Additionally, these investigators provide evidence that in the presence of antioxidants 2-mercaptopropionylglycine (MPG) and 1,10-phenanthroline (Phen), myocytes experience a significant reduction in overall cell death, cytochrome *c* release, and nuclear condensation. A simplified schematic illustrating this pathway can be seen in Figure 2.

Following post-ischemia reperfusion, myocytes experience calcium overload, ATP and adenine nucleotide depletion, increased phosphate levels, and oxidative stress. Independent from ischemia/reperfusion, these conditions have been shown to result in cell death (Duchen, 2004; Halestrap, 2006). Damaging oxidant release, including peroxynitrite and hydroxyl radicals, is partially responsible for myocardial damage following ischemia/reperfusion injury and is primarily associated with the intrinsic cell death pathway and opening of the MPTP. Since acetaminophen is effective at attenuating post-reperfusion production of oxidants (i.e. hydroxyl radicals and peroxynitrite), we hypothesized that its reported cardioprotective effects were mediated, in part, via inhibition of

the intrinisic/mitochondrial pathway of apoptosis. (Merrill and Goldberg, 2001; Merrill, 2002)

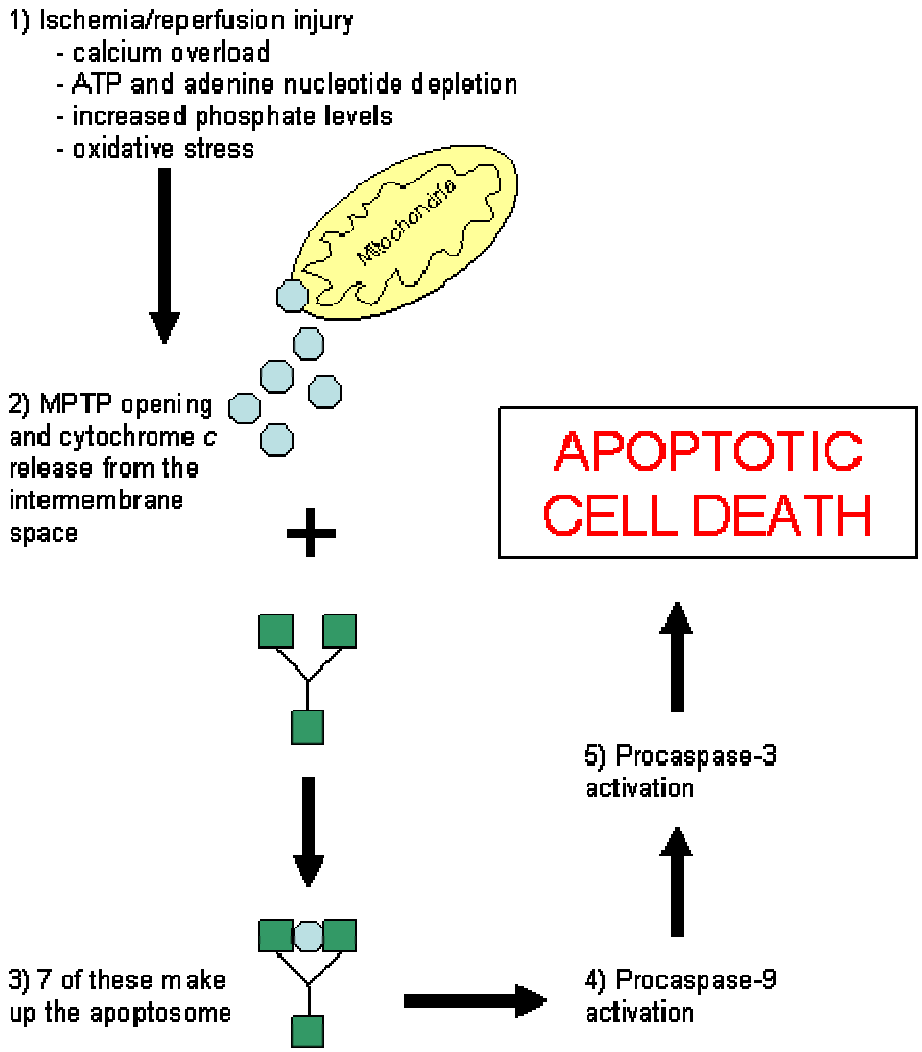


Figure 2. *Schematic of the intrinsic/mitochondrial pathway for apoptotic cell death.* Apoptotic initiating factors, such as those present following ischemia/reperfusion injury, result in MPTP opening and subsequently cytochrome *c* release from the mitochondrial intermembrane space. Seven cytochrome *c* molecules bind to seven dATP activated Apaf-1 molecules to form the apoptosome. This complex binds and activates procaspase-9 molecules which then activate downstream procaspase-3 to carry out apoptosis (Gewies, 2003).

1.5 The mitochondrial permeability transition pore

Since its discovery by Hunter *et al*. (1963), the mitochondrial permeability transition pore (MPTP) has become the focus of much investigation. Evidence that prolonged pore opening results in cell death suggests that this structure is an important physiologic target in the clinical environment (Halestrap et al., 2004). Opening of the MPTP is mediated by conditions similar to those experienced following ischemia/reperfusion and hypoxia/reoxygenation injury and includes calcium overload, depletion of adenine nucleotides, high phosphate levels, and oxidative stress. As a result, the cell will experience mitochondrial ATP hydrolysis, uncoupling of oxidative phosphorylation, cytochrome *c* release from the intermembrane space, mitochondrial swelling, and ultimately cell death (Halestrap et al., 2004).

Mitochondrial permeability transition may be either directly or indirectly inhibited. Agents such as cyclosporin A and sanglifehrin A have been established as direct inhibitors of the transition pore via an interaction with cyclophilin-D, a detachable matrix element (Broekemeier et al., 1989; Halestrap et al., 2004). Figure 3 is a schematic of the MPTP. While there is some controversy regarding complete structural elucidation of the MPTP, it is widely accepted that the adenine nucleotide translocase (ANT) and cyclophilin-D element are crucial components of the MPTP (Javadov and Karmazyn, 2007).

In addition to directly targeting the pore itself, indirect inhibitors may also be used to prevent mitochondrial permeability transition by attenuating upstream

mediators of pore opening, such as damaging oxidants. Numerous antioxidants have been employed to this end. Ginkgo biloba extract, propofol, and pyruvate have exhibited antioxidant behaviors in preventing ischemia/reperfusion-related injury, likely leading to increased closure of permeability transition pores (Morin *et al*., 2001). The mechanism of action, although not completely elucidated, is likely attributable to the free radical scavenging properties of these compounds.

In addition to the similarity between the effects of ischemia/reperfusion and hypoxia-reoxygenation and the causes of mitochondrial permeability transition, the fact that there is a relationship between these two events is suggested by electronmicrograph analysis. Merrill *et al*. (2001) showed noticeably swollen mitochondria in the isolated perfused vehicle-treated guinea pig heart as a result of ischemia/reperfusion. Similar swelling is visible as a result of prolonged mitochondrial pore opening (Halestrap *et al*., 2004). However, treatment with acetaminophen (0.35 mM) resulted in inhibition of cellular damage, including mitochondrial swelling following ischemia/reperfusion.

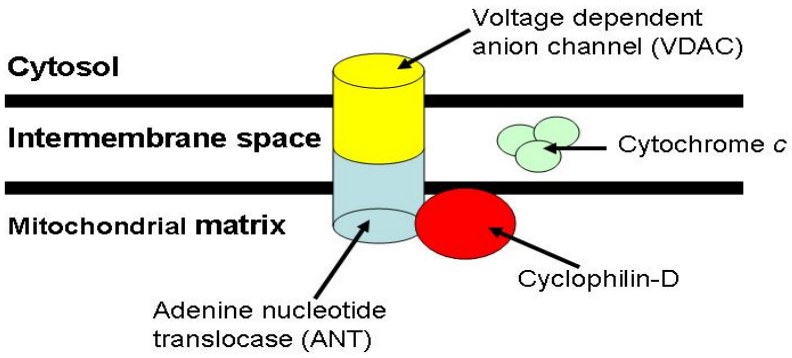


Figure 3. *Schematic of the minimal MPTP structure.* In the open conformation, the 3 nm pore diameter allows for diffusion of molecules less than 1.5 kDa.

2. Purpose

2.1 A mechanistic elucidation of acetaminophen-mediated cardioprotection

Previous studies from our laboratory report acetaminophen-mediated cardioprotection following myocardial ischemia/reperfusion injury in isolated perfused guinea pig hearts (Merrill et al., 2001; Merrill, 2002). While these data clearly demonstrate functional preservation of the myocardium in response to acute treatment (0.35 mM), they only suggest that protection is mediated in response to the antioxidant nature of the compound. The purpose of the following studies was to more definitively examine the mechanistic nature of the compound in attenuating functional myocardial damage.

2.2 Acetaminophen and its effects on the mitochondrial permeability transition pore

The purpose of the first part of the study was to investigate the effects of acetaminophen treatment on one of the more upstream events in the mitochondrial-mediated apoptotic cascade. We examined the capacity of acetaminophen to attenuate MPTP opening following myocardial ischemia/reperfusion injury. Previously reported acetaminophen-mediated attenuation of both hydroxyl radicals and peroxynitrite production following ischemia/reperfusion suggests that those biochemical pathways initiated by

these oxidants will also be affected in response to treatment (Merrill and Goldberg, 2001; Merrill, 2002). Initial studies examined light scattering in isolated mitochondrial from whole heart homogenates as an index of mitochondrial swelling in response to pore opening. Electron micrograph analysis was used to visually corroborate these results. Results from this part of the study have provided the first evidence of a role for acetaminophen in the mitochondrial-mediated pathway of apoptosis.

2.3 Acetaminophen and its effects on mitochondrial cytochrome *c* release

As a result of MPTP opening and ultimately outer mitochondrial membrane rupture, cytochrome *c* is released into the cytosol as an apoptotic trigger. The purpose of the second part of this study was to further delineate the mechanism of acetaminophen-mediated cardioprotection by examining its role in altering mitochondrial cytochrome *c* release. We examined both mitochondrial and cytosolic cytochrome *c* content in vehicle- and acetaminophen-treated hearts following ischemia/reperfusion injury. Results from this part of the study have provided additional evidence of a role for acetaminophen in the mitochondrial mediated pathway of apoptosis in addition to further delineating its mechanism.

2.4 Acetaminophen and its effects on mitochondrial-mediated apoptosis

Mitochondrial permeability transition pore opening and subsequent mitochondrial cytochrome *c* release ultimately lead to mitochondrial-mediated apoptosis (Javadov et al., 2000; Halestrap, 2006). If the MPTP remains open, ATP levels will be depleted and the cell will undergo necrotic cell death. However, transient pore opening, as is the case in less severe forms of ischemia/reperfusion injury (i.e. low versus no flow ischemia), will result in cytochrome *c* release and apoptotic cell death (Halestrap et al., 2004; Halestrap, 2006). The purpose of the third part of this study was to determine if the observed cardioprotection resulting from acetaminophen treatment is, in part, due to a role in apoptotic attenuation.

II. MATERIALS AND METHODS

1. Experimental preparation

1.1 Animals and Langendorff preparation

Hartley strain male guinea pigs (400 ± 25 g) were obtained from Elm Hill Laboratories (Wilmington, MA, USA) and allowed a minimum of three days to acclimate to their new environment. Following IACUC review and approval, guinea pigs were anesthetized using isoflurane in accordance with National Institutes of Health and United States Department of Agriculture guidelines.

Hearts were isolated and perfused *in situ* via the cannulated aorta and subsequently extracted and attached to a Langendorff perfusion apparatus as previously described (Bunger *et al*., 1975a; Bunger *et al*., 1975b; Merrill *et al*., 2001). Pacing electrodes were placed at the base of the right ventricle to control heart rate at approximately 200 beats per minute (model S44, Grass-Telefactor; West Warwick, RI, USA), and physiologic heart temperature was monitored using a thermistor probe (model BAT-12, Physitemp; Clifton, NJ, USA). Coronary perfusion pressure was controlled hydrostatically (55 ± 5 mmHg).

1.2 Perfusate and Perfusion

Hearts were perfused with a modified Krebs-Henseleit physiological salt solution/buffer (KHB) containing (in mM): 128.0 NaCl, 4.7 KCl, 1.5 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, 10.0 glucose, 2.0 pyruvate, and 200 μU/ml insulin. Perfusate was warmed to 37ºC, equilibrated with a 95% O_2,

5% CO_2 gas mixture (pH 7.40 ± 0.02), and delivered from a water-jacketed perfusion reservoir. Flow was allowed to vary naturally and continuously monitored ultrasonically (model T106 flow meter, Transonic Systems; Ithaca, NY, USA). A schematic of the experimental setup is depicted in Figure 4.

Guinea pigs were randomly assigned to vehicle (KHB) or acetaminophen (0.35 mM dissolved in KHB) treatment groups. Following extraction and suspension from the Langendorff apparatus, all hearts remained untreated and were perfused with KHB for the first 15 minutes of the baseline stabilization period. Subsequently, hearts were treated with either acetaminophen (dissolved in KHB and added to the perfusate reservoir, 0.35 mM) or vehicle for the remainder of the 30-minute baseline period and for the duration of the Langendorff perfusion. Low-flow global myocardial ischemia (1 ml/min) was then induced for 30 minutes followed by 60 minutes of reperfusion (Figure 5). Animal choice, age, and the use of low-flow ischemia were employed in order to be consistent with previous reports from our laboratory which establish the functional cardioprotective capacity of acetaminophen (Merrill and Goldberg, 2001; Merrill, 2002).

Monitored variables included heart rate (HR; beats/minute), coronary perfusate flow (CPF; ml/min/g), and coronary perfusion pressure (CPP; mmHg). A data acquisition system (model 214, iWorx/CB Sciences; Dover, NH, USA) in series with a personal computer (Compaq Evo running LabScribe software version 6.0) was used to record monitored variables. Metabolic data including

pH, PO_2 (mmHg), and PCO_2 (mmHg) were recorded using a standard blood-gas analyzer (model 248, Chiron Diagnostics; Norwood, MA, USA).

1.3 Statistical analysis

Reported values are shown as mean ± SEM. Data were analyzed for significance ($p < 0.05$) using ANOVA followed by Tukey's Multiple Comparison Test (InStat, GraphPad; San Diego, CA).

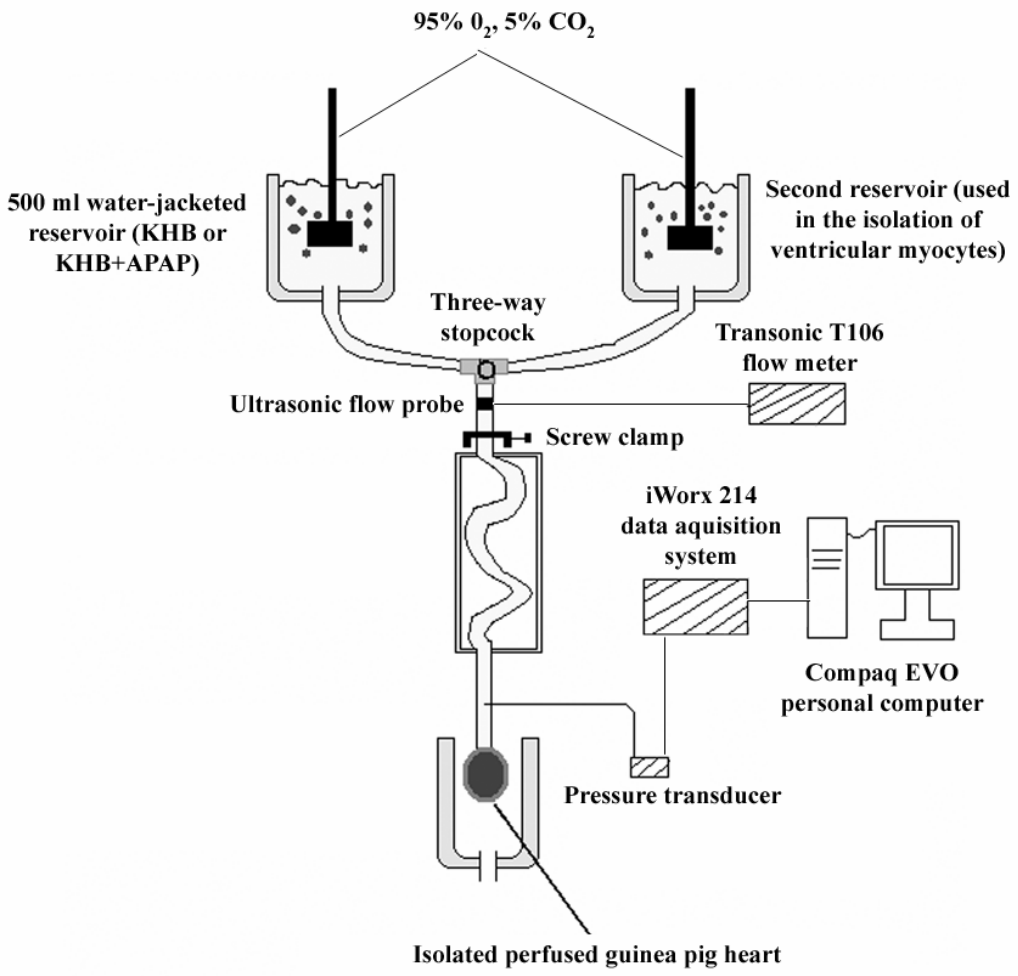


Figure 4. *Schematic of modified Langendorff perfusion apparatus.* The reservoir on the right was only used during ventricular myocyte isolation to deliver calcium-free KHB and subsequently calcium-free KHB with dissolved collagenase to the heart.

A

15 mins. stabilization (KHB perfusion)	15 mins. KHB perfusion	30 mins. low-flow ischemia	60 mins. reperfusion

B

15 mins. stabilization (KHB perfusion)	15 mins. KHB + 0.35 mM APAP perfusion	30 mins. low-flow ischemia	60 mins. reperfusion

Figure 5. *Schematic of experimental ischemia/reperfusion timeline.* Isolated perfused guinea pig hearts were subjected to a 30 minute low-flow global ischemia, 60 minute reperfusion protocol. Treatment (vehicle, A or acetaminophen, B) was administered following 15 minutes baseline.

2. Experimental protocols

2.1 Myocardial homogenization and fractionation

Hearts were randomly divided into vehicle and acetaminophen treatment groups and exposed to the ischemia/reperfusion protocol described in section 1.2. Monitored variables and metabolic data were collected just prior to perfusion termination (i.e. at 15 minutes baseline for control hearts or the end of reperfusion). Following termination of Langendorff perfusion, hearts were immersed in homogenization buffer (10 ml/g) containing (in mM): 210.0 mannitol, 7.0 sucrose, and 5.0 4-morpholinopropanesulfonic acid, pH 7.4., 37ºC, 1 tablet/10 ml buffer protease inhibitor tablets (complete mini, Roche Diagnostics; Indianapolis, IN, USA). Hearts were then homogenized using both Polytron blade (model PT 2100, Kinematica; Littau-Lucerne, Switzerland) and Teflon (model JR4000, Arrow Engineering; Hillside, NJ, USA) homogenizers. Separation of cytosolic and mitochondrial fractions was modified from previously described procedures (Tokarska-Schlattner *et al*., 2005; Bopassa *et al*., 2006). The homogenate was centrifuged at 1000 x *g* for 10 minutes at 4°C and the resulting supernatant was centrifuged at 7,000 x *g* for 10 minutes at 4°C. The pellet from the second centrifugation represented the mitochondrial fraction and was resuspended in 10 mM sodium phosphate, pH 9.0. The supernatant represented the cytosolic fraction. An additional group of hearts was homogenized following 15 minutes of baseline perfusion as a control.

2.2 Mitochondrial swelling

Mitochondrial suspensions were assayed spectrophotometrically (540 nm) at 25°C for changes in light scattering (Hunter *et al*., 1963; Gadelha *et al*., 1997; Bosetti *et al*., 2004; Rousou *et al*., 2004; Tokarska-Schlattner *et al*., 2005). Mitochondrial fractions of heart homogenate were assessed following ischemia/reperfusion from both vehicle- and acetaminophen-treated hearts subsequent to a Bradford assay to determine total protein concentration. Light absorbance values were expressed as a percentage with respect to the average of baseline hearts.

2.3 Myofibrillar ultrastructure

A separate group of hearts was used to assess myofibrillar ultrastructure as previously described (Golfetti *et al*., 2002). Hearts were randomly assigned to one of two termination groups (15 minutes baseline or reperfusion) corresponding to the experimental period following which the Langendorff perfusion would be terminated. The reperfusion group was further divided into either vehicle- or acetaminophen-treatment groups.

Hearts were perfused with Karnovsky's fixative for two minutes at the end of baseline or reperfusion conditions. Hearts were then submerged in fixative and 2-3 mm^3 blocks of myocardium were removed longitudinally from the anterior

free wall of the left ventricle midway between the left ventricular and left anterior descending branches of the left main coronary artery, equidistant from base to apex. Blocks were subsequently fixed using 1% osmium tetroxide and dehydrated in graded ethanol (Golfetti *et al*., 2003; Rork *et al*., 2004). Samples were embedded in Epon-Araldite cocktail, sectioned with a diamond knife ultramicrotome (model LKB-2088, LKB; Bromma, Sweden), and viewed with an electron microscope (model JEM-100CXII, JEOL USA; Peabody, MA), using standard protocols (Bazzola and Russel, 1999).

2.4 Mitochondrial cytochrome *c* release

Following termination of Langendorff perfusion, hearts were freeze-clamped in liquid nitrogen using a modified Wollenberger clamp and stored at -80ºC until homogenization (Rork et al., 2006). A Bradford assay (Biorad Protein Assay, Biorad; Hercules, CA, USA) was used to determine total protein concentration. Cytosolic and mitochondrial fractions from baseline, vehicle- and acetaminophen-treated ischemia/reperfused heart homogenates were then loaded randomly into wells with the investigator blinded for band density quantification. Proteins were resolved on a 15% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was then probed with a mouse monoclonal antibody to cytochrome *c* (clone 7H8.2C12, 1:1000; Stressgen Bioreagents; Victoria, BC, Canada). Cytosolic and mitochondrial fractions were further probed for rabbit anti-α-actin (1:2500; Sigma-

Aldrich; St. Louis, MO, USA) and rabbit anti-voltage-dependent anion channel/Porin (VDAC, 1:2500; Sigma-Aldrich; St. Louis, MO, USA), respectively, as loading controls. Film was scanned, and quantification was carried out through optical density analysis using imaging software (Scion Corporation; Frederick, MD, USA).

2.5 Isolation of ventricular myocytes and fluorescence-activated cell sorting

Hearts were randomly divided into vehicle and acetaminophen treatment groups and exposed to the ischemia/reperfusion protocol described above. Hemodynamic and metabolic data were collected at 15 minutes baseline, 30 minutes ischemia, and 60 minutes reperfusion. Isolation of ventricular myocytes was a modification of previously described methods (Piper and Isenberg, 1989; Huang *et al*., 1996). Briefly, following ischemia/reperfusion, hearts were perfused with calcium-free KHB for 2-3 minutes to arrest contractions. Hearts were then perfused with 0.08% collagenase Type 2 (Worthington Biochemical Corporation; Lakewood, NJ, USA) dissolved in calcium-free KHB in a re-circulating mode for approximately 10-15 minutes. Subsequently, hearts were removed from the perfusion apparatus and ventricles cut longitudinally into 6-8 slices and incubated while mildly agitated with 15 ml of KHB plus 0.08% collagenase at 37ºC for five minutes. Cells were centrifuged at 10,000 x *g* for 50 seconds and washed two times in KHB. An additional group of hearts was digested following 15 minutes of baseline perfusion as a control.

Following isolation, myocytes were re-suspended in annexin V binding buffer, loaded with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), and analyzed using a fluorescence-activated cell sorter (FACS model FC500 flow cytometer, Beckman Coulter; Fullerton, CA, USA) according to the manufacturer's protocol (Vybrant Apoptosis Assay Kit #3, Molecular Probes; Carlsbad, CA, USA). Early and late stage apoptotic myocytes were characterized as annexin V-FITC positive or both annexin V-FITC and PI positive, respectively.

III. RESULTS

1. Hemodynamic and metabolic parameters

Hemodynamic and metabolic data were collected just prior to perfusion termination (baseline or reperfusion) for hearts subjected to homogenization (MPTP light scattering and cytochrome *c* release studies). In addition to baseline and reperfusion collection, these data were also recorded at ischemia for hearts subjected to myocyte isolation. There were no significant differences between vehicle- and acetaminophen-treated hearts or between baseline and reperfused hearts during any sample time. Expected hemodynamic differences in CPF were observed between baseline and ischemic hearts in the myocyte isolation studies (Table 2).

Table 2. Hemodynamic and metabolic data during myocardial ischemia/reperfusion.

A

	15 minutes baseline		Reperfusion	
	V	**A**	**V**	**A**
pO_2 (mmHg)	507 ± 9	535 ± 6	514 ± 19	524 ± 15
pCO_2 (mmHg)	30 ± 1	33 ± 1	31 ± 1	31 ± 1
pH	7.41 ± 0.01	7.39 ± 0.01	7.41 ± 0.01	7.41 ± 0.01
CPF (ml/min/g)	7.5 ± 1.0	6.8 ± 0.6	9.5 ± 2.5	8.2 ± 1.0

B

	15 minutes baseline		Ischemia		Reperfusion	
	V	**A**	**V**	**A**	**V**	**A**
pO_2 (mmHg)	515 ± 10	525 ± 8	520 ± 10	534 ± 17	528 ± 15	515 ± 15
pCO_2 (mmHg)	31 ± 1	33 ± 1	32 ± 1	31 ± 1	30 ± 1	30 ± 1
pH	7.40 ± 0.01	7.40 ± 0.01	7.41 ± 0.01	7.41 ± 0.01	7.42 ± 0.01	7.41 ± 0.01
CPF (ml/min/g)	7.0 ± 1.0	7.2 ± 1.0	0.9 ± 0.1*	1.0 ± 0.1*	9.0 ± 2.0	8.5 ± 1.0

Data are mean ± SEM. (A) Arterial samples were collected and data recorded at the completion of baseline or ischemia/reperfusion in vehicle- and acetaminophen-treated hearts (n=4 per group). Following perfusion termination hearts were homogenized and separated into cytosolic and mitochondrial fractions. (B) Arterial samples were collected and data recorded following 15 minutes baseline, ischemia, and reperfusion in vehicle- (n=4) and acetaminophen-treated (n=4) hearts. Following perfusion termination, hearts

were digested with collagenase and myocytes were isolated in this study. V, vehicle-treated hearts; A, APAP-treated hearts; pO_2, partial pressure of oxygen; pCO_2, partial pressure of carbon dioxide; HR, heart rate; CPF, coronary perfusate flow. *$p<0.05$, as determined by ANOVA followed by Tukey's Multiple Comparison Test relative to corresponding baseline value. Table used with permission (Hadzimichalis *et al*., 2007).

2. Acetaminophen treatment inhibits mitochondrial swelling following myocardial ischemia/reperfusion

Decreases in light absorbance are representative of increases in mitochondrial matrix volume as a result of the opening of MPTPs, subsequent water influx, and mitochondrial swelling (Ruiz-Meana *et al*., 2003; Kaasik *et al*., 2007). In the present study, we examined differences in light absorbance of isolated mitochondria following baseline and ischemia/reperfusion as an index of MPTP opening. Mitochondrial and cytosolic fractions were each probed for VDAC, a mitochondrial outer membrane protein, to confirm mitochondrial membrane integrity. The lack of VDAC in the cytosolic fraction of vehicle-treated baseline heart homogenates, compared to its presence in mitochondrial fractions, demonstrates that mitochondrial membranes were intact (Figure 6A). Our spectrophotometric results indicate a significant decrease in mitochondrial light absorbance of vehicle-treated ischemia/reperfused mitochondrial fractions (0.66±0.04) when compared to either baseline or post-reperfusion acetaminophen-treated mitochondrial fractions (1.27±0.09). However, there were no significant changes in light absorbance between acetaminophen-treated mitochondrial fractions following ischemia/reperfusion and baseline values, suggesting that acetaminophen treatment attenuates ischemia/reperfusion-induced mitochondrial swelling via inhibition of the MPTP (Figure 6B).

To assess whether acetaminophen preserves myofibrillar ultrastructure during cardiac ischemia/reperfusion, we examined electron micrographs in both

vehicle- and acetaminophen-treated hearts following injury and compared to baseline hearts. As shown in Figure 7, myofibrillar ultrastructure from vehicle-treated hearts displayed extensive post-reperfusion tissue damage when compared to myocardial sections from either baseline or acetaminophen-treated hearts. As indicated by arrows, mitochondria from left ventricular free wall sections appear dense and intact in baseline and acetaminophen-treated ischemia/reperfused hearts. However, mitochondria from vehicle-treated ischemia/reperfused hearts are visually swollen and structurally more rounded, indicating increased cellular damage. These data further support the conclusion that acetaminophen treatment inhibits MPTP-induced mitochondrial swelling following ischemia/reperfusion.

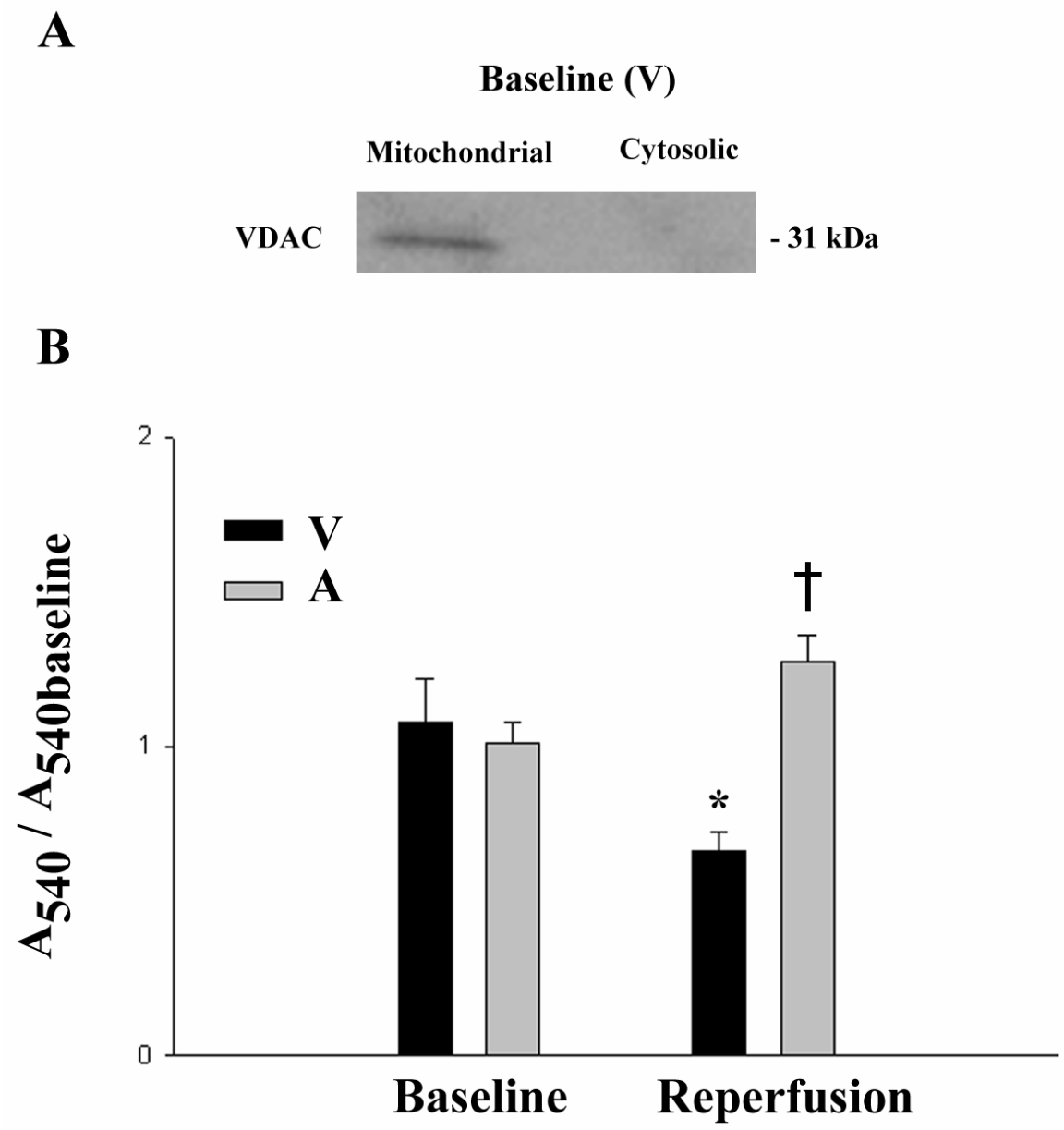


Figure 6. *Spectrophotometric analysis of mitochondrial swelling.* (A) Western blot showing the lack of VDAC in the cytosolic fraction of a representative vehicle-treated baseline heart following homogenization and centrifugation. (B) Hearts were homogenized and mitochondria were isolated via centrifugation following baseline and ischemia/reperfusion in vehicle- and acetaminophen-

treated hearts (n=4 per group). Mitochondrial swelling was measured as a decrease in light scattering at 540 nm and expressed as a percentage of the average corresponding baseline value (protein density approximately 1.0 μg/μl). V, vehicle-treated hearts; A, APAP-treated hearts. *$p<0.05$ as determined by ANOVA followed by Tukey's Multiple Comparison Test compared to corresponding baseline hearts. †$p<0.05$ as determined by ANOVA followed by Tukey's Multiple Comparison Test compared to ischemia/reperfused vehicle-treated hearts. Figure used with permission (Hadzimichalis *et al*., 2007).

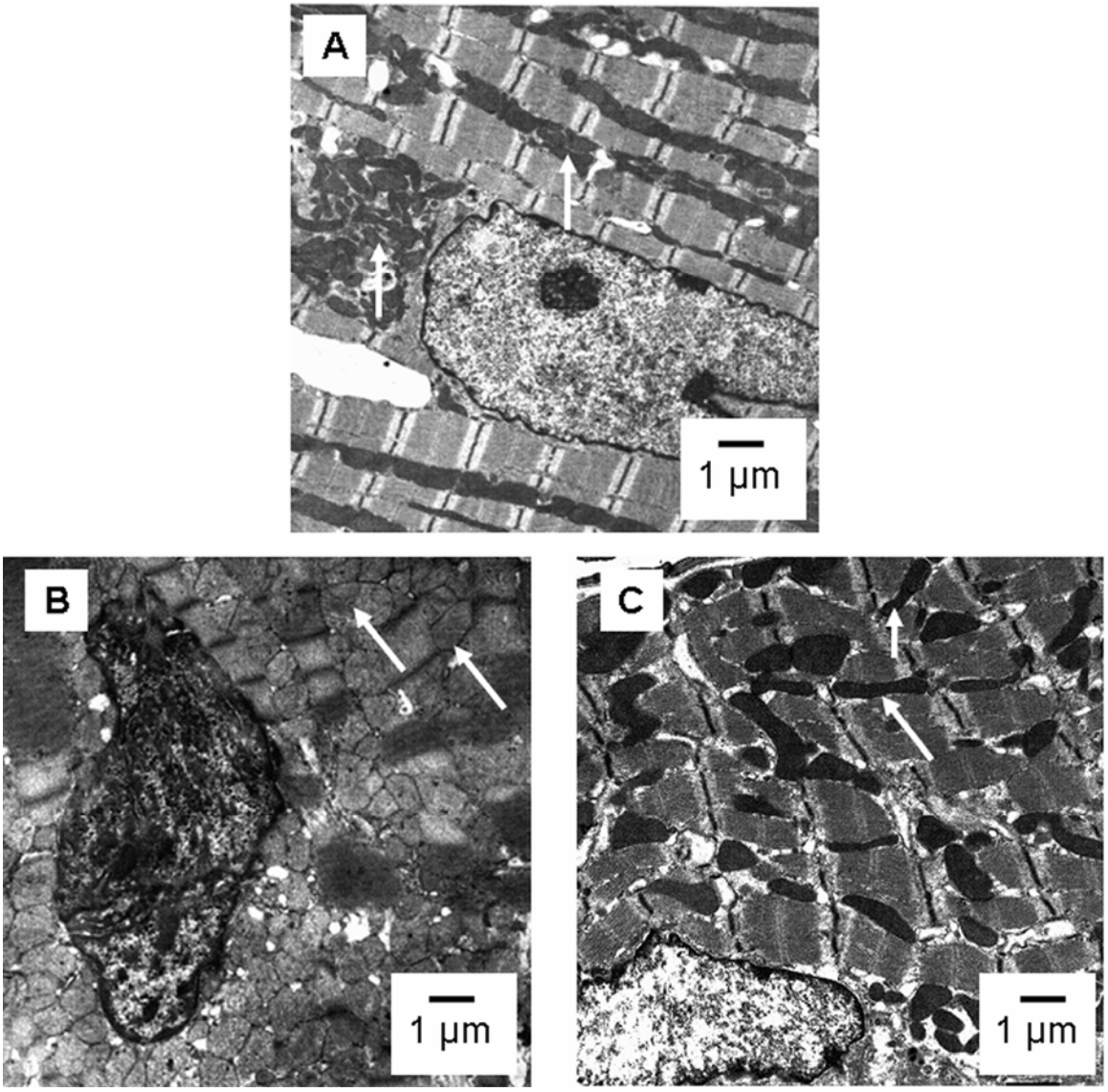


Figure 7. *Electron micrograph analysis of left ventricle free wall.* Representative electron micrographs following (A) baseline, (B) vehicle ischemia/reperfusion, and (C) acetaminophen ischemia/reperfusion (n=2 per group). Swollen mitochondria (white arrows in B relative to A and C) imply the opening of MPTPs. The presence of acetaminophen during ischemia/reperfusion appears to attenuate permeability transition and consequently mitochondrial swelling. Note the similarity in mitochondrial color and shape between (A) baseline and (C)

acetaminophen-treated ischemia/reperfused hearts. Figure used with permission (Hadzimichalis *et al*., 2007).

3. Molecular consistency between vehicle-treated hearts

Previous studies from our laboratory that have examined the effects of acetaminophen following cardiac injury have reported mostly descriptive and functional data (Merrill *et al*., 2001; Golfetti *et al*., 2002; Merrill, 2002; Golfetti *et al*., 2003; Rork *et al*., 2004). Drug- and vehicle-treated hearts were considered similar if the hemodynamic and metabolic parameters collected at baseline were not statistically different. In the current study, we further explored the molecular consistency between discrete Langendorff preparations at baseline by comparing cytosolic cytochrome *c* content. We found quantitative consistency in cytosolic cytochrome *c* content between vehicle-treated hearts following 15 minutes of baseline perfusion (Figure 8). These data show the first evidence that our preparations are biochemically consistent.

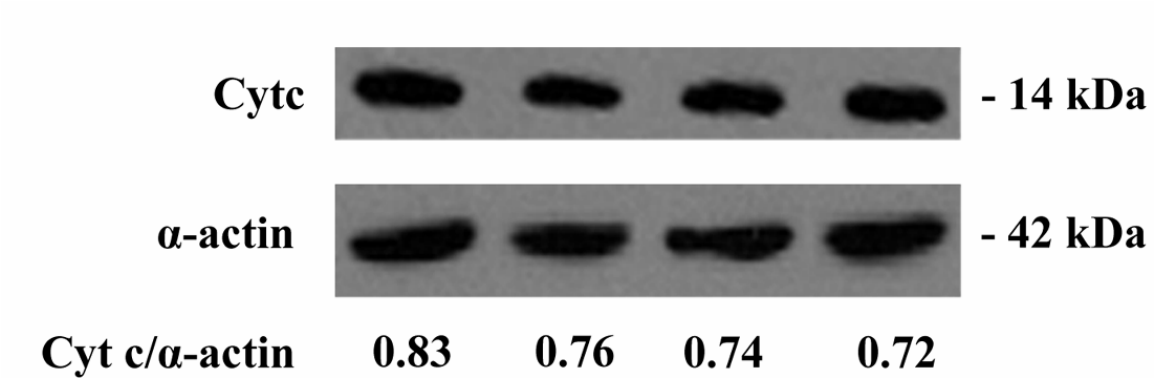
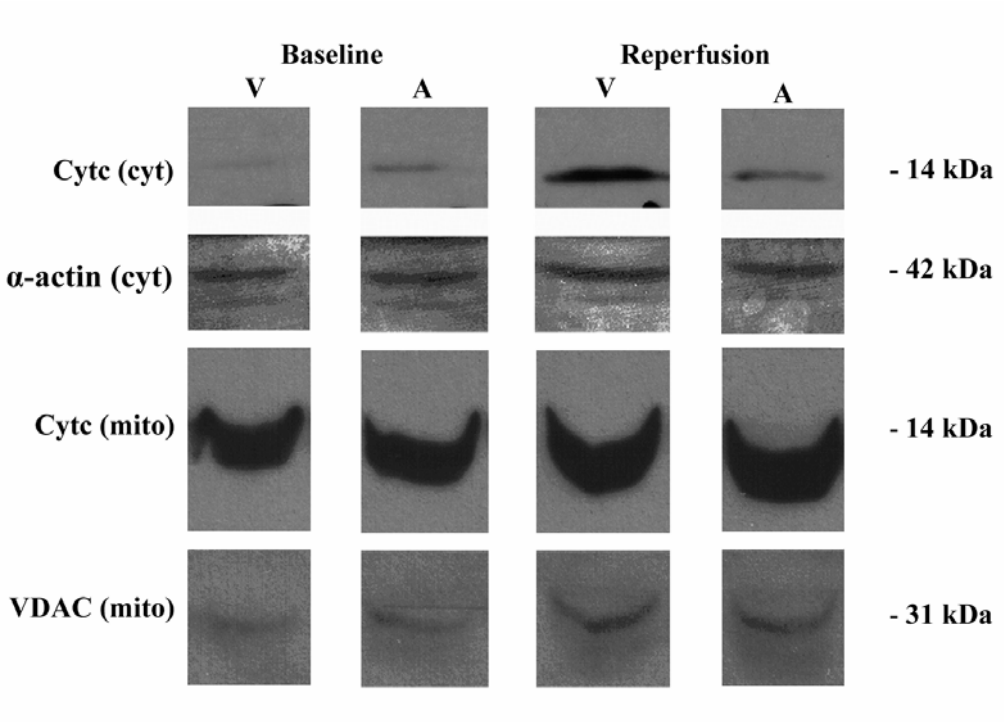


Figure 8. *Western blot analysis of cytosolic cytochrome c content following 15 minutes of baseline perfusion.* Hearts were freeze-clamped and homogenized immediately following 15 minutes of baseline perfusion with KHB (n=4). Approximately 10 μg of protein from each heart was resolved on a 15% SDS polyacrylamide gel and probed for cytochrome *c* and α-actin by Western blotting. Ratio of cytosolic cytochrome *c*/α-actin band intensity was 0.76 ± 0.03 in baseline hearts. Figure used with permission (Hadzimichalis *et al*., 2007).

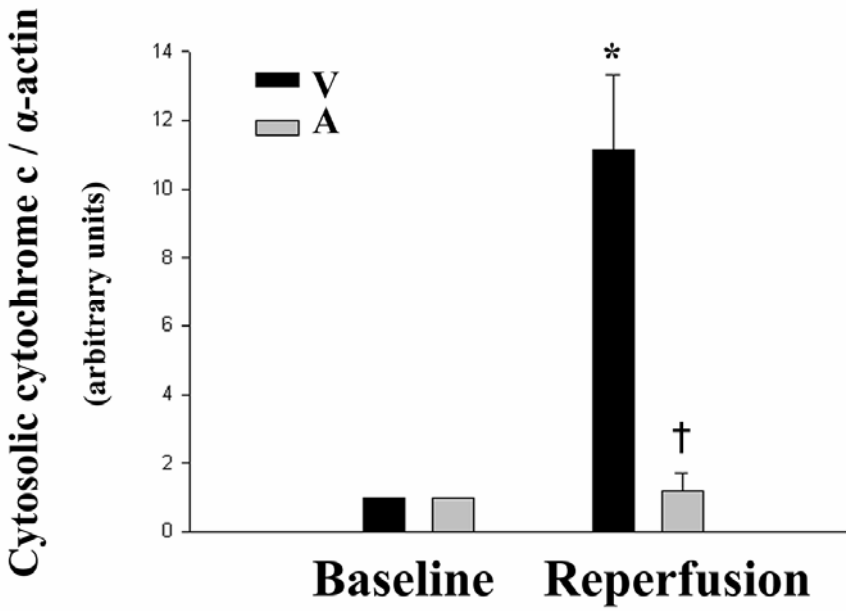
4. Acetaminophen treatment inhibits mitochondrial cytochrome *c* release following myocardial ischemia/reperfusion

Following MPTP opening, cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol thus triggering the intrinsic apoptotic cascade (Weiss *et al*., 2003). In the current study, we analyzed cytosolic and mitochondrial cytochrome *c* content following baseline and ischemia/reperfusion. Mitochondrial cytochrome *c* release was defined by increases in cytosolic cytochrome *c* content concomitant with decreases in mitochondrial cytochrome *c* content. Our results indicate that following ischemia/reperfusion in vehicle-treated hearts there was a significant increase in mitochondrial cytochrome *c* release compared to corresponding baseline values. However, no differences were noted between mitochondrial or cytosolic cytochrome *c* content of acetaminophen-treated hearts when compared to corresponding baseline samples (Figure 9). In addition, treatment with acetaminophen resulted in significant attenuation of cytochrome *c* release post-reperfusion when compared to vehicle-treated hearts. Cytosolic cytochrome *c* levels (normalized to α-actin and baseline hearts) were 11.15±2.18 and 1.21±0.48 in vehicle- and acetaminophen-treated hearts, respectively. These data show that acetaminophen treatment significantly and completely inhibits the mitochondrial cytochrome *c* release normally observed following myocardial ischemia/reperfusion.

A


Baseline
Reperfusion
V
A
V
A
Cytc (cyt)
- 14 kDa
α-actin (cyt)
- 42 kDa
Cytc (mito)
- 14 kDa
VDAC (mito)
- 31 kDa

B


Cytosolic cytochrome c / α-actin
(arbitrary units)
14
12
10
8
6
4
2
0
V
A
*
†
Baseline
Reperfusion

C

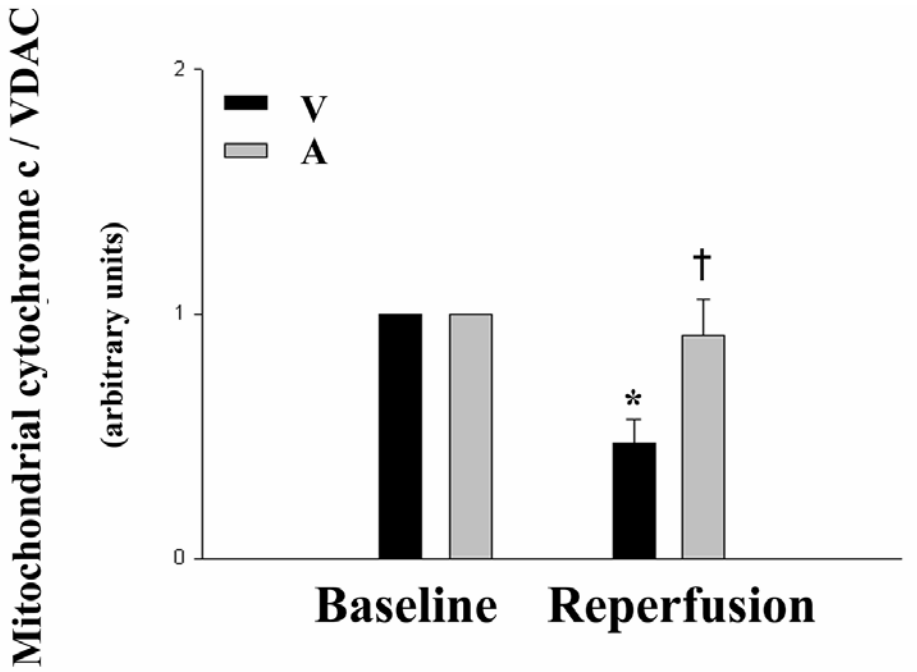


Figure 9. *Western blot analysis of cytosolic and mitochondrial cytochrome c heart homogenate fractions following ischemia/reperfusion.* Hearts were freeze-clamped, homogenized, and separated into mitochondrial and cytosolic fractions following 15 minutes of baseline perfusion or following ischemia/reperfusion in vehicle- and acetaminophen-treated hearts (n=4 per group). Approximately 10 µg of protein from each heart was resolved on a 15% SDS polyacrylamide gel and probed for cytochrome *c* and the appropriate loading control (either α-actin or VDAC) by Western blotting. (A) Representative Western blots from cytosolic (cyt) and mitochondrial (mito) heart homogenate fractions. (B) Statistical analysis of cytosolic cytochrome *c* content normalized to α-actin and the

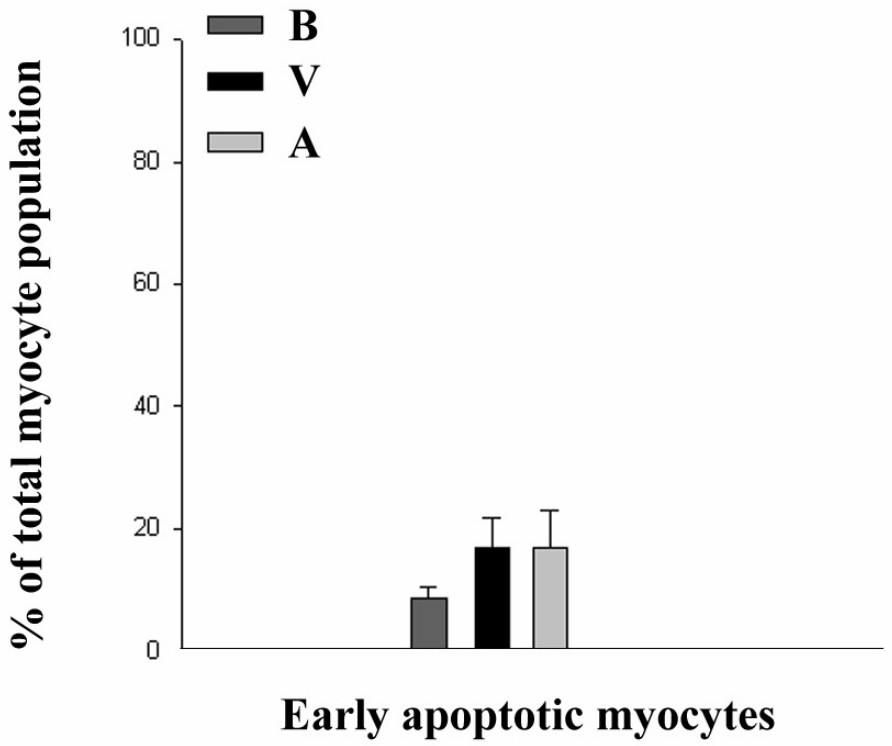
corresponding baseline. (C) Statistical analysis of mitochondrial cytochrome *c* content normalized to VDAC and the corresponding baseline. V, vehicle-treated hearts; A, APAP-treated hearts. *$p<0.05$ as determined by ANOVA followed by Tukey's Multiple Comparison Test compared to corresponding baseline hearts. †$p<0.05$ as determined by ANOVA followed by Tukey's Multiple Comparison Test compared to ischemia/reperfused vehicle-treated hearts. Figure used with permission (Hadzimichalis *et al*., 2007).

5. Acetaminophen treatment attenuates the number of late-stage apoptotic myocytes following myocardial ischemia/reperfusion

Ischemia/reperfusion injury can induce both necrotic and apoptotic cell death (Honda *et al*., 2005). Our group has previously reported that acetaminophen mediates attenuation of necrotic cell death following myocardial infarction; however, acetaminophen's specific role in myocardial apoptosis has not yet been explored (Merrill *et al*., 2004). To address whether inhibition of apoptosis plays a role in the mechanism of acetaminophen-mediated cardioprotection, we isolated ventricular myocytes following baseline and ischemia/reperfusion. We then loaded myocytes with annexin V-FITC and PI and analyzed fluorescent intensity using flow cytometry. As shown in Figure 10, the total percentage of late apoptotic cells following ischemia/reperfusion was significantly reduced in acetaminophen- versus vehicle-treated hearts (58±1% vs. 81±5%, respectively). However, no significant differences were noted between treatment groups during early apoptosis (17±5% vs. 17±6% for vehicle- and acetaminophen-treated hearts, respectively). Additionally, significant increases in late stage apoptotic myocytes were observed in both treatment groups following reperfusion when compared to data from baseline hearts. These data suggest that our preparation was successful at inducing late stage apoptosis and that acetaminophen may play a cardioprotective role by attenuating the progression of apoptosis in cardiomyocytes following ischemia/reperfusion. Figure 11 is a representative analysis of cellular distribution (viable, early

apoptotic, late apoptotic, and necrotic) in a vehicle-treated heart following ischemia/reperfusion.

A


B
V
A
100
80
60
40
20
0
% of total myocyte population
Early apoptotic myocytes

B

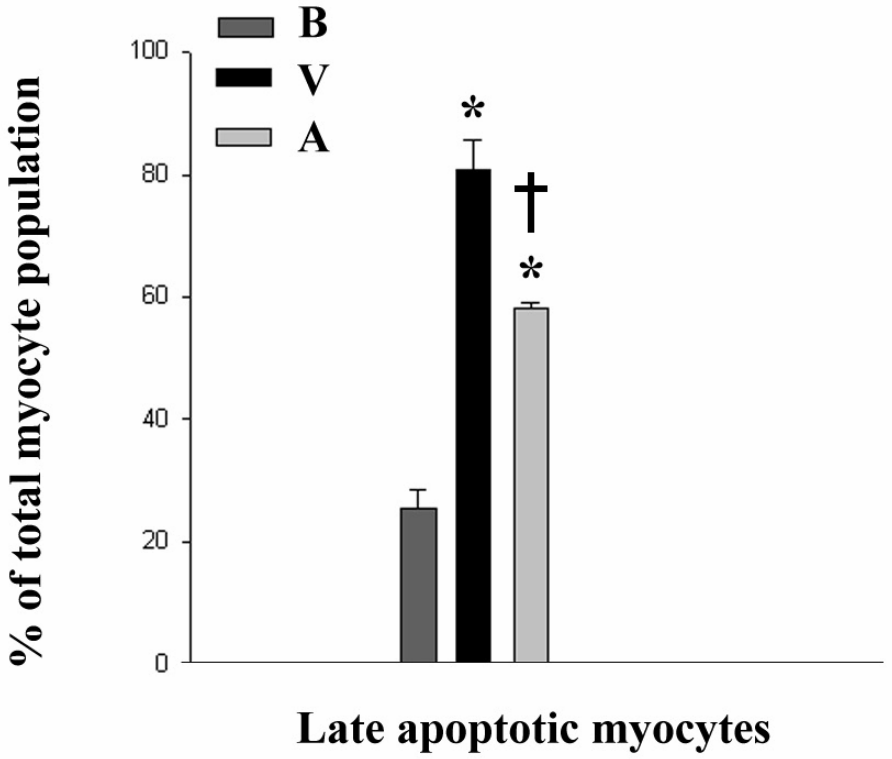

B
V
A
100
80
60
40
20
0
*
†
*
% of total myocyte population
Late apoptotic myocytes

Figure 10. *FACS analysis of post-ischemia/reperfused ventricular myocytes.* Hearts were digested with collagenase and myocytes were isolated and loaded with annexin V-FITC and propidium iodide following 15 minutes baseline or ischemia/reperfusion in vehicle- and acetaminophen-treated hearts (n=4 per group). Flow cytometry was used to determine percentage of (A) early and (B) late stage apoptotic myocytes in vehicle- and acetaminophen-treated hearts following ischemia/reperfusion. B, baseline hearts; V, vehicle-treated hearts; A, APAP-treated hearts. *$p<0.05$ as determined by ANOVA followed by Tukey's Multiple Comparison Test compared to myocytes from baseline hearts in the same stage of apoptosis. †$p<0.05$ as determined by ANOVA followed by Tukey's Multiple Comparison Test compared to myocytes from vehicle-treated hearts in the same stage of apoptosis. Figure used with permission (Hadzimichalis *et al*., 2007).

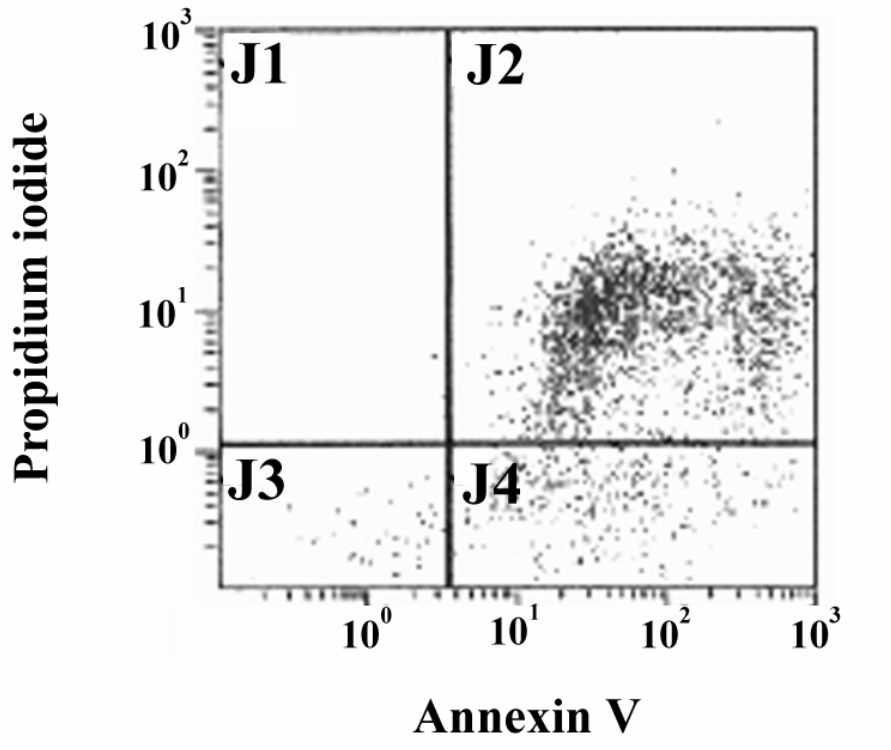


Figure 11. *Representative FACS analysis of vehicle-treated ischemia/reperfused heart.* J1, necrotic cells; J2, late apoptotic cells; J3, viable cells; J4, early apoptotic cells. Figure used with permission (Hadzimichalis *et al*., 2007).

IV. DISCUSSION

1. Rationale

With the marked rise in heart disease, the need for preventative cardiac care has become essential. Many groups have investigated the protective capacity of a variety of compounds in inhibiting myocardial ischemia/reperfusion-induced injury. Studies by Varga *et al*. (2004) investigated the effects of pretreatment with dexamethasone, a potent glucocorticoid, on post-ischemia/reperfusion. They reported that dexamethasone inhibits ventricular fibrillation *via* attenuation of mitochondrial cytochrome *c* release. Kovacs et al. (2001) administered non-specific caspase inhibitors at the onset of reperfusion to examine their ability to maintain cardiac function and limit both infarct size and apoptosis. Additional reports from Das *et al*. (2005) examined the cardioprotective effects of pretreatment with palm tocotrienol, a vitamin E isomer, following myocardial ischemia/reperfusion. They demonstrated that treatment with tocotrienols, derived from a tocotrienol-rich fraction of palm oil, results in attenuation of ischemia/reperfusion-induced damage via inhibition c-Src phosphorylation and maintenance of proteasomal activity. However, despite efforts to discover and/or synthesize new cardioprotective compounds, very little effort has focused on examining the potential cardioprotective effects of historically safe drugs, including acetaminophen (Bi *et al*., 2007).

In this study, we examined the mechanistic basis for acetaminophen-mediated functional cardioprotection. Previous studies reported that in an *in vivo* canine preparation of myocardial infarction, acetaminophen treatment results in a

significant reduction of necrotic tissue (Merrill *et al*., 2004). In the current study, we proposed that acetaminophen might also have an effect on the mitochondrial pathway of apoptosis following ischemia/reperfusion. Specifically, we explored whether therapeutic concentrations of acetaminophen can attenuate MPTP opening, cytochrome *c* release, and apoptotic cell death. The major finding of our study is that following myocardial ischemia/reperfusion, acetaminophen treatment completely blocks opening of the MPTP and mitochondrial swelling as well as mitochondrial cytochrome *c* release. Furthermore, although acetaminophen attenuates late stage apoptosis, it does not completely block it. These results suggest that acetaminophen inhibits the MPTP-induced pathway of apoptosis; however, other pathways leading to apoptosis may not be affected by acetaminophen.

Acetaminophen, when taken at therapeutic concentrations, has been established as a safe antipyretic and analgesic drug (Prescott, 2001). More recently, this compound has also been established as an effective cardioprotective agent during myocardial ischemia/reperfusion injury (Merrill *et al*., 2001; Merrill and Goldberg, 2001; Merrill, 2002; Halestrap *et al*., 2004). Mechanistically, the phenolic hydroxyl group of acetaminophen likely donates its hydrogen atom to aid in the reported reduction of free radicals, namely peroxynitrite and hydroxyl radicals, post-reperfusion (Merrill *et al*., 2001; Merrill and Goldberg, 2001; Prescott, 2001; Merrill, 2002). Ischemia/reperfusion-induced oxidative stress is a well-known trigger for MPTP opening, mitochondrial cytochrome *c* release, and downstream apoptotic cell death pathway activation

(Weiss *et al*., 2003; Halestrap *et al*., 2004; Gateau-Roesch *et al*., 2006; Orrenius *et al*., 2007). We hypothesize that acetaminophen-mediated inhibition of ROS generation results, in part, in attenuation of reperfusion-induced myocardial injury via a reduction in MPTP opening, mitochondrial cytochrome *c* release, and apoptotic cell death.

2. The Langendorff perfusion

2.1 Advantages and limitations of the Langendorff preparation

Since its conception over 100 years ago, the Langendorff-perfused mammalian heart preparation still remains one of the most popular methods for studying cardiac metabolism, hemodynamics, metabolic and pharmacological interventions, electrical activity, and global myocardial ischemia and hypoxia (Langendorff, 1895; Sutherland and Hearse, 2000). Modification of the preparation in this study, including perfusate composition, temperature, pressure, and pacing rate, was established based on previously published reports from our laboratory (Merrill *et al*., 2001; Merrill and Goldberg, 2001; Merrill, 2002).

The isolated perfused Langendorff heart preparation provides an efficient and highly reproducible means of collecting widespread physiologic data during global myocardial ischemia and reperfusion (Hearse and Sutherland, 2000). Denervation presents a unique opportunity to study cardiac function devoid of sympathetic and vagal stimulation (Sutherland and Hearse, 2000). However, while there are countless advantages, this preparation also introduces several limitations. Myocardial extraction and *ex vivo* placement result in restricted clinical application and continual tissue deterioration (i.e. 5-10% decrement in contractile function/hour) over prolonged periods (Sutherland and Hearse, 2000). Nevertheless, the Langendorff-perfused heart presented the most optimal compromise between quality and quantity of data for our studies.

2.2 The Langendorff-perfused guinea pig heart model

This study was an identification of the mechanism underlying previously reported acetaminophen-mediated functional cardioprotection following ischemia/reperfusion. As such, it was essential to employ an identical animal model, namely the Langendorff-perfused guinea pig heart. However, certain factors were taken into consideration when initially choosing an animal model.

Firstly, measures were taken to establish the best compromise between clinical relevance, cost, data quality and quantity, and reproducibility (Hearse and Sutherland, 2000). In addition, it was noted that guinea pigs, similar to humans, are unable to synthesize ascorbic acid, an organic acid exhibiting antioxidant properties. Deficiency in the enzyme required to synthesize this compound suggests that the isolated perfused guinea pig heart would be a remarkably valuable model to study the effects of a drug on post-reperfusion injury (Meister, 1994).

3. Acetaminophen; therapeutic dosages and experimental concentrations

Therapeutic concentrations of acetaminophen in human plasma samples may range from 10-100 µg/ml, with hepatatoxicity occurring at concentrations >300 µg/ml (Prescott, 2000; Spiler *et al*., 2005). Clinically, administration of 1000 mg of acetaminophen every 4 hours for 4 doses (50-70 kg patient), will result in fluctuating plasma concentrations within the therapeutic range (Rumack, 2004). In these studies acetaminophen (0.35 mM) was dissolved into the perfusate and delivered continuously. HPLC analysis from our laboratory reveals net extraction of acetaminophen by the myocardium, with arterial and venous concentrations ranging from 45-50 µg/ml (Spiler *et al*., 2005). Hence, concentrations used in our studies fall well within the effective therapeutic range and far below the toxic range.

4. Acetaminophen-mediated inhibition of mitochondrial swelling and MPTP opening following ischemia/reperfusion

Reports indicate that mitochondrial swelling is indicative of MPTP opening and ultimately results in outer mitochondrial membrane (OMM) rupture (Halestrap *et al*., 2004; Di Lisa and Bernardi, 2006). Increases in mitochondrial swelling, as assessed by decreases in light absorbance, would therefore imply downstream cytochrome *c* release and activation of the mitochondrial-mediated pathway of apoptosis (Di Lisa and Bernardi, 2006; Kaasik *et al*., 2007). Central to the successful analysis of mitochondrial light scattering was the isolation of purified intact mitochondria. Voltage dependent anion channel, an outer mitochondrial membrane protein, was used as a loading control in isolated mitochondrial fractions of whole heart homogenate.

We found a significant decrease in the light absorbance of isolated mitochondria from vehicle-treated ischemia/reperfused hearts when compared to either baseline, and acetaminophen treatment completely reversed this effect (Figure 1B). These results suggest that our model of ischemia/reperfusion (30 minutes low-flow global ischemia and 60 minutes reperfusion) successfully induced mitochondrial permeability pore opening at the completion of reperfusion, and that the presence of acetaminophen resulted in inhibition of this opening (Figure 6B). These data are further strengthened by electron micrograph analysis showing preserved myofibrillar ultrastructure and intact mitochondria in acetaminophen-treated hearts, similar to baseline controls, and

visually swollen mitochondria post-ischemia/reperfusion in vehicle-treated hearts (Figure 7). These data suggest that acetaminophen completely attenuates pore opening following ischemia/reperfusion in our model.

5. Acetaminophen-mediated inhibition of mitochondrial cytochrome *c* release following ischemia/reperfusion

Following ischemia/reperfusion-induced OMM rupture in response to MPTP opening and mitochondrial swelling, cytochrome *c* is released into the cytosol to initiate the intrinsic pathway of apoptosis (Halestrap et al., 2004). We found a significant increase in cytosolic cytochrome *c* content, with a concomitant decrease in mitochondrial cytochrome *c* content following ischemia/reperfusion in vehicle-treated hearts. This suggests that our model of ischemia/reperfusion was successful at inducing mitochondrial cytochrome *c* release at the completion of reperfusion. In addition, acetaminophen treatment resulted in a significant and complete inhibition of cytochrome *c* release following injury when compared to vehicle-treated hearts. These data suggest that acetaminophen treatment completely inhibits mitochondrial cytochrome *c* release following ischemia/reperfusion in our model. We have shown (Figures 1 and 2) that the observed inhibition of cytochrome *c* release is likely a response to the complete upstream inhibition of MPTP opening; however, it is possible that acetaminophen also exhibits functional cardioprotection via other pathways upstream to cytochrome *c* release.

6. Acetaminophen-mediated attenuation of late stage apoptosis following ischemia/reperfusion

In our protocol, early stage apoptotic myocytes were defined as those cells that were stained with annexin V-FITC. This population was comprised of myocytes that had externalized phosphatidylserine residues and active caspases but no DNA degradation or loss of membrane integrity. Late stage apoptotic myocytes were defined as those cells that were both annexin V-FITC and PI positive. This myocyte population had active caspases and permeabilized cell membranes (Schmid et al., 2007). We found that acetaminophen treatment significantly inhibited the number of late stage apoptotic myocytes when compared to vehicle-treated hearts at the completion of reperfusion. However, there was also a significant increase in late stage apoptotic myocytes between baseline and acetaminophen-treated ischemia/reperfused hearts. This increasing index of damage following ischemia/reperfusion in acetaminophen-treated hearts was not as apparent as mitochondrial swelling or cytochrome *c* release. It is possible that the changes in apoptotic cell death noted between treatment groups at reperfusion are due, in part, to acetaminophen-mediated MPTP inhibition and cytochrome *c* release. However, these data also suggest that while acetaminophen may abolish permeability pore transition and cytochrome *c* release following ischemia/reperfusion, other pathways of apoptosis, unaffected by acetaminophen treatment, are still active during injury. We propose that acetaminophen-mediated cardioprotection is, at least in part,

specific to inhibition of MPTP-induced cytochrome *c* release and apoptosis. A schematic of these data are shown in Figure 12.

Damaging post-reperfusion oxidants, including peroxynitrite and hydroxyl radicals, activate the intrinsic pathway of apoptosis, and the efficacy of acetaminophen in attenuating these compounds makes it a likely inhibitor of this pathway (Merrill, 2002). However, it is possible that the incomplete attenuation of apoptosis in response to treatment is the result of ischemia/reperfusion-induced activation of other apoptotic cell death pathways, including the extrinsic pathway of apoptosis.

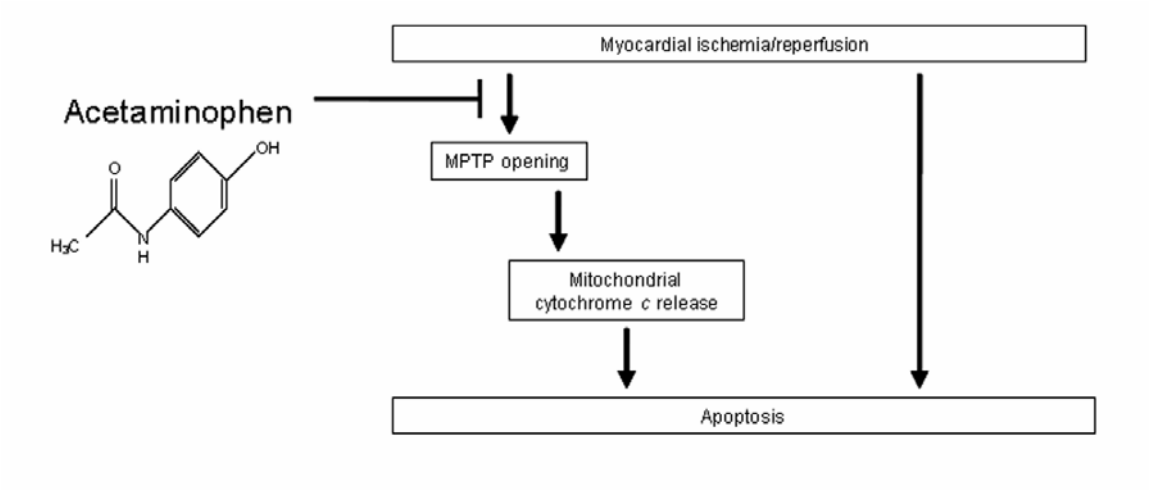


Figure 12. *Schematic of proposed mechanism of action of acetaminophen following myocardial ischemia/reperfusion.* Our studies imply that while acetaminophen completely inhibits MPTP opening and mitochondrial cytochrome *c* release, apoptosis is not completely blocked. Thus, additional apoptotic pathways are still active following insult. Figure used with permission (Hadzimichalis *et al*., 2007).

7. Future directions

These data (Table 3) begin to explain the mechanism underlying previously reported acetaminophen-mediated cardioprotection following ischemia/reperfusion. They suggest that administration of acetaminophen just prior to an ischemic attack can result in attenuation of functional damage via inhibition of MPTP opening and cytochrome *c* release-induced apoptotic cell death following ischemia/reperfusion. While these data are promising in that they offer a historically safe alternative to preventative cardiac care, additional pathway details must be elucidated prior to clinical application. Future studies should examine the specific role of acetaminophen in the extrinsic versus intrinsic apoptotic pathways.

We have reported that while acetaminophen completely inhibits ischemia/reperfusion-induced MPTP opening, mitochondrial swelling, and cytochrome *c* release, it only partially attenuates apoptosis. Future studies may also aim to employ the use of acetaminophen in conjunction with caspase inhibitors or other anti-apoptotic drugs, in order to further prevent apoptotic cell death post-reperfusion.

Our laboratory has already begun investigating the effects of acetaminophen following other cardiovascular injuries caused, in part, by damaging oxidants. Preliminary data suggest that functional cytoprotectivity may also be observed in other organ systems, including the brain following cerebral ischemia/repefusion. We encourage other laboratories to examine the effects of

acetaminophen in an attempt to expand the spectrum of uses for this historically safe drug.

Table 3. Summary table of study findings as they relate to the mechanism of acetaminophen-mediated cardioprotection following ischemia/reperfusion.

	Acetaminophen vs. Baseline	Vehicle vs. Baseline	Acetaminophen vs. Vehicle
MPTP opening (Spectrophotometry and electron microscopy)	ns	*	*
Mitochondrial cytochrome *c* release (Western blotting)	ns	*	*
Apoptosis			
Early	ns	ns	ns
Late (Flow cytometry)	*	*	*

Acetaminophen completely inhibits MPTP opening and mitochondrial cytochrome *c* release and partially attenuates apoptosis when compared to vehicle-treated hearts following ischemia/reperfusion. *$p<0.05$; ns, no significance. Figure used with permission (Hadzimichalis et al., 2007).

V. REFERENCES

Bazzola JJ, Russel LD (1999) Electron microscopy: principles and techniques for biologists. , 2nd Edition. Sudbury, MA: Jones and Bartlett Publishers.

Bi W, Cai J, Liu S, Baudy-Floc'h M, Bi L (2007) Design, synthesis and cardioprotective effect of a new class of dual-acting agents: Phenolic tetrahydro-beta-carboline RGD peptidomimetic conjugates. Bioorg Med Chem 15:6909-6919.

Bopassa JC, Vandroux D, Ovize M, Ferrera R (2006) Controlled reperfusion after hypothermic heart preservation inhibits mitochondrial permeability transition-pore opening and enhances functional recovery. Am J Physiol Heart Circ Physiol 291:H2265-2271.

Borutaite V, Jekabsone A, Morkuniene R, Brown GC (2003) Inhibition of mitochondrial permeability transition prevents mitochondrial dysfunction, cytochrome c release and apoptosis induced by heart ischemia. J Mol Cell Cardiol 35:357-366.

Bosetti F, Baracca A, Lenaz G, Solaini G (2004) Increased state 4 mitochondrial respiration and swelling in early post-ischemic reperfusion of rat heart. FEBS Lett 563:161-164.

Botting RM (2000) Mechanism of action of acetaminophen: is there a cyclooxygenase 3? Clin Infect Dis 31 Suppl 5:S202-210.

Brodie BB, Axelrod J (1948a) The estimation of acetanilide and its metabolic products aniline, N-acetyl p-aminophenol and p-aminophenol (free and total conjugated) in biological fluids and tissues. J Pharmacol Exp Ther 94:22-28.

Brodie BB, Axelrod J (1948b) The fate of acetanilide in man. J Pharmacol Exp Ther 94.

Broekemeier KM, Dempsey ME, Pfeiffer DR (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J Biol Chem 264:7826-7830.

Bunger R, Haddy FJ, Gerlach E (1975a) Coronary responses to dilating substances and competitive inhibition by theophylline in the isolated perfused guinea pig heart. Pflugers Arch 358:213-224.

Bunger R, Haddy FJ, Querengasser A, Gerlach E (1975b) An isolated guinea pig heart preparation with in vivo like features. Pflugers Arch 353:317-326.

Chou TM, Greenspan P (2002) Effect of acetaminophen on the myeloperoxidase-hydrogen peroxide-nitrite mediated oxidation of LDL. Biochim Biophys Acta 1581:57-63.

Cotter J, Wooltorton E (2005) New restrictions on celecoxib (Celebrex) use and the withdrawal of valdecoxib (Bextra). Cmaj 172:1299.

Das S, Powell SR, Wang P, Divald A, Nesaretnam K, Tosaki A, Cordis GA, Maulik N, Das DK (2005) Cardioprotection with palm tocotrienol: antioxidant activity of tocotrienol is linked with its ability to stabilize proteasomes. Am J Physiol Heart Circ Physiol 289:H361-367.

Di Lisa F, Bernardi P (2006) Mitochondria and ischemia-reperfusion injury of the heart: fixing a hole. Cardiovasc Res 70:191-199.

Duchen MR (2004) Roles of mitochondria in health and disease. Diabetes 53 Suppl 1:S96-102.

Foo RS, Mani K, Kitsis RN (2005) Death begets failure in the heart. J Clin Invest 115:565-571.

Gadelha FR, Thomson L, Fagian MM, Costa AD, Radi R, Vercesi AE (1997) Ca2+-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. Arch Biochem Biophys 345:243-250.

Gateau-Roesch O, Argaud L, Ovize M (2006) Mitochondrial permeability transition pore and postconditioning. Cardiovasc Res 70:264-273.

Gewies A (2003) Introduction to Apoptosis. ApoReview:1-26.

Golfetti R, Rork T, Merrill G (2003) Chronically administered acetaminophen and the ischemia/reperfused myocardium. Exp Biol Med (Maywood) 228:674-682.

Golfetti R, VanDyke K, Rork T, Spiler N, Merrill G (2002) Acetaminophen in the post-ischemia reperfused myocardium. Exp Biol Med (Maywood) 227:1031-1037.

Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94:1621-1628.

Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626-629.

Green DR, Kroemer G (2005) Pharmacological manipulation of cell death: clinical applications in sight? J Clin Invest 115:2610-2617.

Hadzimichalis NM, Baliga SS, Golfetti R, Jaques KM, Firestein BL, Merrill GF (2007) Acetaminophen-mediated cardioprotection via inhibition of the mitochondrial permeability transition pore-induced apoptotic pathway. Am J Physiol Heart Circ Physiol.

Halestrap AP (2006) Calcium, mitochondria and reperfusion injury: a pore way to die. Biochem Soc Trans 34:232-237.

Halestrap AP, Clarke SJ, Javadov SA (2004) Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res 61:372-385.

Haunstetter A, Izumo S (1998) Apoptosis: basic mechanisms and implications for cardiovascular disease. Circ Res 82:1111-1129.

Hearse DJ, Sutherland FJ (2000) Experimental models for the study of cardiovascular function and disease. Pharmacol Res 41:597-603.

Hemler M, Lands WE (1976) Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. J Biol Chem 251:5575-5579.

Hinsberg O, Treupel G (1894) 33:216-250.

Honda HM, Korge P, Weiss JN (2005) Mitochondria and ischemia/reperfusion injury. Ann N Y Acad Sci 1047:248-258.

Huang XD, Horackova M, Pressler ML (1996) Changes in the expression and distribution of connexin 43 in isolated cultured adult guinea pig cardiomyocytes. Exp Cell Res 228:254-261.

Hunter FE, Jr., Gebicki JM, Hoffsten PE, Weinstein J, Scott A (1963) Swelling and lysis of rat liver mitochondria induced by ferrous ions. J Biol Chem 238:828-835.

Javadov S, Karmazyn M (2007) Mitochondrial permeability transition pore opening as an endpoint to initiate cell death and as a putative target for cardioprotection. Cell Physiol Biochem 20:1-22.

Javadov SA, Lim KH, Kerr PM, Suleiman MS, Angelini GD, Halestrap AP (2000) Protection of hearts from reperfusion injury by propofol is associated with inhibition of the mitochondrial permeability transition. Cardiovasc Res 45:360-369.

Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P, Debatin KM (2000) Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. Circulation 102:915-920.

Kaasik A, Safiulina D, Zharkovsky A, Veksler V (2007) Regulation of mitochondrial matrix volume. Am J Physiol Cell Physiol 292:C157-163.

Kis B, Snipes JA, Busija DW (2005) Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fictions, and uncertainties. J Pharmacol Exp Ther 315:1-7.

Kovacs P, Bak I, Szendrei L, Vecsernyes M, Varga E, Blasig IE, Tosaki A (2001) Non-specific caspase inhibition reduces infarct size and improves post-ischaemic recovery in isolated ischaemic/reperfused rat hearts. Naunyn Schmiedebergs Arch Pharmacol 364:501-507.

Krijnen PA, Nijmeijer R, Meijer CJ, Visser CA, Hack CE, Niessen HW (2002) Apoptosis in myocardial ischaemia and infarction. J Clin Pathol 55:801-811.

Langendorff O (1895) Untersuchungen am uberlebenden Saugethierherzen. Pflugers Arch fur die Gesamte Physiologie des Menschen and der Tiere 61:291-332.

Logue SE, Gustafsson AB, Samali A, Gottlieb RA (2005) Ischemia/reperfusion injury at the intersection with cell death. J Mol Cell Cardiol 38:21-33.

Luo C, He ML, Bohlin L (2005) Is COX-2 a perpetrator or a protector? Selective COX-2 inhibitors remain controversial. Acta Pharmacol Sin 26:926-933.

MacLellan WR, Schneider MD (1997) Death by design. Programmed cell death in cardiovascular biology and disease. Circ Res 81:137-144.

Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci U S A 91:3228-3232.

Meister A (1994) Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 269:9397-9400.

Mering vJ (1893) Beitrage zur Kenntniss der Antipyretica. Therapeutishe Monatshefte 7:577-578.

Merrill G, McConnell P, Vandyke K, Powell S (2001) Coronary and myocardial effects of acetaminophen: protection during ischemia-reperfusion. Am J Physiol Heart Circ Physiol 280:H2631-2638.

Merrill GF (2002) Acetaminophen and low-flow myocardial ischemia: efficacy and antioxidant mechanisms. Am J Physiol Heart Circ Physiol 282:H1341-1349.

Merrill GF, Goldberg E (2001) Antioxidant properties of acetaminophen and cardioprotection. Basic Res Cardiol 96:423-430.

Merrill GF, Rork TH, Spiler NM, Golfetti R (2004) Acetaminophen and myocardial infarction in dogs. Am J Physiol Heart Circ Physiol 287:H1913-1920.

Morin D, Hauet T, Spedding M, Tillement J (2001) Mitochondria as target for antiischemic drugs. Adv Drug Deliv Rev 49:151-174.

Nachtigal M, Mayer E, Ghaffar A, Greenspan P (2005) The effect of acetaminophen on the development of aorta athersclerotic lesions in apoE deficient mice. Experimental Biology, San Diego California (abstract 60510).

Orrenius S, Gogvadze V, Zhivotovsky B (2007) Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol 47:143-183.

Piper H, Isenberg G (1989) Isolated adult cardiomyocytes. Boca Raton, FL: CRC Press, Inc.

Prescott LF (2000) Paracetamol: past, present, and future. Am J Ther 7:143-147.

Prescott LF (2001) Paracetamol (Acetaminophen): A Critical Bibliographic Review., 2nd Edition. New York: Taylor & Francis.

Rogers L, Taylor A, Raya R, Gest A, Smith C (1999) Acetaminophen lowers fatty streak formation and lipid oxidation in hypercholesterolemic rabbits. In: Free Radic Biol Med, p S108.

Rork TH, Van Dyke K, Spiler NM, Merrill GF (2004) Acetaminophen in the hypoxic and reoxygenated guinea pig myocardium. Exp Biol Med (Maywood) 229:1154-1161.

Rork TH, Hadzimichalis NM, Kappil MA, Merrill GF (2006) Acetaminophen attenuates peroxynitrite-activated matrix metalloproteinase-2-mediated troponin I cleavage in the isolated guinea pig myocardium. J Mol Cell Cardiol 40:553-561.

Rousou AJ, Ericsson M, Federman M, Levitsky S, McCully JD (2004) Opening of mitochondrial KATP channels enhances cardioprotection through the modulation of mitochondrial matrix volume, calcium accumulation, and respiration. Am J Physiol Heart Circ Physiol 287:H1967-1976.

Ruiz-Meana M, Garcia-Dorado D, Pina P, Inserte J, Agullo L, Soler-Soler J (2003) Cariporide preserves mitochondrial proton gradient and delays ATP depletion in cardiomyocytes during ischemic conditions. Am J Physiol Heart Circ Physiol 285:H999-1006.

Rumack BH (2004) Acetaminophen misconceptions. Hepatology 40:10-15.

Salzberg DJ, Weir MR (2007) COX-2 inhibitors and cardiovascular risk. Subcell Biochem 42:159-174.

Schmid I, Uittenbogaart C, Jamieson BD (2007) Live-cell assay for detection of apoptosis by dual-laser flow cytometry using Hoechst 33342 and 7-amino-actinomycin D. Nat Protoc 2:187-190.

Seibert K, Masferrer JL (1994) Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor 4:17-23.

Spiler NM, Rork TH, Merrill GF (2005) An old drug with a new purpose: cardiovascular actions of acetaminophen (paracetamol). Curr Drug Targets Cardiovasc Haematol Disord 5:419-429.

Sutherland FJ, Hearse DJ (2000) The isolated blood and perfusion fluid perfused heart. Pharmacol Res 41:613-627.

Tokarska-Schlattner M, Zaugg M, da Silva R, Lucchinetti E, Schaub MC, Wallimann T, Schlattner U (2005) Acute toxicity of doxorubicin on isolated perfused heart: response of kinases regulating energy supply. Am J Physiol Heart Circ Physiol 289:H37-47.

Vanden Hoek TL, Qin Y, Wojcik K, Li CQ, Shao ZH, Anderson T, Becker LB, Hamann KJ (2003) Reperfusion, not simulated ischemia, initiates intrinsic apoptosis injury in chick cardiomyocytes. Am J Physiol Heart Circ Physiol 284:H141-150.

Vane JR, Botting RM (1997) Mechanism of action of aspirin-like drugs. Semin Arthritis Rheum 26:2-10.

Varga E, Nagy N, Lazar J, Czifra G, Bak I, Biro T, Tosaki A (2004) Inhibition of ischemia/reperfusion-induced damage by dexamethasone in isolated working rat hearts: the role of cytochrome c release. Life Sci 75:2411-2423.

Weiss JN, Korge P, Honda HM, Ping P (2003) Role of the mitochondrial permeability transition in myocardial disease. Circ Res 93:292-301.

Xie WL, Chipman JG, Robertson DL, Erikson RL, Simmons DL (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci U S A 88:2692-2696.

Zhu YZ, Chong CL, Chuah SC, Huang SH, Nai HS, Tong HT, Whiteman M, Moore PK (2006) Cardioprotective effects of nitroparacetamol and paracetamol in acute phase of myocardial infarction in experimental rats. Am J Physiol Heart Circ Physiol 290:H517-524.

Zidar N, Jera J, Maja J, Dusan S (2007) Caspases in myocardial infarction. Adv Clin Chem 44:1-33.

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Merrill GF, Rork TH, **Spiler NM**, and Golfetti R. Acetaminophen and myocardial infarction in dogs. Am J Physiol Heart Circ Physiol 287: H1913-1920, 2004.

Rork TH, Van Dyke K, **Spiler NM**, and Merrill GF. Acetaminophen in the hypoxic and reoxygenated guinea pig myocardium. Exp Biol Med (Maywood) 229: 1154-1161, 2004.

Spiler NM, Rork TH, and Merrill GF. An old drug with a new purpose: Cardiovascular actions of acetaminophen (paracetamol). Curr Drug Targets Cardiovasc Haematol Disord 5: 419-429, 2005.

Rork TH, **Hadzimichalis NM**, Baliga SS, Golfetti R, and Merrill GF. New Perspectives on Acetaminophen. Current Cardiology Reviews 2: 131-146, 2006.

Rork TH, **Hadzimichalis NM**, Kappil MA, and Merrill GF. Acetaminophen attenuates peroxynitrite-activated matrix metalloproteinase-2-mediated troponin I cleavage in the isolated guinea pig myocardium. J Mol Cell Cardiol 40: 553-561, 2006.

Merrill GF, Merrill JH, Golfetti R, Jaques KM, **Hadzimichalis NM**, Baliga SS, and Rork TH. Antiarrhythmic properties of acetaminophen in the dog. Exp Biol Med (Maywood) 232: 1245-1252, 2007.

Hadzimichalis NM, Baliga SS, Golfetti R, Jaques KM, Firestein BL, and Merrill GF. Acetaminophen-mediated cardioprotection via inhibition of the mitochondrial permeability transition pore-induced apoptotic pathway. Am J Physiol Heart Circ Physiol, in press, 2007.

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