Antioxidant Modulation of Acute Organ Injury

Ph.D. Thesis

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MOTTO

It is both near and far
Both within and without every creature
It moves and is unmoving
In its subtlety it is beyond comprehension
It is indivisible, yet appears divided in separate creatures
Know it to be the creator, the preserver, and the destroyer
Dwelling in every heart, it is beyond darkness
It is called the light of lights
The object and goal of knowledge
And **knowledge** itself.

- The Bhagavad Gita: 13:12, pp. 15-17

ABBREVIATIONS

Akt	Protein kinase B
ALI	Acute lung injury
ALT	Alanine amino transferase
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ASK	Apoptosis signal regulating kinase
AST	Aspartate aminotransferase
BPD	Bronchopulmonary dysplasia
CI	Cardiac index
CO	Carbon monoxide
COHb	Carboxyhaemoglobin
CSFM	Complete serum free medium
EA	Ethacrynic acid
EDTA	Ethylene-diamine tetra-acetate
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
EVLW	Extra vascular lung water
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
GST	Glutathione S transferase
ICG	Indocyanine green
IPC	Ischemic preconditioning
IR	Ischemia-reperfusion
ITBV	Intrathoracic blood volume

JNK	c-Jun N-terminal kinase
LiMON	Transcutaneous non-invasive liver monitor
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MAPK	Mitogen activated protein kinases
MIP	Macrophage inflammatory protein
MSOF	Multisystem organ failure
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAC	N-acetylcysteine
NF-kB	Nuclear factor-kappa B
OA	Oleic acid
OFR	Oxygen free radical
PBS	Phosphate buffered saline
PDR	Plasma disappearance rate
PEEP	Positive end expiratory pressure
PiCCO	Pulse contour cardiac output
PIP	Peak inspiratory pressure
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
SPSS	Statistical program for social sciences
SVRI	Systemic vascular resistance index
TNF	Tumour necrosis factor

Chapter 1

INTRODUCTION

1.1. Acute organ injury and oxidative stress

The complex biology of critical illness not only reflects the initial insult that brought the patient to the intensive care unit but also, and perhaps even more importantly, it reflects the consequences of the many clinical interventions initiated to support life during a time of lethal organ system insufficiency [1]. The latter may amplify or modify the response to the former and are eminently amenable to modulation by changes in practice. However, they rarely figure in conceptual models of critical illness and are almost never accounted for in preclinical models of disease.

Inflammatory events culminate in the production of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical. Under normal physiological conditions, the formation of oxidants is kept in balance with their efficient removal by a series of endogenous anti-oxidant pathways. However, when ROS generation exceeds the capacity of these defence systems, the cells experience severe oxidative stress, that involves secondary oxidation reactions with small molecular weight metabolites and all kinds of macromolecules including lipids, proteins and DNA, which in turn initiates a cellular adaptive/survival response or leads to cell death.

Although there are multiple biochemical and pathophysiological consequences of oxidative stress, there are special considerations for the development of acute organ injury:

i) Lipid peroxidation: ROS are known to induce oxidation of polyunsaturated fatty acids, initiating a chain reaction that gives rise to degradation products, including malondialdehyde. Frequently, accumulation of these substances is taken as evidence of peroxidative damage

affecting the structural, functional and enzymatic organization of the membranes and thus cell viability.

ii) Activation of redox sensitive intracellular signalling pathways. Many of these are dormant in resting pulmonary cells or exhibit only background activity. Upon stimulation by a variety of oxidants, many signal transduction systems particularly protein phosphorylation cascades become activated resulting in transmission of cytoplasmic signals to the cell nucleus. Prominent examples of such activation are the mitogen activated protein kinases (MAPK), (particularly the p38 MAPK system) and the nuclear factor-kappa B (NF-kB) transcription factor.

iii) Genetic response to oxidative stress leads to either adaptive survival or injury and death. Classical examples of pro-inflammatory and redox sensitive genetic activation are expression of various adhesion molecules such as VCAM-1 and ICAM-1 on the cell surface and the release of cytokines. These classes of molecules play major roles in orchestrating the inflammatory cell to cell and humoral communication in the microenvironment of oxidant activated cells. These are important in both innate and adaptive immune responses and are thus involved in many immunological and inflammatory diseases.

iv) Induction of apoptosis (programmed cell death). In response to severe cell stress, the intrinsic apoptotic pathway may be activated, through oxidation of the mitochondrial pores leading to the release of pro-apoptotic proteins including cytochrome c, which in turn triggers caspase activation and ultimately apoptosis [2]. Initiation of apoptosis may also arise through the activation of specific pro-apoptotic receptors on the cell surface by pro-apoptotic ligands which also lead to caspase activation, in a mitochondrium-independent manner.

Support of acute organ system insufficiency is the *raison d'être* of intensive care and is the embodiment of the remarkable successes of a relatively young discipline. However, organ system support itself can exacerbate the very injury it seeks to support, and despite apparently

successful resuscitation and intensive care unit management of the critically ill, *de novo* organ dysfunction [3], remote to the site of the original insult, commonly evolves in the most seriously ill patients. The intricate interactions of an acute life-threatening insult with the profound homeostatic derangements that follow resuscitation, and the superimposed injury caused by the need for organ system support, are poorly understood; they are largely ignored in our attempts to replicate critical illness using animal models.

1.2. Multiple organ injury, ischemia-reperfusion injury and oxidative stress

In the late 1960s, several reports appeared describing remote organ failure (e.g., pulmonary failure, liver failure) as a complication of severe sepsis. In 1975, a classic editorial by Baue was entitled "Multiple, progressive or sequential systems failure, a syndrome of the 1970s." This concept was formulated as the basis of a new clinical syndrome [4]. Several terms were cloned thereafter, such as multiple organ failure, multiple system organ failure, and multiple organ system failure, to describe this evolving clinical syndrome of otherwise unexplained progressive physiological failure of several interdependent organ systems. More recently, the term multiple organ dysfunction syndrome (MODS) [5] has been proposed as a more appropriate description for an altered organ function in an acutely ill patient requiring medical intervention to achieve homeostasis.

1.2.1. Role of ischemia-reperfusion injury

Ischemia-reperfusion (IR) injury is a complex pathophysiological process involving numerous pathways and body systems. Normal antioxidant defence mechanisms function to limit oxidative injury during times of health. Ischemia is the period that occurs before oxygenated blood is re-introduced and the severity of injury has been shown to correlate with the magnitude and length of ischemia in dogs [6]. During ischemia, there is a build up of

substances (i.e., xanthine oxidase, hypoxanthine, etc.) that, upon re-introduction of oxygen, form ROS which is produced in large part upon reperfusion and can cause extensive damage to DNA, proteins, carbohydrates and lipids. Although mammalian systems are endowed with abundant antioxidant defenses, the generation of large amounts of ROS can overwhelm these mechanisms leading to cell dysfunction and death. Neutrophils play a critical role in IR injury and may mediate the majority of mucosal and microvascular injury that occurs by releasing ROS and proteolytic enzymes. Although experimental studies have been carried out on *in vivo* models there are few clinical studies on companion animals. The pathophysiology of IR injury is complex and involves damage by ROS to all biological membranes. Neutrophils play a major role in IR injury and initiate and propagate much of the damage.

1.2.2 Role of oxidative stress

Oxygen is the primary oxidant in metabolic reactions designed to obtain energy from the oxidation of a variety of organic molecules. Oxidative stress results from the metabolic reactions that use oxygen, and it has been defined as a disturbance in the equilibrium status of pro-oxidant/anti-oxidant systems in intact cells [7]. This definition of oxidative stress implies that cells have intact pro-oxidant/anti-oxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism. When additional oxidative events occur, the pro-oxidant systems outbalance the anti-oxidant, potentially producing oxidative damage to lipids, proteins, carbohydrates, and nucleic acids, ultimately leading to cell death in severe oxidative stress. Mild, chronic oxidative stress may alter the anti-oxidant systems by inducing or repressing proteins that participate in these systems, and by depleting cellular stores of anti-oxidant materials such as glutathione and vitamin E.

A disturbance in pro-oxidant/anti-oxidant systems results from a myriad of different oxidative challenges, including radiation, metabolism of environmental pollutants and administered drugs (these are xenobiotics, i.e., foreign materials), and immune system response to disease or infection [8]. The immune response is especially interesting since many toxic oxidative materials are generated in order to kill invading organisms. Evidence for the role of a variety of chemicals called radicals in these processes has led to interest in the reactions of partially reduced oxygen products and radical and non-radical species derived from them. A variety of reactive nitrogen species derived from the reactions of nitric oxide play important roles as well. A radical species is specifically understood to be any atom that contains one or more orbital electrons with unpaired spin states. The radical may be a small gas molecule such as oxygen or nitric oxide, or it may be a part of a large biomolecule such as a protein, carbohydrate, lipid, or nucleic acid [9]. Some radical species are very reactive with other biomolecules and others like the normal triplet state of molecular oxygen are relatively inert.

Oxidative stress has been implicated in human disease by a growing body of facts. However, cells have multiple protective mechanisms against oxidative stress and succeed in preventing cell damage to the extent that these protective mechanisms are effective. Many dietary constituents are important sources of protective agents that range from anti-oxidant vitamins and minerals to food additives that might enhance the action of natural anti-oxidants. Indeed, at least part of the beneficial effects of a high fruit and vegetable diet is thought to derive from the variety of plant anti-oxidants that might act as beneficial supplements in humans [10]. On the other hand, materials such as pesticides, polyunsaturated lipids, and a variety of plant and microorganism-derived toxins might produce pro-oxidant effects in man.

1.3. Organ manifestations

Signalling pathways, such as those provided by cytokines and their receptors, may have protective or injurious effects depending on the site of challenge and whether the inflammation is local or systemic. The unknown aspects of these complex events are particularly relevant to acute organ injury that is induced as a part of systemic disorders such as sepsis, multiple trauma, and shock but also may be critical in order to understand the pathologic responses to diffuse organ injury caused by direct insults.

1.3.1. Liver injury

Hepatocyte injury is common worldwide. Acute hepatic injury can be recognized by the presence of jaundice or nonspecific symptoms of acute illness accompanied by increases in the activities of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) [11]. An estimated 80% of individuals with acute viral hepatitis are never diagnosed clinically, although some may be detected by increased aminotransferases in the face of nonspecific or absent clinical symptoms. AST and ALT activities are seldom >10 times the upper reference limit in liver diseases other than acute hepatic injury. With the decreasing incidence of acute viral hepatitis, other liver diseases are more commonly encountered as causes of increased AST or ALT activities; as many as 25% of those with AST >10 times the reference limit may have obstruction as a cause [12].

IR injury of liver is a clinically significant manifestation of several surgical procedures, such as liver transplantation, partial hepatic resection, hepatic tumour, or trauma repair. In intensive care, it plays a significant role in acute liver failure. Figure 1 shows acute liver failure in case of paracetamol overdose and the important role of N-acetylcysteine (NAC).

The degree of liver cell damage that occurs as a consequence of these procedures depends in part on primary injury that occurs during ischemia and in part on secondary damage that occurs during reperfusion. Severe hepatic IR injury not only causes liver failure but damage to other organs. Inflammatory events that occur during reperfusion lead to disruption of the integrity of the vascular endothelium and sinusoids, platelet aggregation, immunocyte activation (monocytes/macrophages, Kupffer cells, neutrophils), chemokine and cytokine secretion, and complement activation.

In liver injury, profound, directly lethal oxidative stress is rarely encountered and is typified by acetaminophen-induced necrosis. Otherwise, it is encountered in response to exogenous

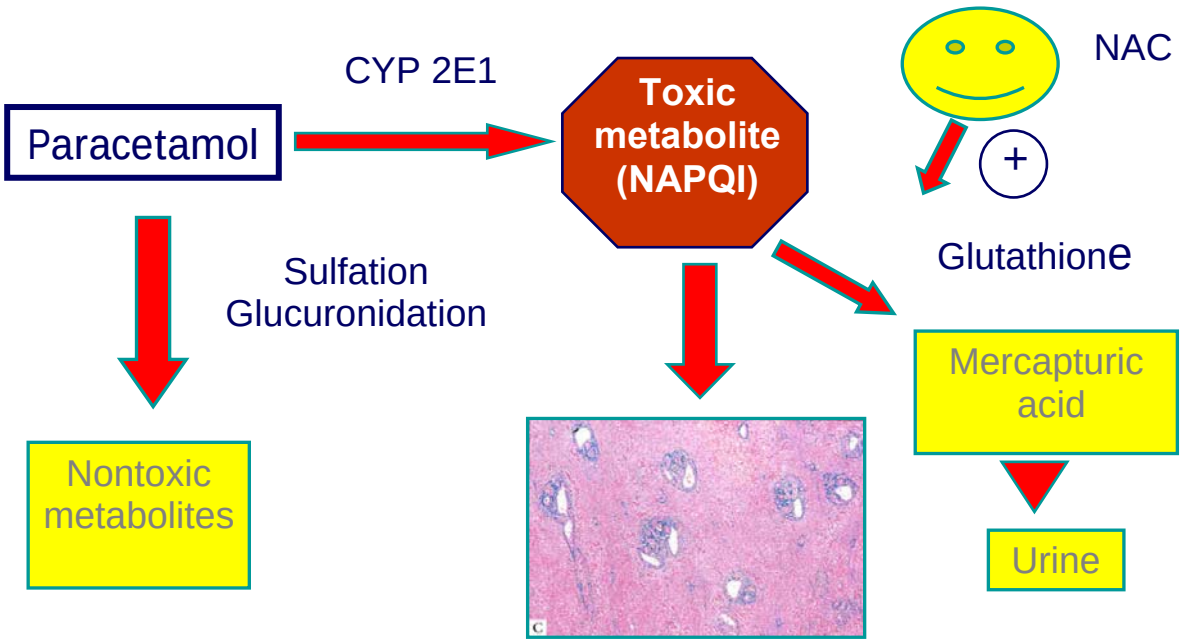


Figure 1

Schematic representation of paracetamol (acetaminophen) metabolism. The primary metabolic pathway for paracetamol is glucuronidation yielding non-toxic substances. A small amount of the drug is metabolized via the cytochrome P-450 (CYP2E1) pathway into the toxic NAPQI (N-acetyl-p-benzo-quinone imine) which may cause fulminant liver failure in paracetamol overdose if not treated with antidote NAC (N-acetylcysteine).

sources, such as infiltrating inflammatory cells or tumour necrosis factor (TNF) action. The most important consequence of oxidative stress in the pathogenesis of liver disease is the promotion of inflammation through the activation of transcription of cytokines, chemokines, and adhesion molecules.

Due to its multiple energy-dependent functions, liver has a high mitochondrial metabolic rate, and is heavily engaged in detoxification mechanisms that involve redox- enzyme systems. Since these are major sources of endogenous free radicals, ROS production is higher in liver as compared with most other organs. Hence, hepatocytes are equipped with abundant antioxidant enzymes to counterbalance this oxidative challenge. However, this borderline equilibrium makes liver highly susceptible to the pro-oxidant injury induced by pathological conditions that disrupt this balance, by enhancing ROS formation, by impairing the antioxidant defences, or both. Indeed, oxidative stress is a common feature in most hepatopathies including hepatic IR injury following partial hepatectomy or liver transplantation.

1.3.1.1. Attenuation of liver injury by N-acetylcysteine

NAC is a glutathione precursor which increases glutathione levels in hepatocytes. Increased glutathione levels, in turn, limit the production of ROS which cause hepatocellular injury.

NAC is a small molecule which, by being freely filterable, has prompt access to the intracellular compartments. This drug has a diversity of applications, largely because of the chemical properties of the thiol moiety present in its structure. The ability of the reduced thiol moiety to sweep ROS is well-established with NAC. In addition to this marked antioxidant capacity, NAC exerts an indirect protection to the liver by being hydrolyzed into cysteine. Therefore it serves as a substrate for reduced glutathione and assists in increasing its levels.

1.3.2. Lung injury

Acute Lung injury (ALI) is thought to develop when pulmonary or systemic inflammation leads to systemic release of cytokines and other proinflammatory molecules. The cytokines activate alveolar macrophages and recruit neutrophils to the lungs, which in turn release leukotrienes, oxidants, platelet-activating factor, and proteases. These substances damage capillary endothelium and alveolar epithelium, disrupting the barriers between capillaries and airspaces. Oedema fluid, protein, and cellular debris flood the alveoli and interstitial space, causing disruption of surfactant, alveolar collapse, ventilation-perfusion mismatch, shunting, stiffening of the lungs with decreased compliance, and pulmonary hypertension. The injury is distributed heterogeneously but mainly affects dependent lung zones.

ALI's more severe form, the acute respiratory distress syndrome (ARDS), also known as respiratory distress syndrome (RDS) or adult respiratory distress syndrome is considered as a serious reaction to various forms of injuries to the lung. ARDS is a severe lung disease caused by a variety of direct and indirect issues. It is characterized by inflammation of the lung parenchyma leading to impaired gas exchange with concomitant systemic release of inflammatory mediators causing inflammation, hypoxemia and frequently resulting in multiple organ failure. Figure 2 shows a schematic explanation of the mechanism of lung injury.

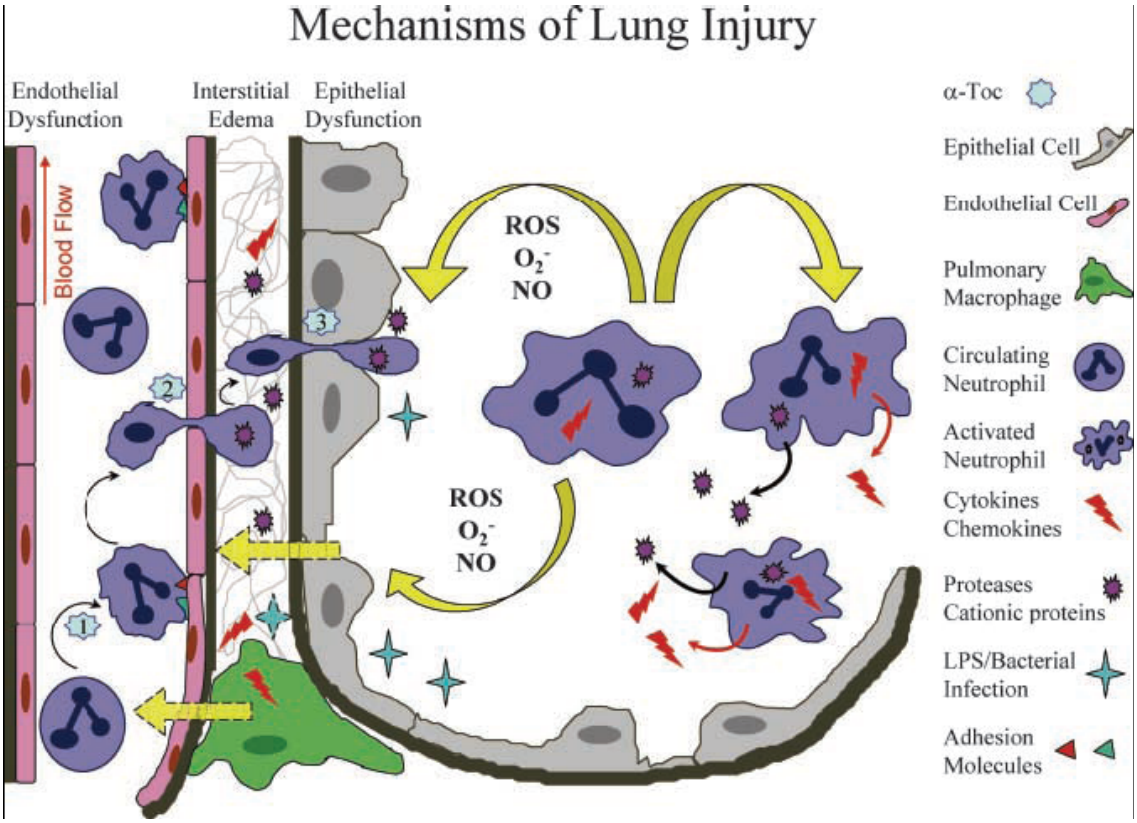


Figure 2

As a result of a bacterial infection and in response to a trigger such as lipopolysaccharide (LPS) production, the pulmonary macrophages and endothelium become activated and upregulate surface expression of adhesion molecules. This leads to neutrophil adhesion and subsequent transmigration from the intravascular space into the alveolus. The activated neutrophil produces a plethora of inflammatory mediators that include reactive oxygen species (ROS) such as OH and O_2 and nitric oxide (NO), cytokines, chemokines, proteases, and cationic proteins. In addition to the activated neutrophil including the pulmonary monocytes and macrophages, the pulmonary endothelial and epithelial cells also contribute to the production of inflammatory mediators. The neutrophil is a major source of ROS production in acute lung injury and ARDS (Chow et al. Am. J. Respir. Cell Mol. Biol., 2003).

The lung, because of its interface with the environment, is a major target organ for injury by exogenous oxidants such as environmental pollutants, cigarette smoke, drugs, chemotherapeutic agents and hyperoxia, as well as by endogenous ROS generated by inflammatory cells. In addition, many pulmonary diseases [e.g. ARDS, chronic obstructive pulmonary disease (COPD), bronchopulmonary dysplasia (BPD)] require supplemental

oxygen therapy to maintain optimal lung function by oxygenation, which further increases the oxidant burden of the lung. It is believed that the damaging effects of oxygen are mediated by superoxide radical, H_2O_2, and hydroxyl radicals formed by mitochondrial epithelial cells, neutrophil and macrophage NADPH oxidase, but the mechanisms are still not clear.

1.3.2.1. Attenuation of lung injury by carbon monoxide

The ubiquitous gas, carbon monoxide (CO), is of substantial biological importance, but apart from its affinity for reduced transition metals, particularly heme-iron, it is surprisingly nonreactive—as is the ferrous-carbonyl—in living systems. CO does form strong complexes with heme proteins for which molecular O_2 is the preferred ligand and to which are attributed diverse physiological, adaptive, and toxic effects. Lately, it has become apparent that both exogenous and endogenous CO produced by heme oxygenase engender a pro-oxidant milieu in aerobic mammalian cells which initiates signalling related to ROS generation. ROS signalling contingent on CO can be segregated by CO concentration-time effects on cellular function, by the location of heme proteins, e.g. mitochondrial or non-mitochondrial sites, or by specific oxidation-reduction (redox) reactions. The fundamental responses to CO involve overt physiological regulatory events, such as activation of redox-sensitive transcription factors or stress-activated kinases, which institute compensatory expression of antioxidant enzymes and other adaptations to oxidative stress.

1.3.3. Cardiac injury

The extent of cell death (necrosis) caused by an acute coronary occlusion not only depends on the size of the area at risk, but also on the severity and duration of ischemia. In recent years, there has been genuine medical progress that has significantly helped to improve survival and quality of life among patients with coronary disease. This has consisted in the development of treatment capable of restoring blood flow (reperfusion therapy) in patients with acute myocardial infarction.

It is well known that the survival of ischemic cells depends on various factors, and that the period cells undergo ischemia until the restoration of blood flow is the main factor determining the success of reperfusion therapy. This knowledge has led to great effort being invested in reducing the time from the moment a patient feels thoracic pain to the beginning of reperfusion therapy.

Reperfusion therapy, whether by thrombolysis or invasive procedures, does not guarantee the survival of ischemic cells, and numerous research studies conducted in the last two decades have unquestionably established that, although revascularization is the only possible alternative to salvage the ischemic cells from certain death, cell death is partly precipitated, paradoxically, by restoration of the flow itself.

A constant supply of oxygen is indispensable for cardiac viability and function. However, the role of oxygen and oxygen-associated processes in the heart is complex, and they can be either beneficial or contribute to cardiac dysfunction and death. As oxygen is a major determinant of cardiac gene expression, and a critical participant in the formation of ROS and numerous other cellular processes, consideration of its role in the heart is essential in

understanding the pathogenesis of cardiac dysfunction. Figure 3 shows a map of the mechanisms by which ROS can alter the structure and function of cardiac muscle.

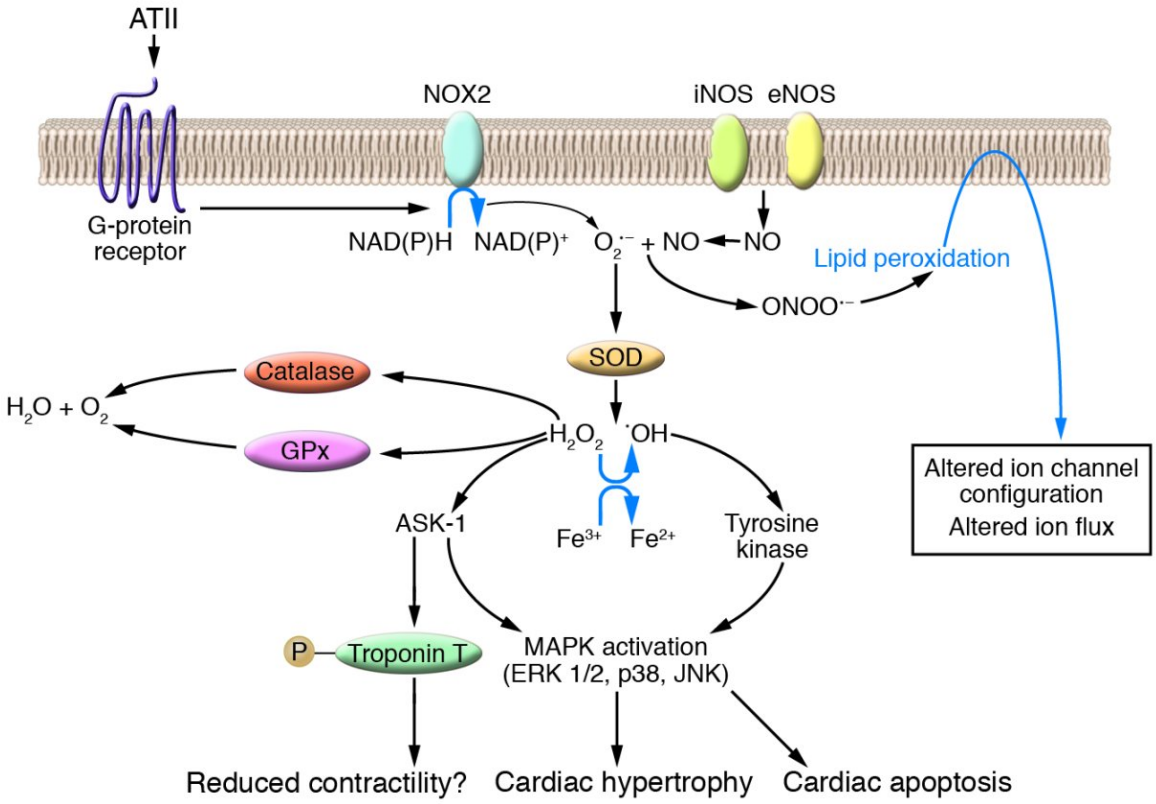


Figure 3

Mechanisms by which reactive oxygen species (ROS) can alter the structure and function of cardiac muscle. Angiotensin (AT II) binds a G-protein receptor, initiating a cascade of events that involves activation of O_2^- production by the NAD(P)H oxidase (NOX2). O_2^- is converted by superoxide dismutase (SOD) into H_2O_2 and $^{\cdot}OH$ that mediates activation of mitogen-activated protein kinase (MAPKs) via a tyrosine kinase. MAPK activation can lead to cardiac hypertrophy or to apoptosis. The ROS that is generated can also signal through apoptosis signal-regulating kinase 1 (ASK-1) to induce cardiac hypertrophy, apoptosis, or phosphorylate troponin T, an event that reduces myofilament sensitivity and cardiac contractility. NO production by the NO synthases iNOS and eNOS can interact with O_2^- to form $ONOO^-$. $ONOO^-$ can cause lipid peroxidation, an event that can alter ion channel and ion pump function. Catalase and glutathione reductase (GPx) are shown as enzymatic pathways to produce water and oxygen from H_2O_2 (Giordano J. Clin Invest, 2005).

1.3.3.1. Attenuation of cardiac injury by glutathione S-transferase

Glutathione plays several essential roles in the protection of the cell. There are three distinct families of glutathione S- transferase (GST) in mammals: cytosolic (which forms the largest), mitochondrial and membrane-associated proteins. As well as secondary oxidant and xenobiotic detoxification, GST's are also thought to be involved in modulation of cell proliferation and apoptotic signalling pathways [13].

Seven classes of the GST family are recognised in mammalian species, designated Mu, Pi, Theta, Alpha, Sigma, Omega and Zeta. Those within a particular class typically share > 40% identity but at least five of the human GST genes display functional polymorphisms. The GSTM1 and GSTT1 polymorphisms are deletions, with homozygous variant patients producing no active protein. The GSTM1 gene is homozygously deleted in 50% of Caucasian individuals and null mutations of GSTT1 are also frequent (approximately 20% of population). Significant variations in GST Mu and Pi isoforms in particular, have been identified in heterogeneous populations of critically ill patients. It has been suggested that these polymorphisms likely contribute to interindividual differences in response to xenobiotics and clearance of oxidative stress products and thus may determine susceptibility to various inflammatory pathologies including cancer, cardiovascular diseases [14].

Chapter 2

OBJECTIVES

The objectives of our studies were to explore injuries in an acute setting and their organ manifestations in a variety of in vivo and in vitro models.

In the first setting, an in vivo canine liver model was used to evaluate the possibility that repeated ischemic preconditioning or NAC could prevent IR injury as determined by indocyanine green plasma disappearance rate (ICG-PDR) or has favourable hemodynamic effects during reperfusion. In order to confirm ischemic injury along with basic parameters, invasive hemodynamic parameters were measured. Hepatic injury was measured with plasma disappearance rate of indocyanine green and serum levels of AST and ALT.

We hypothesized that ischemic preconditioning along with NAC would dramatically eliminate the harmful effects of IR injury and improve liver function and haemodynamic parameters in the process.

In the second setting, we evaluated the effects of inhaled CO in three different in vivo mouse models of ALI. In order to mimic an intensive care unit scenario, anaesthetized mice were ventilated with oxygen in the presence or absence of CO before lung injury was induced by intravenous/intratracheal lipopolysaccharide (LPS) or intravenous oleic acid (OA) administration. Ventilation was then continued with the same gases and haemodynamic and respiratory parameters monitored throughout. In order to confirm lung injury, alveolar inflammation was detected by measuring lavage fluid neutrophils, total protein, and cytokines. Vascular lung injury was seen, with plasma tumour necrosis factor (TNF) and increased neutrophil activation using surface Mac-1 upregulation and L-selectin shedding and

sequestration within the pulmonary vasculature. Lung function was analysed using changes in respiratory mechanics and blood gases and lavage fluid neutrophil accumulation.

Our hypothesis was that CO exposure would attenuate cytokine response, improve haemodynamic and lung function parameters and show beneficial effects by preventing the progression of ALI.

In the third setting, we used an in vitro model to study the role of GST on oxidative stress induced apoptosis in cardiomyocytes. Mitochondrial viability in cardiac cells was measured using an MTT assay, Annexin V and propidium iodide using flow cytometry. Phosphorylation of extracellular signal-regulated protein kinase (ERK ½), c-Jun N-terminal kinase (JNK), and p38 MAPK was also studied. Our concept was to study the biological role played by GST in cardiac myocytes under oxidative stress conditions. The biological role played by GST represented by the effect of GST inhibition with administration of ethacrynic acid (EA) when cells are exposed to various stress components of ischemia and or reperfusion were studied.

Our hypothesis was that GST activity would improve survival of cardiomyocytes by attenuating the severity of IR injury as a response to oxidative stress.

Chapter 3

THE EFFECT OF N-ACETYLCYSTEINE AND ISCHEMIC PRECONDITIONING DURING HEPATIC ISCHEMIA-REPERFUSION IN A CANINE MODEL.

3.1. Introduction

Hepatic IR injury as a result of hepatic inflow obstruction is a major cause of morbidity and mortality during liver surgery but despite continuing research few effective therapies have been identified. In recent years, NAC and ischemic preconditioning (IPC) have been reported to have hepatoprotective effects in several animal models of liver injury [15,16,17,18] During partial liver resection, 'Pringle's manoeuvre' (figure 4) is often carried out in order to minimise intraoperative bleeding [19]. Several studies have shown that intermittent clamping of the portal triad results in better outcome than continuous inflow obstruction [20,21,22]. The beneficial effects of intermittent clamping might be related to a preconditioning effect [23,24], which involves a short period of ischemia followed by a brief reperfusion before a sustained ischemic insult. In a rodent IR model of preconditioning, reperfusion prevented fatal outcome in mice subjected to total hepatic ischemia [25]. In human studies, the serum levels of AST and ALT were significantly lower in preconditioned groups compared to controls 24 hours after surgery [26]. As Kupffer cell and neutrophil activation in hepatic IR injury leads to increased oxygen free radical (OFR) activity, many attempts have been made to attenuate this process. Superoxide dismutase, allopurinol, α-tocopherol and NAC have all been shown to inhibit OFR damage during hepatic I-R injury [1,12].

The aim of the study was to investigate the usefulness of normothermic IPC and NAC to ameliorate the haemodynamic effects of reperfusion and to prevent IR injury in canine liver in a prospective, randomised animal study.

3.2. Materials and methods

The principles of laboratory animal care were carried out in accordance with the guidelines of the University of Pécs Animal Rights Protection Board (1301-7/1999), the Hungarian Animal Protection Law (1998) and the approval of the institutional ethics committee.

3.2.1. Animal preparation

Fifteen purpose-bred dogs (~ 20 kg body weight) from Juhász Limited (Pécs, Hungary) were housed in the Unit for Laboratory Animal quarters for at least 1 week before surgery. Canines were used for the study due to established liver IR protocols by several groups [27, 28].

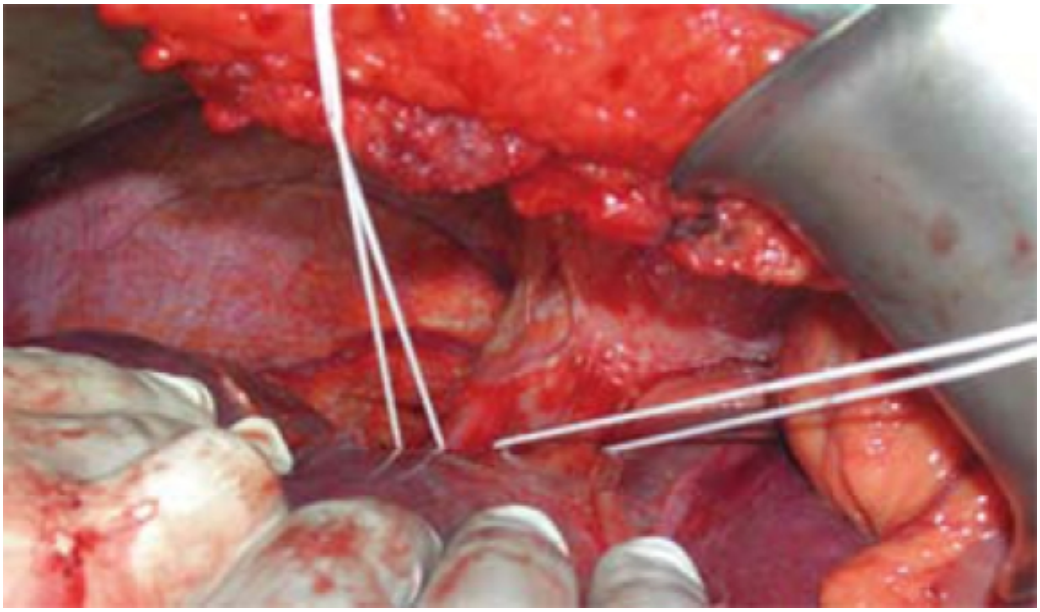


Figure 4

Isolation of the hepatoduodenal ligament for Pringle's manoeuvre.

Canines have an advantage because they are gentle and easy to handle and monogastric like humans. All rodent models have the disadvantage of having different metabolisms and kinetics of drugs, eliciting responses different from those seen in humans, the secondary

testing in higher models such as dogs have responses close to human [29]. A major disadvantage though is group size [30]. Prior to the experiment, the dogs were randomly divided into three groups and fasted for 24 hours with access to water *ad libitum*.

Table1:

Measured parameters and groups for hepatic ischemia-reperfusion. NAC: N-acetylcysteine, IPC: ischemic preconditioning,MAP: mean arterial pressure, SpO_2: oxygen saturation, CVP: central venous pressure, CI: cardiac index, EVLWI: extravascular lung water index, SVRI: systemic vascular resistance index, dP/dT max: rate of rise of left ventricular pressure, ICG-PDR: indocyanine green plasma disappearance rate, ABG: arterial blood gas, ALT: alanine amino transferase, AST: aspartate aminotransferase.

Groups	Physiological Parameters Measured	Haemodynamic parameters with PiCCO	Liver function parameters with LiMON	Blood sample measurements
Controls (n=5) 60 min. of hepatic ischemia followed by reperfusion	Heart rate,MAP, SpO_2, ECG, CVP, Temperature	CI, ITBVI, EVLWI, SVRI, dP/dT max	ICG-PDR	ABG, AST, ALT
NAC (n=5) 150 mg/kg i.v. NAC for 2 min then 60 min of warm ischemia followed by reperfusion.	Heart rate,MAP, SpO_2, ECG, CVP, Temperature	CI, ITBVI, EVLWI, SVRI, dP/dT max	ICG-PDR	ABG, AST, ALT
IPC (n=5) 10 min of ischemia then 10 min of reperfusion x 3 times then 60 min of warm ischemia followed by 180 min reperfusion.	Heart rate,MAP, SpO_2, ECG, CVP, Temperature	CI, ITBVI, EVLWI, SVRI, dP/dT max	ICG-PDR	ABG, AST, ALT

3.2.2. Experimental design

The control group (n = 5) underwent 60 minutes of hepatic ischemia. The NAC group (n = 5) received 150 mg/kg of NAC (Zambon Group, Vincenza, Italy) intravenously for 2 minutes before inducing 60 minutes of warm ischemia (summarized in table 1). In the IPC group (n =

5) the randomised animals received ischemic preconditioning (10 minutes of ischemia followed by 10 minutes of reperfusion repeated three times) before inducing 60 minutes of warm ischemia followed by 180 minutes reperfusion.

3.2.3. Anaesthesia and physiological measurements

All the dogs were premedicated with droperidol 1 mg/kg, fentanyl 0.005 mg/kg, atropine 0.004 mg/kg intramuscularly. After a peripheral vein was canulated each dog received thiopentone 10 mg kg-1 and atracurium 0.5 mg/kg. Physiological parameters were monitored (table 1). Following tracheal intubation, the lungs were ventilated with halothane (1.0 -2.0 % v/v) in a mixture of 50% oxygen and 50% nitrous oxide. Once the anaesthesia was established, the right internal jugular vein and the right femoral artery were canulated with a central venous catheter and a flexible 4-French arterial catheter with an integrated thermistor (Pulsiocath 4F, PV 2014L16A, Pulsion Medical Systems, Munich, Germany) respectively under direct vision. The central venous line was connected to a pressure transducer while the arterial catheter was used with a PiCCO® monitor to monitor haemodynamic parameters at baseline (t_0), 5 minutes before reperfusion (t_{m5}), and 5, 30, 60, 180 minutes after reperfusion (t_{p5}, t_{p30}, t_{p60}, t_{p180}). Intrathoracic blood volume (ITBV) and extravascular lung water (EVLW) measurements were obtained by injection of a 10 ml bolus of 0.9 % saline <10 °C into the central venous line. The mean value of three consecutive measurements was used for analysis. For inter-individual comparison, absolute values for ITBV and EVLW were normalised as indexed by body surface area (ITBVI) and body weight (EVLWI), respectively. All injections were made manually and not synchronised with the respiratory cycle. Cardiac index (CI), and systemic vascular resistance index (SVRI) were recorded according to standard formulas. Ventricular contractility during systole dP/dt_{max} was calculated automatically. ECG analysis was continuously performed in all the dogs. Median laparotomy was performed, the portal

triad was isolated and clamped (Pringle's manoeuvre) according to randomisation. Heart rate, mean arterial pressure, central venous pressure and arterial blood gas were measured at baseline, 5 minutes before reperfusion, and 5, 30, 60, 180 minutes after reperfusion. Electrocardiogram analysis was continuous in all the dogs.

To maintain mean arterial pressure (MAP) > 60 mmHg, Ringer-lactate and succinylated gelatine, Gelofusine® (modified fluid gelatine, average molecular weight 30,000 dalton, Braun Ltd, Budapest, Hungary) were administered.

Pulse-dye densitometry (LiMON®, Pulsion Medical Systems, Munich, Germany) was used to measure blood ICG concentration [31]. A bolus of ICG (0.5 mg/kg) was injected intravenously, and blood ICG concentrations were monitored at every pulse interval via an optical probe attached to the animals' tongue. The elimination rate constant, ICG-PDR, was calculated automatically by the blood ICG concentration time course. Blood samples for determining AST and ALT levels were drawn at these assessment points. In each group following 180 minutes reperfusion the animals were euthanized by using potassium chloride intravenously.

3.2.4. Statistics

All data are expressed as mean ± SD. To test normal distribution the Kolgomorov-Smirnov test with the Lilliefors modification was used. Analysis of variance (ANOVA) was used for testing the significance levels between the different groups, and ANOVA for repeated measures was used for testing significance levels between the measurement stages. To investigate the relationship between the observed variables Pearson's correlation test was performed. With type I alpha of 5% and type II (power) of 80%, we calculated that we would need about 5 dogs per group. For statistical analysis the Statistical Program for Social

Sciences (SPSS®, Chicago, Il, USA, version 10.0) software for Windows was used. Statistical significance was considered at $p<0.05$.

3.3. Results

3.3.1. Haemodynamic and metabolic analysis

14 dogs survived the study period. 1 dog in the NAC group died due to circulatory failure unresponsive to inotropic drugs before the last assessment point. The haemodynamic parameters were not significantly different at any assessment point between the three groups (Table 1). Arterial blood gases were well maintained within normal limits throughout the procedures.

The CI (Control: 2.1±0.61; Preconditioned: 3.5±1) and ITBVI (Control: 390±84.7; Preconditioned: 533±92.7) were significantly higher in the preconditioning group compared to the NAC group throughout the study period (Table 1). The amount of fluid which was administered during the study period was similar in each group (Control: 3225±570 ml, NAC: 3467±686 ml, Preconditioned: 3542±768 ml, respectively).

3.3.2. Indocyanine green pulse spectrophotometry

ICG clearance as determined by PDR was higher in the IPC group 5 minutes after reperfusion. At other time points it was similar in each group throughout the study (Table 2). The serum ALT and AST levels sketched in figure 4 and 5 respectively, showed no significant differences between the groups. Arterial blood gases did not show major differences between the groups.

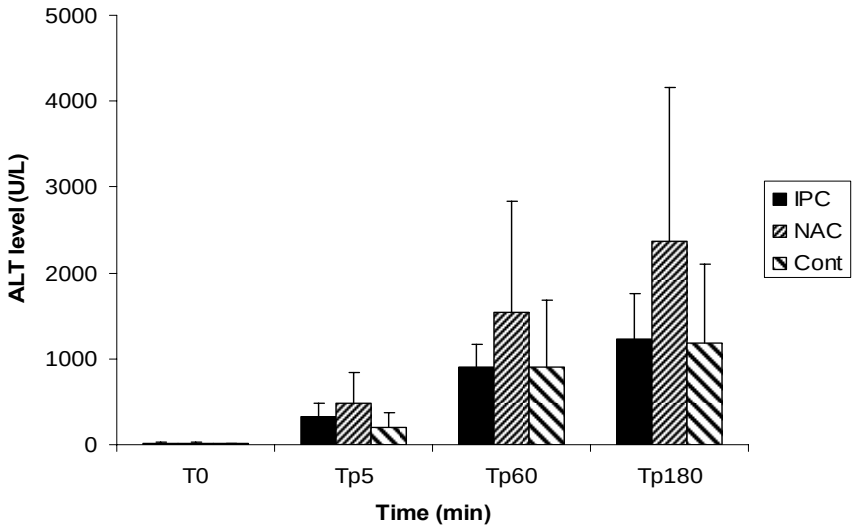


Figure 5

Serum levels of alanine aminotransferase (ALT), Results are expressed as mean ± SD in units per litre, before (T0) and 5, 60 and 180 minutes (Tp5, Tp60 and Tp180, respectively) after Pringle's manoeuvre. n = 5 per group. IPC = ischemic preconditioning; NAC = N-acetylcysteine; Cont = controls. There were no significant differences between the groups.

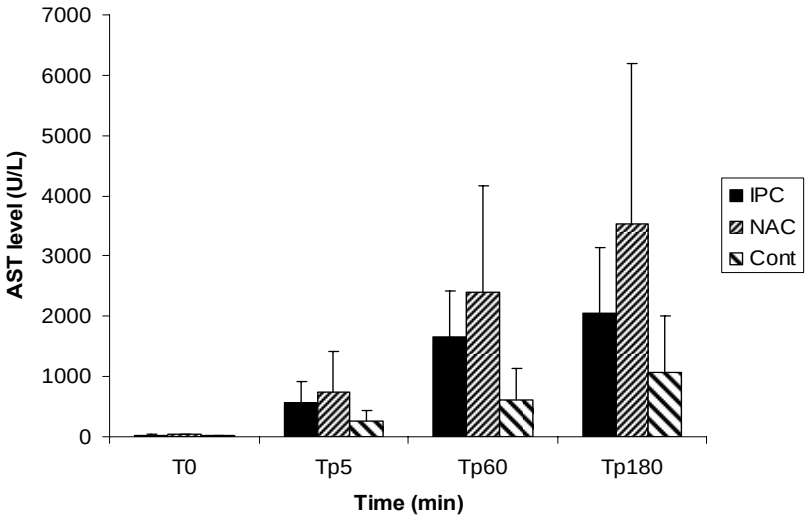


Figure 6

Serum levels of aspartate aminotransferase (AST). Results are expressed as mean ± SD in units per litre, before (T0) and 5, 60 and 180 minutes (Tp5, Tp60 and Tp180, respectively) after Pringle's manoeuvre. N = 5 per group. IPC = ischemic preconditioning; NAC = N-acetylcysteine; Cont = controls. There were no significant differences between the groups.

Table 2: *Haemodynamic variables with PiCCO in ischemic preconditioning (IPC), N-acetylcysteine (NAC) and controls measured over time T at 0 min, 5 min before (m5) ischemia and following reperfusion (p5, p30, p60, p180). Data presented as mean ± SD. * P<0.05 compared to controls. N=5 for all observations. For statistical analysis ANOVA was used. Cardiac index (CI); intrathoracic blood volume index (ITBVI); extravascular lung water index (EVLWI); systemic vascular resistance index (SVRI); rate of rise of left ventricular pressure (dP/dtmax).*

Time	Group	CI (L m^{-2})	ITBVI (mL m^{-2})	EVLWI (mL kg^{-1})	SVRI (dyn s cm^5)	dP/dt_{max} (mmHg s^{-1})
T_0	IPC	3.04±0.68	552±91	9±4	3545±1660	858±237
T_0	NAC	2.49±0.72	495±67	10±4	3445±793	813±203
T_0	**Cont**	**2.49±0.57**	**450±118**	**10±3**	**2891±1378**	**850±108**
T_{m5}	IPC	2.31±0.50*	430±73*	8±2	2849±1665	604±148
T_{m5}	NAC	1.18±0.67	320±1061	1±5	5065±1934	500±135
T_{m5}	**Cont**	**1.18±0.57**	**292±76**	**9±4**	**2870±1725**	**630±241**
T_{p5}	IPC	4.51±0.72*	565±54*	8±2	2016±1124	828±142
T_{p5}	NAC	3.29±1.16	466±39	12±6	1823±660	713±164
T_{p5}	**Cont**	**2.37±0.71**	**447±79**	**8±2**	**1921±1263**	**820±187**
T_{p30}	IPC	4.04±1.07*	559±98*	8±3	2176±1147	894±183
T_{p30}	NAC	2.84±0.61	437±27	10±4	2302±507	773±172
T_{p30}	**Cont**	**2.86±1.27**	**470±157**	**8±2**	**1573±566**	**884±196**
T_{p60}	IPC	3.69±1.00*	539±102*	8±2	2501±1393	844±113
T_{p60}	NAC	2.18±0.18	410±71	11±4	2564±374	707±51
T_{p60}	**Cont**	**2.18±0.22**	**380±34**	**9±3**	**1996±1436**	**796±125**
T_{p180}	IPC	3.48±2.00*	553±138*	9±2	2467±552	870±145
T_{p180}	NAC	1.68±0.24	349±15	12±5	3119±250	810±192
T_{p180}	**Cont**	**1.55±0.32**	**301±44**	**9±2**	**3064±1363**	**736±95**

Table 3: *Hemodynamic, metabolic parameters with LiMON and blood gas analysis in animals treated with IPC and NAC. HR, heart rate; MAP, mean arterial pressure, CVP, central venous pressure; ICG-PDR, indocyanine green-plasma disappearance rate; Measurements were taken over time T at 0 min, 5 min before (m5) ischemia and following reperfusion (p5, p30, p60, p180). N=5 for all observations. Data are presented as: mean ± SD. HR: heart rate; MAP: mean arterial pressure; CVP: central venous pressure; ICG-PDR: indocyanine green plasma disappearance rate. No significant differences were measured between the groups.*

		HR (min^{-1})	**MAP** (mmHg)	**CVP** (mmHg)	**ICG-PDR** (% min^{-1})	**pH**	**pO2** (mmHg)	**pCO2** (mmHg)
Cont	**T_0**	**108±9**	**90±9**	**2±2**	**7.44±1.1**	**7.37±0.1**	**116.2±10.9**	**33.8±2.7**
IPC	T_0	134±26	100±22	2±2	7.45±2.5	7.4±0.1	143.9±27.2	30.2±7.9
NAC	T_0	131±17	96±5	2±2	7.5±0.8	7.43±0.1	130.7±31.2	28.9±4.1
Cont	**T_{m5}**	**138±11**	**52±13**	**2±1**	-	**7.17±0.1**	**144.7±52.3**	**27.5±3.3**
IPC	T_{m5}	153±28	69±31	3±2	-	7.15±0.1	135.9±21.2	30±10.8
NAC	T_{m5}	152±16	59±5	2±2	-	7.27±0.1	128.8±8.5	25.7±3.3
Cont	**T_{p5}**	**122±18**	**75±12**	**2±1**	**6.06±1.1**	**7.02±0.1**	**100.9±36.4**	**37.5±3.6**
IPC	T_{p5}	138±21	82±25	4±3	8.73±4.8	7.07±0.1	143.3±41.9	39.7±14.9
NAC	T_{p5}	131±20	68±3	4±2	5.23±1.1	7.06±0.1	134.4±25.4	33.3±5.1
Cont	**T_{p30}**	**132±23**	**75±10**	**2±2**	-	**7.07±0.04**	**133.9±11.9**	**37.5±3**
IPC	T_{p30}	145±21	85±19	4±3	-	7.13±0.1	123.9±24.8	36.3±12.0
NAC	T_{p30}	137±18	77±2	4±3	-	7.09±0.1	159.3±32.6	32±4.8
Cont	**T_{p60}**	**131±19**	**74±7**	**3±1**	**6.40±1.6**	**7.16±0.04**	**123.1±12.2**	**34±3.7**
IPC	T_{p60}	149±17	83±22	4±3	6.80±1.9	7.18±0.07	139.9±40.7	33.5±8.1
NAC	T_{p60}	141±16	70±6	4±2	6.67±0.5	7.12±0.1	134.1±23.7	28.9±2.8
Cont	**T_{p180}**	**141±20**	**77±4**	**3±1**	**7.26±2.2**	**7.21±0.04**	**151.8±30.8**	**33.1±2.2**
IPC	T_{p180}	128±28	79±10	4±3	7.83±2.5	7.29±0.05	156.2±61.7	24.5±4.5
NAC	T_{p180}	140±40	66±9	3±2	6.17±1.2	7.15±0.1	140.1±28.8	30.5±6.1

3.4. Discussion

It has been suggested that decreasing IR injury during liver resections may be achieved by both ischemic preconditioning and OFR scavengers. The present study attempted to evaluate the effects of repeated IPC and NAC on hemodynamic and liver function by ICG clearance in an IR injury model. No significant differences were seen amongst haemodynamic or hepatic function variables.

Ischemic preconditioning has been shown to dramatically eliminate harmful effects of IR injury in experimental as well as clinical studies. Most of these studies applied one cycle of brief ischemia (usually 5 to 10 minutes) followed by 5 to 10 minutes of reperfusion [32]. However, based on previous studies by our group on different organs [33, 34] pilot experiments with a similar setting and consultation with liver surgeons, we decided to introduce a preclamping procedure which would suit the need for short term effects consisting of three cycles of 10 minutes of ischemia followed by 10 minutes of reperfusion. Neither the ICG-PDR nor the observed AST and ALT levels showed significant differences between the groups following the procedure. However, ICG-PDR values were constantly the highest in the IPC group.

It has been shown that ICG-PDR is an independent predictor of hepatic dysfunction and outcome during large hepatic resections, and also that IPC improved liver function on the first and third postoperative day [9]. This study attempted to address the question whether IPC or NAC administered before inducing liver ischemia may alter the course of ICG clearance. Our study was designed to evaluate the short term effects of preconditioning; therefore the possible later developing differences were undetectable.

There is evidence that oxygen intermediates are generated during hepatic IR injury; however, their physiological relevance remains unclear. Free radical scavengers have been proposed to ameliorate the effects of OFRs. NAC is said to be a strong protective agent in liver ischemia

and reperfusion, by its direct antioxidant effect and by restoring glutathione levels in hepatic cells [12]. In this study, NAC treated animals did not show any significant difference when compared to controls.

There are no data in this study to explain the lack of protection of NAC in our model, although, it is likely that 60 minutes of ischemia may not be long enough to deplete stores of glutathione in hepatic cells. Nakano and co-workers have shown that depletion of glutathione stores up to 90% before inducing hepatocellular ischemia does not significantly enhance liver injury [35]. Therefore a protecting effect caused by NAC improving those reserves is not obvious. According to reported comparisons regarding OFRs and liver injury it seems unlikely that free radicals are the primary mechanism of parenchymal cell injury during reperfusion [36]. Because mitochondria are one of the main sources of free radicals, the maintenance of cellular and mitochondrial integrity suggest that the occurrence of OFR is a concomitant event rather than a cause of IR injury directly.

In conclusion, we were unable to find any significant difference between the three groups regarding hepatic cell functions following warm ischemia as monitored by ICG-PDR and AST, ALT levels and based on these results a larger prospective trial is warranted to determine whether preconditioning before inducing hepatic ischemia may enhance liver cell survival in a longer run as determined by ICG-PDR.

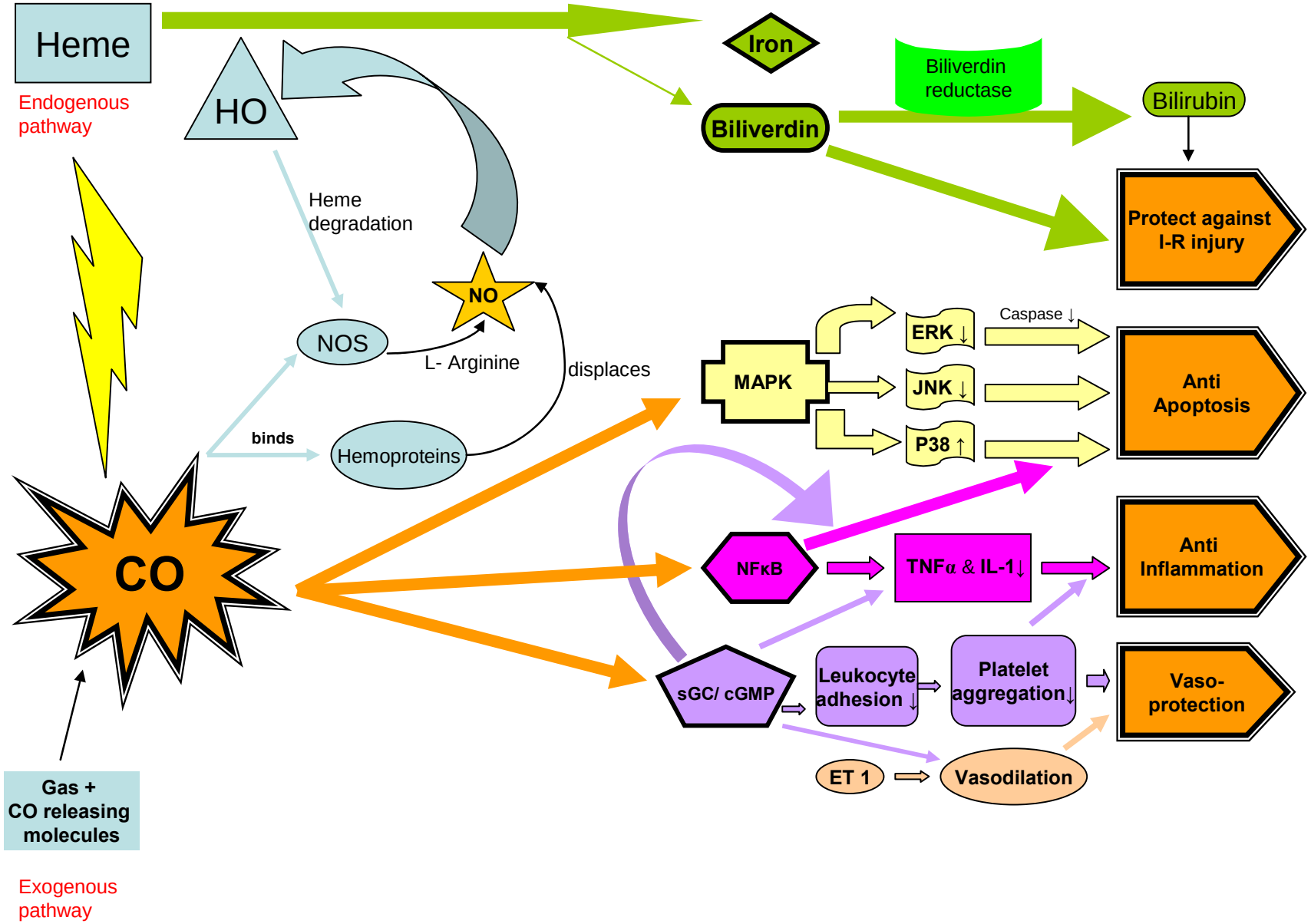
Chapter 4

EFFECTS OF INHALED CARBON MONOXIDE ON ACUTE LUNG INJURY IN MICE

4.1. Introduction

ALI and its more severe form ARDS are major causes of mortality and morbidity in the intensive care unit. These syndromes are characterized by the development of hypoxemia, alveolar-capillary barrier damage, and pulmonary inflammation, occurring within hours to days of a predisposing insult which may be of either pulmonary (e.g., pneumonia, aspiration of gut contents) or extrapulmonary (sepsis, acute pancreatitis) nature [37]. Despite intense research, few studies have identified therapies that show a beneficial impact on the outcome of ALI, with the exception of the use of lung protective ventilatory strategies [38, 39]. CO has long been known in biology and medicine as a toxic compound due to its ability to bind haemoglobin with a much higher affinity than oxygen [40].

Despite this image as a deadly gas, recent evidence indicates that CO may in fact have a cytoprotective function at low doses. CO is endogenously produced during heme metabolism by the enzyme heme oxygenase, which is one of the major antioxidant cytoprotective enzymes within the body [41,42] (Figure 6). Several investigators have reported that exogenously administered CO by inhalation may exert anti-inflammatory effects, providing protection in various animal models of tissue injury. In particular, the lung appears ideally placed for such treatment, and inhaled CO at concentrations of 250–500 parts per million (ppm) has been shown to be beneficial in a number of lung injury models, including hyperoxic injury [43, 44], allergen-induced inflammation [45], ischemia-reperfusion injury [46], lung transplant rejection [47], and ventilator-induced lung injury [48].


Heme
Endogenous pathway
HO
Heme degradation
Iron
Biliverdin
Biliverdin reductase
Bilirubin
Protect against I-R injury
NO
NOS
L- Arginine
displaces
binds
Hemoproteins
CO
MAPK
ERK ↓
JNK ↓
P38 ↑
Caspase ↓
Anti Apoptosis
NFκB
TNFα & IL-1↓
Anti Inflammation
sGC/ cGMP
Leukocyte adhesion ↓
Platelet aggregation↓
Vaso-protection
ET 1
Vasodilation
Gas + CO releasing molecules
Exogenous pathway

However, the effectiveness of CO treatment in attenuating lung injury is not a completely universal experimental finding [49], and as the majority of previous studies have investigated relatively subacute, slowly developing models of injury, it is unclear whether inhaled CO may be useful in attenuating more rapidly progressing pulmonary inflammation in ALI and ARDS. We therefore investigated the potential anti-inflammatory effects of inhaled CO in three in vivo mouse models of ALI, each of which display different pathophysiology to encompass the heterogeneous nature of human ALI. Initially, ALI induced by either intratracheal or intravenous LPS was studied, as models of ALI/ARDS induced by both direct (pulmonary aetiology) and indirect (extrapulmonary aetiology) processes. Although CO has been reported to attenuate cytokine responses and improve mortality in LPS-induced endotoxin shock in mice [50, 51], the possible impact of CO on LPS-induced ALI in vivo is unclear. In addition, we investigated the impact of CO inhalation using the OA model of ALI, which produces a clinical picture similar to human ARDS, and has previously been used to investigate potential therapies for ALI/ARDS [52]. We were however unable to demonstrate anti-inflammatory effects or beneficial impact on physiological and immunological signs of ALI due to CO exposure in any of the models used.

4.2. Materials and methods

4.2.1. Animal preparation

All experimental protocols were reviewed and approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK. Male C57BL/6 mice (Charles River, Margate, UK) aged 9–13 wk (20–30 g) were used throughout. Physiological parameters were monitored (table 4). Mice were anesthetised by intraperitoneal injection of 2.5 ml/kg Hypnorm (Fentanyl 0.8 mg/kg, Fluanisone 25 mg/kg) and 2.5 ml/kg Midazolam (12.5 mg/kg) and then acutely instrumented as described previously [53, 54, 55]. In brief,

mice were mechanically ventilated via tracheotomy using a custom-made mouse ventilator-pulmonary function testing system [56]. The left carotid artery was cannulated for monitoring of arterial blood pressure (BP), blood-gas analysis, and infusion of fluids. The right jugular vein was also cannulated in some animals for administration of LPS or OA. Anaesthesia was maintained by further administration of the anaesthetic mix via a catheter placed in the intraperitoneal cavity. Throughout the experiment, rectal temperature was monitored and maintained within the normal range by use of a heating pad.

4.2.2. Experimental design

All animals were ventilated with tidal volume (VT) of 9–10 ml/kg, 2–2.5 cmH_2O positive end-expiratory pressure, and respiratory rate of 120 breaths/min, with either 100% O_2 or a premixed 98% O_2, 2% N_2, 500 ppm CO gas mixture (Carburos Metalicos, Barcelona, Spain). CO content of the inspired gas was confirmed with an electrochemical CO analyzer (Testo 315-2; Testo, Alton, UK). After 1-h pre-exposure to either O_2 or O_2+CO, animals were allocated to the following protocols.

4.2.2.1. Intratracheal lipopolysaccharide

LPS (20 µg, *Escherichia coli* O111:B4; Sigma, Poole, UK) in 25 µl of sterile saline was instilled directly into the trachea through the endotracheal tube. Animals were then ventilated with the same inspired gases and ventilator settings as described above for a further 3 h period, after which animals were killed by anaesthetic overdose. Lung lavage was carried out with 750-µl aliquots of sterile saline as described previously [53] for analysis of protein and cytokine concentrations and for differential cytology using Diff-Quikstained samples prepared by Cytospin (Shandon, Runcorn, UK). A blood sample was removed via cardiac puncture for determination of carboxyhaemoglobin (COHb) levels using a CO oximeter (IL

Synthesis 45; Instrumentation Laboratory, Warrington, UK) and for measurement of plasma tumour necrosis factor-α (TNF-α).

Table 4: *Measured parameters and groups for acute lung injury. I.t.: intratracheal, I.v.: intravenous, LPS: lipopolysaccharide, OA: oleic acid, HR: heart rate, MAP: mean arterial pressure, SpO_2: oxygen saturation, CVP: central venous pressure, PIP: peak inspiratory pressure, COHb: carboxyhemoglobin, TNF-α: tumour necrosis factor, ABG: arterial blood gas, PMN: neutrophils, MIP-2: macrophage inflammatory protein, IL-8: interleukin.*

Groups	Physiological Parameters	Blood samples	Lung lavage	Lung cell suspension
Controls (n=5)	HR, MAP, SpO_2, ECG, CVP, Temperature, PIP, airway flow,compliance, resistance.	COHb, TNF-α, ABG	Total protein, PMN, TNF-α, MIP-2, IL-8	PMN,L-selectin, Mac-1
I.t. LPS (n=5)	HR, MAP, SpO_2, ECG, CVP, Temperature, PIP, airway flow,compliance, resistance.	COHb, TNF-α, ABG	Total protein, PMN, TNF-α, MIP-2, IL-8	PMN,L-selectin, Mac-1
I.v. LPS (n=5)	HR, MAP, SpO_2, ECG, CVP, Temperature, PIP, airway flow,compliance, resistance.	COHb, TNF-α, ABG	Total protein, PMN, TNF-α, MIP-2, IL-8	PMN,L-selectin, Mac-1
I.v. OA (n=5)	HR, MAP, SpO_2, ECG, CVP, Temperature, PIP, airway flow,compliance, resistance.	COHb, TNF-α, ABG	Total protein, PMN, TNF-α, MIP-2, IL-8	PMN,L-selectin, Mac-1

4.2.2.2. Intravenous lipopolysaccharide

LPS (20 μg) in a volume of 200 μl of sterile saline was infused by a syringe infusion pump over 15 min into the jugular vein. Animals were then ventilated with the same inspired gases for a further 2 h. At 1 h post-LPS infusion, a blood sample (70–80 μl) was removed via the carotid artery cannula for analysis of plasma TNF-α levels, and the volume was replaced with sterile saline. After death at the end of the protocol, the right lung was removed for flow cytometric analysis to investigate the processes of intravascular sequestration and activation of neutrophils within the lung, which occur before transepithelial neutrophil migration into the alveoli [54]. The left lung was lavaged using 400-μl aliquots of sterile saline for differential cytology.

4.2.2.3. Intravenous oleic acid

A modification of our previously described mouse model of OA-induced lung injury was used [57]. OA (0.07 ml/kg, Sigma) was infused over 1 min via the jugular vein by a syringe infusion pump at a rate of 0.1 ml/h. Animals were then ventilated with the same inspired gases for a further 2 h period. At the end of the procedure animals were euthanised, and the lungs were lavaged with 750 μl of saline for analysis of protein and cytokine levels and for differential cytology.

4.2.3. Physiological measurements

In all animals, airway pressure, airway flow, and mean BP were monitored continually throughout the experiments. Blood gas analyses were performed at baseline and at the end of the protocols. Respiratory system compliance and resistance were measured by the end-inflation occlusion technique [54, 56]). Sustained inflation of 30 cmH2O for 5 s was performed every 30 min throughout experiments to avoid atelectasis. No animals died during any of the protocols used.

4.2.4. Proteins and cytokine measurements

The protein concentration in lung lavage fluid was determined by the Bradford method [58] using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hemel Hempstead, UK) with bovine serum albumin (Sigma) as a standard. Determination of TNF-α concentration in plasma and lavage fluid was carried out using a custom-made sandwich ELISA as described previously [54]. Macrophage inflammatory protein- 2 (MIP-2) levels in lavage fluid were determined using a commercially available sandwich ELISA (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

4.2.5. Pulmonary neutrophil measurements

To evaluate the intravenous LPS-induced inflammatory response in the lung vasculature, a sensitive flow cytometry method recently developed in our laboratory [53] was used. In brief, lung cell suspensions were prepared from the excised lungs of mice by mechanical disruption, and cells were analyzed by flow cytometry (FACScalibur, BD, Oxford, UK). For quantification of pulmonary sequestration of polymorphonuclear neutrophils (PMN), PMN were identified by positive staining for fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Ly-6G (Gr-1) antibody (BD Pharmingen, Oxford, UK) and side scatter properties, and the absolute PMN counts recovered per lung were measured using Perfect-count microspheres (Caltag Medsystems, Towcester, UK) according to the manufacturer's instructions. For assessment of cellular activation, lung-sequestered PMN were analyzed for surface expression of the adhesion molecules L-selectin and Mac-1 (CD11b), by simultaneous staining with phycoerythrin-conjugated rat antimouse L-selectin (MEL-14, BD Pharmingen) or rat anti-mouse Mac-1 (M1/70, BD Pharmingen).

4.2.6. Statistics

The data are expressed as mean values ± SD. Changes in parameters across the course of the experiments and differences between groups were analyzed by one-way ANOVA with Scheffe's tests or Student's *t*-tests, p value of less than 0.05 was considered significant.

4.3. Results

4.3.1. Intratracheal lipopolysaccharide exposure

Physiological measurements and blood gas: To confirm the efficacy of CO exposure, blood COHb levels were determined at the end of the intratracheal LPS protocol. The levels of COHb were undetectable (0% of total haemoglobin) in all animals exposed to O_2 alone (n = 5) and 10.7 ± 1.4% of total haemoglobin in CO-treated animals (n = 5). Intratracheal instillation of 20 μg of LPS produced an initial increase in peak inspiratory pressure (PIP) in all animals due to the presence of fluid within the airways (Table 3). Over the course of the experiments, PIP returned toward pre-instillation values, indicating clearance of intratracheal fluid, rather than any LPS induced deterioration in respiratory mechanics. Arterial BP and blood gases were well maintained within normal limits throughout the procedures. Inhalation of 500 ppm CO had no significant impact on these physiological variables.

Neutrophil infiltration: Instillation of LPS into the lungs produced a significant recruitment of neutrophils into the alveolar space compared with untreated control animals (Figure 7). Exposure of animals to CO had no impact on neutrophil recruitment, when expressed either as % neutrophils or total neutrophil number recovered in lavage fluid.

Protein and cytokine analysis: Intratracheal LPS produced an increase in lavage fluid total protein concentration compared with untreated animals, indicating the development of pulmonary oedema (Figure 8A).

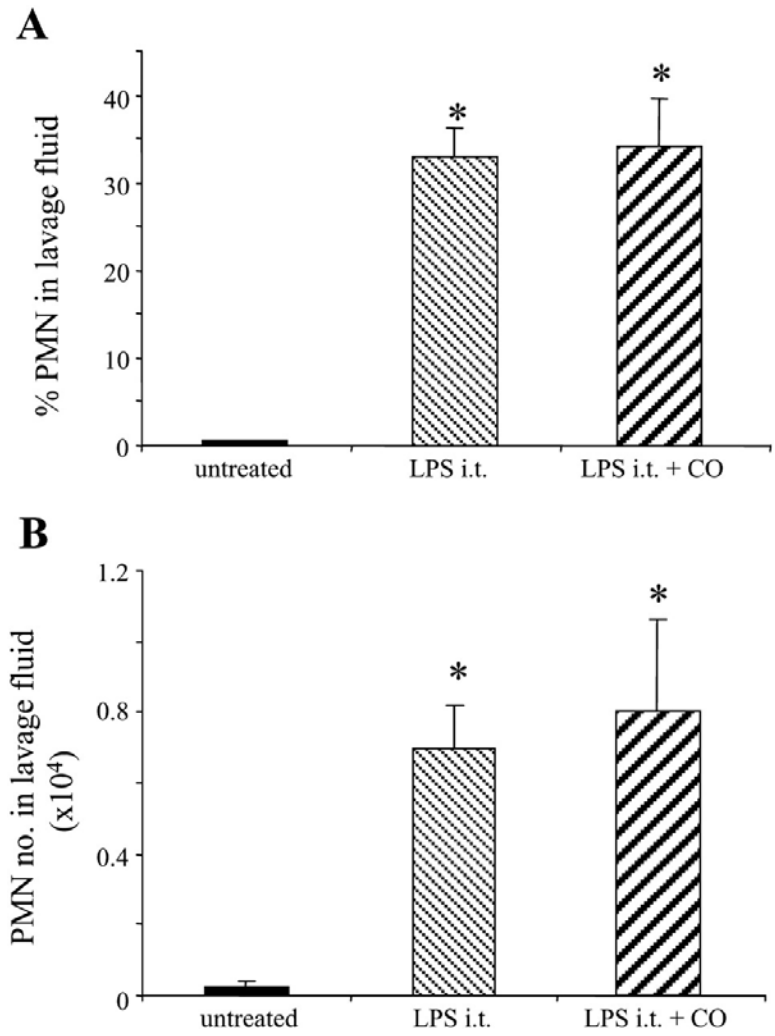


Figure 8

*Recovery of neutrophils (PMN) in lavage fluid from animals administered intratracheal lipopolysaccharide (LPS) and ventilated using either O_2 or O_2+ 500 ppm CO, and in untreated control animals. Results are expressed either as percentage (A) or total number (B) of PMN recovered. *p<0.01 vs. untreated controls. n=5 per group.*

Lavage fluid levels of the cytokines TNF-α and MIP-2, a murine functional homolog of the human neutrophil chemoattractant IL-8, were dramatically increased by intratracheal LPS administration (Figure 8, B and C). CO had no effect on the levels of protein or proinflammatory cytokines in lung lavage fluid. TNF-α was undetectable (<7 pg/ml) in plasma in animals treated with intratracheal LPS.

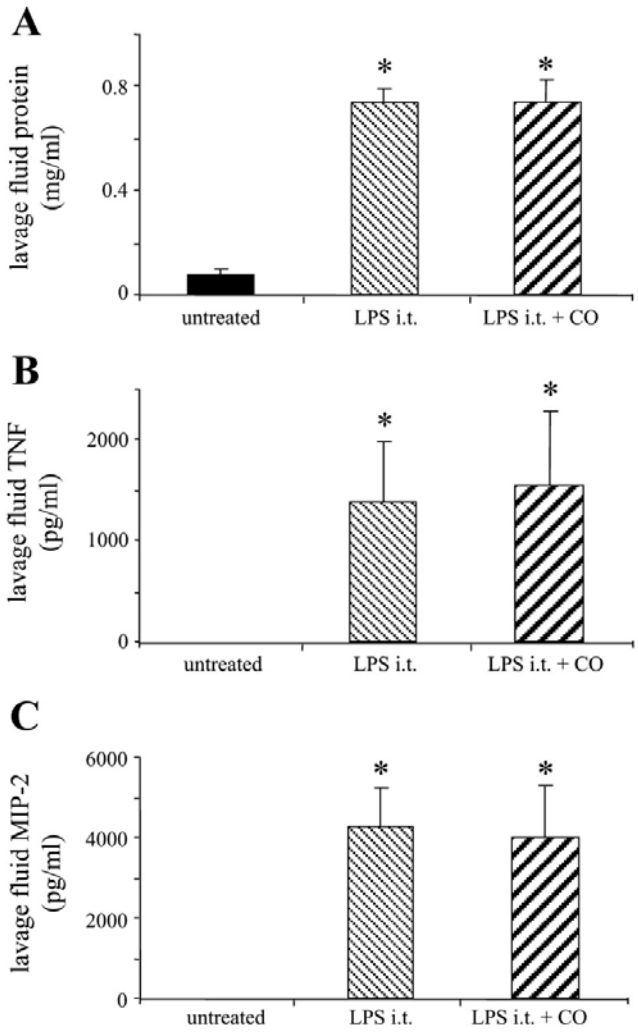


Figure 9

*Total protein (A) and cytokines TNF-α (B) and MIP-2 (C) in lavage fluid from animals administered intratracheal LPS and ventilated using either O_2 or O_2+ 500 ppm CO, and in untreated control animals. *p<0.01 vs. untreated controls. n=5 per group.*

4.3.2. Intravenous Lipopolysaccharide exposure

Physiological measurements and blood gas: Intravenous administration of 20 μg of LPS did not affect PIP but did cause a progressive decrease in arterial BP, indicating endotoxin induced hypotension (Table 4). Blood gases remained within relatively physiological limits throughout, although pH declined over time, consistent with progressive deterioration in organ perfusion with intravenous LPS. Overall there was little sign that physiological variables were affected by CO exposure, although PO_2 was found to be lower in the CO group.

Neutrophil sequestration and activation: Intravenous LPS produced negligible neutrophil migration into the alveolar space in all animals. However, flow cytometric analysis of lung cell suspension demonstrated that intravenous LPS infusion produced a dramatic increase in the number of lung-sequestered PMN compared with untreated controls (Figure 9A). These lung-sequestered PMN were also found to be activated by LPS in terms of increased Mac-1 expression and decreased L-selectin expression, indicating increased L-selectin shedding (Figure 9, B and C). Both lung PMN sequestration and activation were unaffected by CO treatment.

Cytokine analysis: TNF-α levels in plasma 1 h after intravenous LPS infusion were greatly increased compared with the undetectable levels observed in untreated controls and intratracheal LPS animals but were not affected by CO exposure (1,040 ± 410 for O2 animals vs. 1,060 ± 350 pg/ml for O_2 + CO animals, $n = 5$ for each).

Table 5:

Physiological parameters in animals administered intratracheal lipopolysaccharide (LPS). peak inspiratory pressure (PIP); mean arterial blood pressure (MAP). Measurements were taken before LPS administration, and every 60 minutes thereafter. MAP was well maintained within a reasonable range expected for C57BL6 mice anesthetised with Hypnorm and Midazolam. n=5 for all observations. $^{\dagger}p<0.05$, $*p<0.01$ vs. pre LPS; $^{\ddagger}p<0.05$ vs. LPS i.t. group.

	Pre LPS	**LPS + 60 min**	**LPS + 120 min**	**LPS + 180 min**
LPS i.t.				
PIP (cmH_2O)	13.1±2.4	16.0±2.3*	14.8±2.3†	14.6±2.3†
MAP (mmHg)	56±9	63±8	64±9†	61±9
pH	7.41±0.09			7.39±0.05
Pco_2(mmHg)	44.8±9.7			42.9±7.5
PO_2(mmHg)	357±27			353±36
LPS i.t. + CO				
PIP (cmH_2O)	14.5±1.1	16.2±2.3†	15.8±2.0	15.0±1.6
MAP (mmHg)	68±6‡	64±9	64±6	63±6
pH	7.44±0.02			7.38±0.04*
pCO_2(mmHg)	39.9±4.1			38.4±6.4
PO_2(mmHg)	332±20			337±52

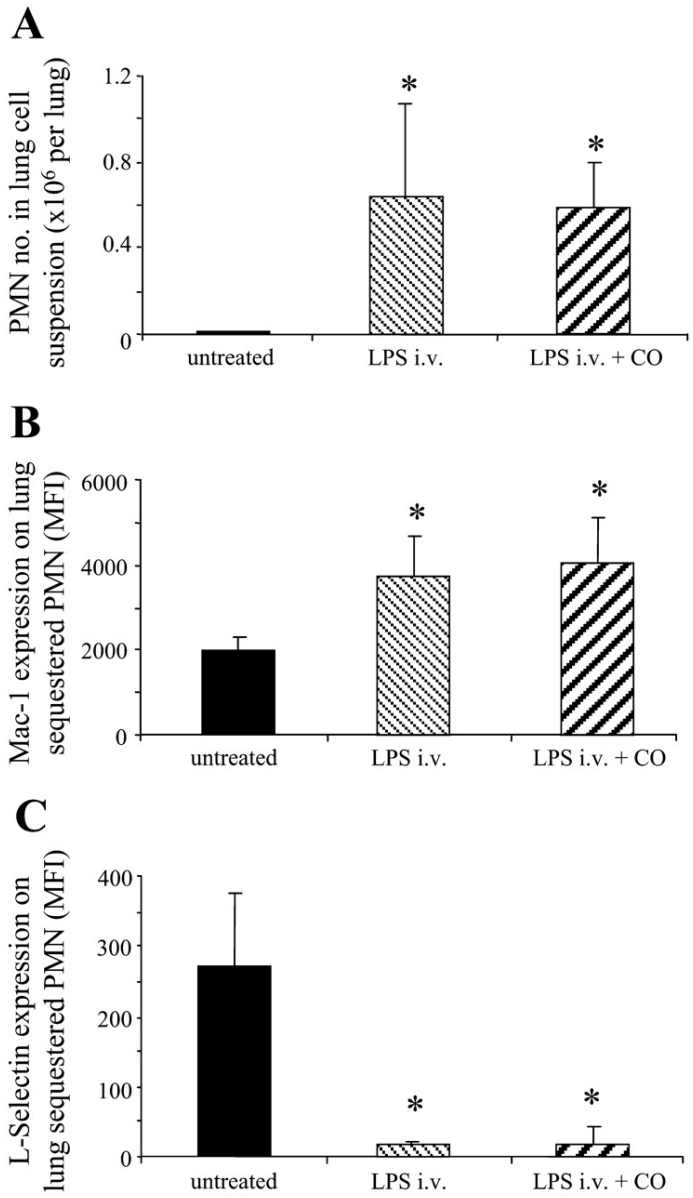


Figure 10

*Flow cytometric analysis of lung sequestered neutrophils (PMN) in animals treated with intravenous lipopolysaccharide (LPS) and ventilated using either O_2 or O_2+ 500 ppm CO, and in untreated control animals. PMN in lung cell suspensions were identified by positive staining for surface Ly-6G antibody and counted using Perfect-count microspheres (A). Cellular activation of lung-sequestered PMN was confirmed by increased surface expression of the adhesion molecule Mac-1 (B), and decreased expression of L-Selectin (C), with data expressed as mean fluorescence intensity (MFI). *p<0.05 vs. untreated controls. n=4-5 per group.*

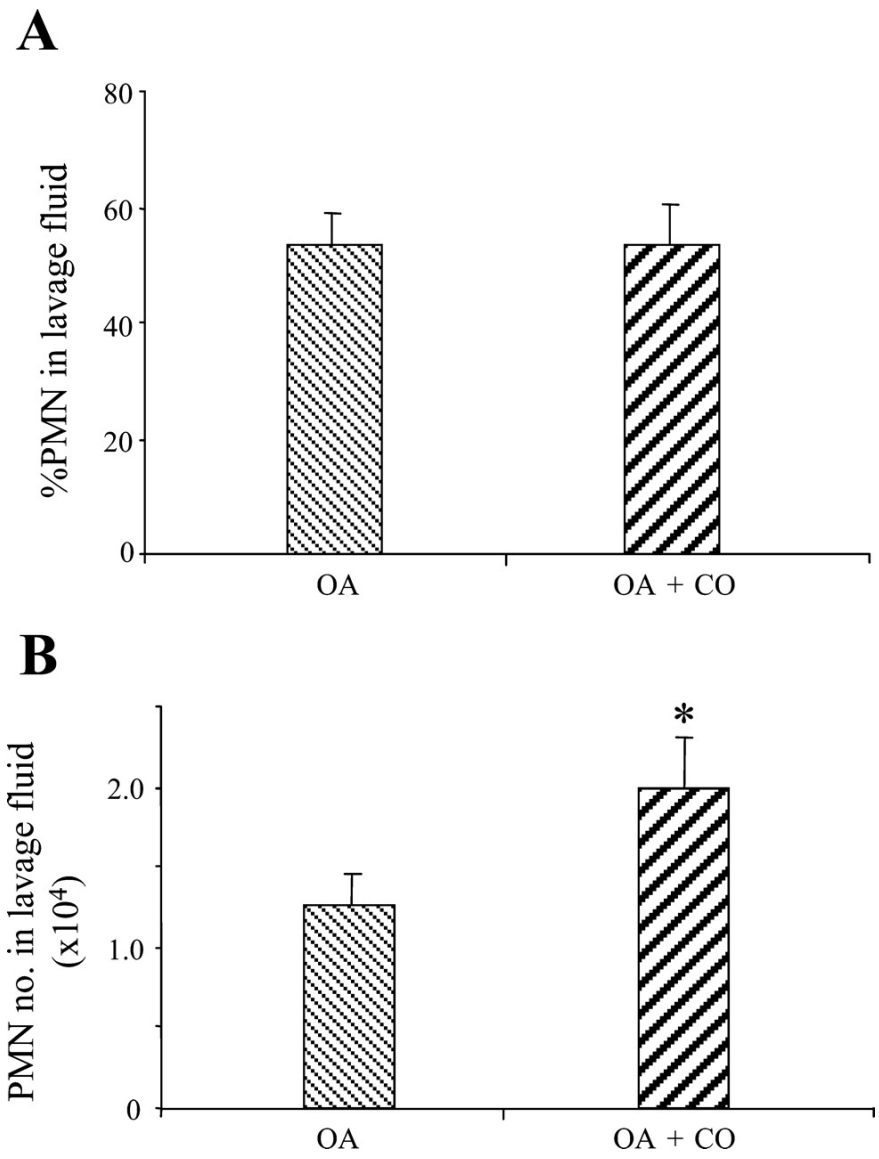


Figure 11

*Recovery of neutrophils (PMN) in lavage fluid from animals administered intravenous oleic acid (OA) and ventilated using either O_2 or O_2+ 500 ppm CO. Results are expressed either as percentage (A) or total number (B) of PMN recovered. *p<0.01 vs. OA group. n=5 per group.*

4.3.3. Intravenous oleic acid exposure

Physiological measurements and blood gas: Intravenous OA administration provoked a significant increase in PIP in all animals (Table 5), which was associated with a decline in respiratory system compliance (47 ± 10% decrease for O_2 animals vs. 52 ± 2% decrease for O_2+CO animals compared with pre-OA values, n = 5 for each).

OA also caused a significant decrease in arterial BP and deterioration of gas exchange, i.e., decreased pH, increased PCO_2, and decreased PO_2 (Table 5). However, these physiological signs of ALI were not significantly different between animals exposed to O_2 alone or O_2 with CO (except that PO_2 was lower in CO group at baseline).

Neutrophil infiltration: Intravenous OA administration induced a dramatic neutrophil infiltration into lavage fluid (Figure 10), which was not attenuated by CO exposure.

Protein and cytokine analysis: Total protein content in lavage fluid was dramatically increased in all OA-treated animals compared with untreated mice, consistent with the development of pulmonary oedema, but this was not affected by CO exposure (4.8 ± 0.4 for O2 animals vs. 4.7 ± 0.9 mg/ml for O_2 + CO animals, n = 5 for each). Lavage fluid concentrations of both TNF-α (5.7 ± 10.8 for O_2 animals vs. 14.1 ± 8.4 pg/ml for O_2 + CO animals, n = 5 for each) and MIP-2 (4.4 ± 1.1 for O_2 animals vs. 3.8 ± 0.6 pg/ml for O_2 + CO animals, n = 5 for each) were very low in all OA-treated animals and unaffected by the presence of CO.

***Table 6*:**

*Physiological parameters in animals administered intravenous lipopolysaccharide (LPS). Peak inspiratory pressure (PIP); mean arterial blood pressure (MAP). Measurements were taken before LPS administration, and every 60 minutes thereafter. n=5 for all observations. $^{\dagger}p<0.05$, *$p<0.01$ vs. pre LPS; $^{\ddagger}p<0.05$ vs. LPS i.t. group.*

	Pre LPS	**LPS + 60 min**	**LPS + 120 min**
LPS i.v.			
PIP (cmH_2O)	10.2±1.2	11.0±1.4†	10.8±1.3
MAP (mmHg)	63±11	51±5	44±4*
pH	7.44±0.07		7.35±0.04*
pCO_2(mmHg)	40.2±8.4		36.2±4.1
pO_2(mmHg)	344±32		342±24
LPS i.v. + CO			
PIP (cmH_2O)	10.7±0.7	10.9±1.0	10.9±0.8
MAP (mmHg)	61±10	51±3	42±2*
pH	7.44±0.06		7.33±0.04*
pCO_2(mmHg)	39.4±8.3		37.7±5.8
pO_2(mmHg)	315±33		304±18‡

Table 7:

Physiological parameters in animals administered intravenous oleic acid. Peak inspiratory pressure (PIP); mean arterial blood pressure (MAP). Measurements were taken before OA administration, and every 60 minutes thereafter. n=5 for all observations. $^{\dagger}p<0.05$, $*p<0.01$ vs. pre OA; $^{\ddagger}p<0.05$ vs. OA group.

	Pre OA	**60 minutes post OA**	**120 minutes post OA**
OA			
PIP (cmH_2O)	10.1±0.4	14.7±1.5*	16.4±2.0*
MAP (mmHg)	62±3	51±3*	50±3*
pH	7.43±0.02		7.24±0.03*
pCO_2(mmHg)	40.3±3.0		63.7±4.2*
pO_2(mmHg)	379±23		133±33*
OA + CO			
PIP (cmH_2O)	10.4±0.3	15.2±1.8*	17.7±1.5*
MAP (mmHg)	58±5	48±4*	47±4*
pH	7.45±0.02		7.24±0.04*
pCO_2(mmHg)	37.8±3.9		59.8±14.0†
pO_2(mmHg)	329±32‡		109±31*

4.4. Discussion

In the current study we investigated the putative anti-inflammatory effects of inhaled CO on three models of ALI of different pathophysiologies in mice. We studied both inflammatory (neutrophil infiltration and cytokine levels in lavage fluid, pulmonary neutrophil sequestration and activation) and physiological (respiratory mechanics, blood gas variables) markers of ALI in each of these three models. We were, however, unable to determine a positive effect of CO exposure on the measured parameters in any of the models used. In recent years it has become apparent that activity of the enzyme heme oxygenase-1 (HO-1) confers protection to tissues against oxidative injury. HO-1 has been demonstrated to possess anti-inflammatory, anti-apoptotic, and anti-proliferative effects [41], although the mechanisms behind this are unclear.

Cytoprotection may be provided by the byproducts of HO-1 catalysis of heme protein, i.e., biliverdin, iron, and CO [42]. CO in particular has been the subject of intense research, as it is a stable molecule, and being gaseous it is easily administered by inhalation. Beneficial effects of low-dose CO inhalation have been shown in various models of organ injury, both in the lung [43,44,45,46,47,48] and in distal organs such as small intestine [59] and liver [60]. The major mechanism by which CO provides cytoprotection is considered to be the attenuation of inflammatory responses [61], with CO exposure reported to reduce LPS-induced production of proinflammatory cytokines including TNF-α, IL-6, IL-1, granulocyte/monocyte colony stimulating factor, and MIP-1, both *in vivo* in mice [50,51] (17,20) and in isolated macrophages [50,51,62]. Inhaled CO therapy may therefore be of great use in the intensive care unit, particularly in the treatment of ALI and ARDS. These syndromes are a major cause of mortality with substantial inflammatory components and, as lung disorders, would seem to be particularly amenable to inhaled gas therapy. To investigate the possible beneficial effects of inhaled CO on ALI, we initially used *in vivo* mouse models of endotoxin induced ALI. As ALI/ARDS is induced by both pulmonary and nonpulmonary etiologies, we studied the

response to intratracheal and intravenous LPS administration. Both models produced substantial pulmonary inflammation, although the pathophysiology involved was different, with the inflammatory response being relatively compartmentalized in either the alveolar or vascular space [63, 64, 65]. Intratracheal LPS induced lung injury with clear alveolar inflammation, characterized by intra-alveolar neutrophil infiltration and increased lavage fluid proinflammatory cytokines (TNF-α and MIP-2). There was also an increase in protein concentration in lavage fluid, indicating increased pulmonary endothelial and epithelial permeability, consistent with previous studies [66,67].

Intravenous LPS induced lung injury of a predominantly vascular nature. Animals injected with intravenous LPS did not show increases in lavage fluid neutrophils within the time frame of this study, consistent with previous demonstrations that transepithelial migration of neutrophils and increases in alveolar space cytokines requires more time and higher doses of intravenous LPS [68]. On the other hand, there was an increase in plasma TNF-α and significant activation and sequestration of neutrophils within the lung vasculature, as assessed by flow cytometry.

In both models, however, we did not observe any in vivo effect of CO on these immunological parameters of neutrophil recruitment and cytokine activation. This may be particularly significant in the case of intratracheal LPS administration, as alveolar macrophages, which are considered to play a major role in producing inflammatory cytokines and triggering neutrophil infiltration in this model [69], would have been directly exposed to inhaled CO. Because there were few physiological signs of lung injury in the above LPS models, we also investigated the impact of CO exposure on OA-induced ALI. This model produces a clinical picture very similar to human ARDS and has been frequently used to study potential treatments for ALI/ARDS [52].

The precise mechanisms by which OA promotes lung injury are not clear but appear to involve the formation of microemboli and production of ROS leading to endothelial and epithelial cell damage [52], induction of apoptosis [70], and fibrin deposition [52], culminating in a rapid pulmonary inflammatory response. It has been suggested that CO enhances fibrinolysis via cGMP signalling in ischemic lung injury [46], possesses anti-apoptotic properties in lung transplant injury [47], and provides protection against oxidative stress [43]. We therefore reasoned that CO may be protective against OA-induced lung injury. After OA infusion, clear signs of physiological lung injury developed, including deteriorating respiratory mechanics and abnormalities in gas exchange, as well as a large influx of neutrophils into the alveolar space. In the presence of 500 ppm CO, however, neither physiological lung injury markers nor the consequent pulmonary inflammation was attenuated.

These results are clearly in contrast to a number of previous studies that have demonstrated beneficial effects of CO on lung injury [45, 48, 46, 43, 44, 47]. This is, however, not the only study that has failed to show a positive impact of CO in vivo. Clayton et al. [49] found that inhaled CO did not significantly attenuate lung injury induced by hyperoxia in a rat model very similar to the one Otterbein et al. [43] used to show a positive effect. Clayton et al. [49] pointed out a lack of concordance of blood COHb values with gas equilibrium calculations in Otterbein's study, in which the COHb levels were unexpectedly high (6.6%) at baseline without CO and increased to 11.3% at only 250 ppm CO. They suggested that the CO measurement in Otterbein's study may not be accurate and that the observed protecting effects of CO in hyperoxic lung injury could be due to higher doses of CO or lower inspired oxygen [49]. In the current study we delivered CO by a ventilator into the animal lungs in the form of a premixed 500 ppm CO/O_2 gas mixture, precluding any potential problem in the inspired CO dose, and found blood COHb levels of ~11%, which is comparable with previously reported

values of COHb (10–14% at CO 200–500 ppm) delivered in 95–98% oxygen [49, 43, 44]. It is possible that higher blood COHb levels, if achieved either by the use of gas mixtures containing air as opposed to 98% O_2 or by the inhalation of higher concentrations of CO, could have led to positive results.

However, such treatment protocols do not have much clinical relevance, because the ventilatory management of ALI/ARDS usually requires high inspired O_2 levels, and inhaled CO 500 ppm might be associated with apoptosis in the brain [49]. Moreover, previous dose response studies of anti-inflammatory effects of inhaled CO have indicated positive effects at doses as low as 10–50 ppm [43, 48, 51]. Taken together, it appears unlikely that the current negative results are related to insufficient CO dose or CO delivery to the animals. Other explanations for the lack of a positive effect of inhaled CO include the possibility that the models used in our study were too severe to show small to moderate effects of inhaled CO, or that the techniques used to assess inflammation were insufficiently sensitive to detect changes induced by CO.

However, we believe it unlikely that these explanations could apply to all of the three models used in the current study. In particular, using similar mouse models of ALI induced by intratracheal LPS, we [53] and others [71] have shown that intravenous CD18 antibody markedly attenuated neutrophil infiltration into the alveolar space. The dose of intravenous LPS used in the current study (0.7–1 mg/kg) is comparable to the intraperitoneal LPS doses used in previous studies demonstrating benefits of CO in mice [50, 51]. Dolinay et al. [48] recently reported in rats that CO exposure did not affect small to moderate lung inflammation induced by either LPS (3 mg/kg iv) or high VT mechanical ventilation but did attenuate more severe injury induced by the combined inflammatory (LPS) and mechanical (ventilatory) insults, although interpretation of such data is complicated because multi-insult lung injury models may potentially produce unexpected and model-specific interactions [72, 73]. In

addition, many of the techniques used (cytokine ELISA, neutrophil accumulation in lavage fluid) have been used previously to show CO-induced organ protection, and we have demonstrated that the flow cytometry based method used in the intravenous LPS model is able to detect small differences in pulmonary neutrophil sequestration between different mechanical ventilatory strategies [53].

Finally, it is possible that CO exposure may have prolonged effects on ALI that could not be detected in the current study, due to the acute (2–3 h) nature of the injury models used. Even if inhaled CO is not effective in reversing or modifying acute overwhelming inflammation in the lung as seen in the ALI models in our study, more prolonged exposure to CO may have some beneficial effects in attenuating subacute to chronic lung inflammation or facilitating the resolution from lung injury.

In clinical ALI/ARDS, the disease process develops more slowly than that in animal models, and hence prolonged exposure to inhaled CO may change the course of ALI, leading to better recovery and survival following the acute episode. This possibility was not excluded by the results of the current study and remains to be further investigated.

Chapter 5

ROLE OF GLUTATHIONE S-TRANSFERASE ON OXIDATIVE STRESS INDUCED APOPTOSIS IN CARDIOMYOCYTES

5.1. Introduction

Cardiovascular diseases remain the leading cause of death in both developed and developing countries, accounting for roughly 20% of all worldwide deaths per year [74]. Administration of exogenous anti-oxidative compounds has been shown to protect against oxidative cardiovascular disorders in animal models [75]. The increasing recognition of the role for oxidative stress in cardiac disorders has led to extensive investigation on the protection by exogenous antioxidants against oxidative cardiac injury [76]. Evidence from research suggests that ROS are crucially involved in the pathogenesis of cardiovascular diseases, such as myocardial IR injury [77]. Consistent with this notion, administration of exogenous anti-oxidative compounds has been shown to provide protection against oxidative cardiac injury [78]. Considerable research now dictates that IR injury is predominantly mediated by an inflammatory response that occurs following reoxygenation of an ischemic tissue. This concept therefore highlights the contribution of both the ischemic and reperfusion phases, with the recognition that reperfusion of an ischemic tissue with oxygenated blood can accelerate the degree of tissue injury.

Recent studies highlight the potentially unique roles of GST enzymes as crucial determinants of the development of IR [79]. In the area of heart, and lung transplantation [80], investigators found an association between donor GST genotype and patient primary graft dysfunction [81]. Although GST may have protective role for developing cardiovascular disorders [82], its role is not clear primarily because few studies have been performed in the field.

EA and it's GSH conjugate have been shown to specifically inhibit the GSTpi isoform [83] with a lower affinity towards the mu and alpha isoforms. Previous studies have suggested that EA-induced cell injury is driven by oxidative stress [84]. During the past few years it has become clear that, in addition to necrosis, apoptosis plays a significant role in myocardial IR injury [85]. However, the precise mechanisms involved in stimulus recognition and progression to apoptosis in response to ischemic injury or ATP depletion remains largely uncertain. Many studies have suggested that the MAPKs as well as PKB/Akt may be important regulators of apoptosis in response to myocardial IR, but reports on their precise roles are still conflicting. Three major MAPKs, namely ERK, p38 and JNK play a pivotal role in the transmission of signals from cell surface receptors to the nucleus [86]. It is important to note that alteration of cellular GSH metabolism moreover activity of GST can influence several signalling pathways [87,88]. Certain types of GST play a key role in regulating MAP kinase pathways involved in cellular response to stress, apoptosis and proliferation [89, 90] moreover altering activity state of apoptotic signal regulating kinase-1 (ASK1) influencing cell fate decision.

Our concept was to study the biological role played by GST in cardiac myocytes under oxidative stress conditions. Therefore we tried to identify the effect of GST inhibition by administrating EA when cells were exposed to various stress components of IR. Since GST activity is a major determinant survival and adaptive response to oxidative stress in the heart, we hypothesized that its inhibition with EA might exacerbate the severity and outcome of IR and oxidative injury.

5.2. Materials and methods

5.2.1. Cell culture

Primary culture of neonatal rat cardiomyocytes was prepared as described previously [91, 92]. Briefly, cells were obtained from ventricular myocytes of 2-4 day-old Wistar rats (Charles-River Ltd., Hungary), using collagenase (Gibco™ Collagenase Type II, Invitrogen Corp., Carlsbad, CA, USA). Isolated cells were plated on collagen I coated plates (Coll Typ 1 cellcoat, Greiner, Germany) at the density of 200 000 per cm^2. Cells were incubated in DMEM/F12 medium (Sigma–Aldrich, USA) supplemented with 10 % of foetal bovine serum (Gibco, USA). The following day, when the cells attached to the plate firmly, the medium was replaced with complete serum free medium (CSFM) containing the following supplements: BSA (2.5 %, AlbuMax 1, Invitrogen), insulin (1 μM), transferrin (5.64 μg/ml), selenium (32 nM) (insulin-transferrin-sodium-selenit media supplement, Sigma, Hungary), sodium pyruvate (2.8 mM, Sigma), 3,3',5'-triiodo-L-thyronine sodium salt (1 nM, Sigma, Hungary), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) (PS solution, Sigma, Hungary). Experiments started 48 hours after incubation with CSFM and the medium was changed every 24 hours.

Cultured cardiomyocytes were randomly assigned to one of six experimental groups: Group I, control group of cells, incubated in CSFM without treatment; Group II, EA 150 μM alone; Group III, cells exposed to 1 mM of H_2O_2; Group IV, cells exposed to IR; Group V, cells treated with 1 mM H_2O_2 together with EA 150 μM; Group VI, cells exposed to IR and EA 150 μM.

Based on our pilot experiments we chose to use a concentration of 150 μM and a treatment time of 5 hours (Figure 12 and 13).

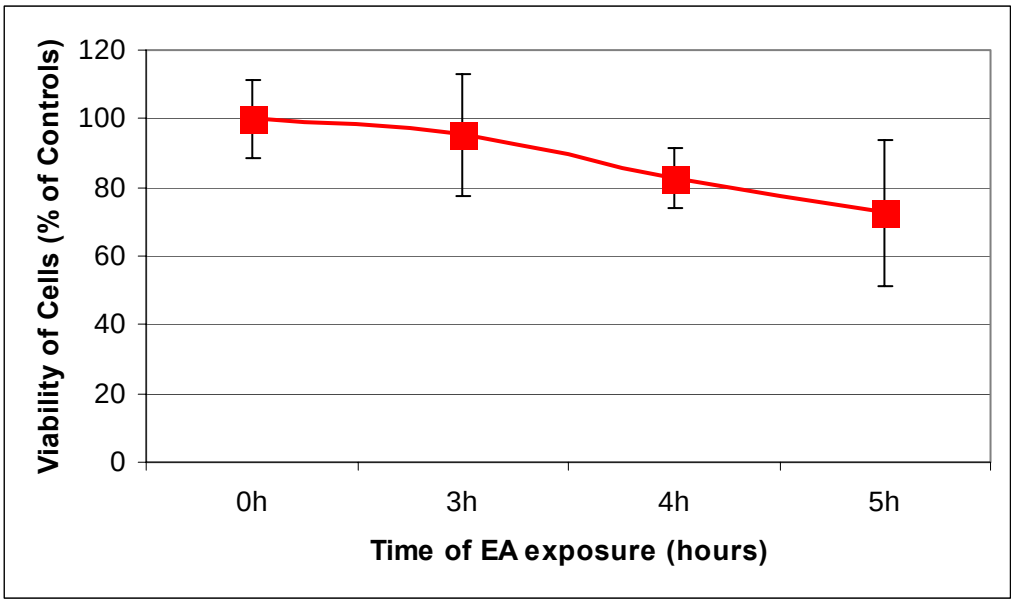


Figure 12: *Survival of cells after exposure to Ethacrynic Acid (EA) with time in hours. Results are expressed as mean ± SD. N=5.*

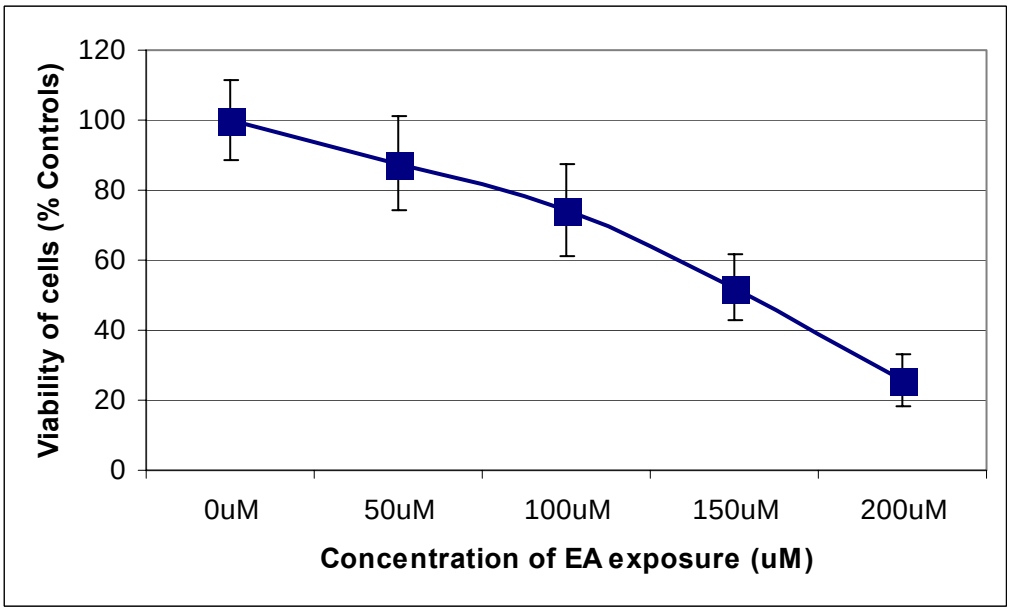


Figure 13: *Survival of cells after exposure to Ethacrynic Acid (EA) with concentration in µM. Results are expressed as mean ± SD. N=5.*

Cells were exposed to mentioned concentration of chemicals for 4 hours MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma]. Evaluation of cell survival was performed immediately after termination of treatments. Assessment of apoptotic

signalling markers was also started after treatments until permeabilization, and samples were stored at -20 ºC until further processing according to the protocol supplied by the manufacturer. Experiments were repeated six times in duplicate wells.

5.2.2. Cell viability test

Viability of cardiomyocytes was determined by colorimetric MTT assay. The assay is based on the reduction of MTT into a blue formazan dye by the functional mitochondria of viable cells. At the end of the treatments, the medium was discarded from plates and the cells were subsequently washed twice with phosphate buffered saline (PBS, Sigma). Cells were then incubated with PBS containing 0.5 mg/ml of MTT for 3 hours at 37 ºC in an atmosphere of 5% CO_2. The solution was aspirated carefully and 1 ml of dimethylsulfoxid was added to dissolve the blue-colored formazan particles. Samples from duplicate wells were transferred to a 96-well plate and adsorbance was measured by an ELISA reader (Sirio microplate reader, Seac Corp. Florence, Italy) at 570 nm representing the values in arbitrary unit (AU). Results are expressed as percentage of control values.

5.2.3. Annexin V and propidium iodide staining of cells

By conjugating a fluorescent group to annexin V, apoptosis can be quantitatively detected using fluorescent microscopy or flow cytometry, and this assay has been used together with other criteria to detect apoptosis in failing hearts [93]. Ratio of apoptosis was evaluated after double staining with FITC-labelled annexin V (BD Biosciences, Pharmingen, USA) and propidium iodide (PI) (BD Biosciences, Pharmingen, USA) using flow cytometry, as described previously [94]. First, the medium was discarded and wells were washed twice with isotonic sodium chloride solution. Cells were removed from plates using a mixture of 0.25 % trypsin (Sigma, Hungary), 0.2 % ethylene-diamine tetra-acetate (EDTA; Serva,

Hungary), 0.296 % sodium citrate, 0.6 % sodium chloride in distillated water. This medium was applied for 15 minutes at 37 ºC. Removed cells were washed twice in cold PBS and were resuspended in binding buffer containing 10 mM Hepes NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$. Cell-count was determined in Bürker's chamber for achieving a dilution in which 1 ml of solution contains 106 cells. 100 µl of buffer (105 cells) was transferred into 5 ml round-bottom polystyrene tubes. Cells were incubated for 15 minutes with FITC conjugated annexin V molecules and PI. After this period of incubation, 400 µl of Annexin-binding buffer (BD Biosciences, Pharmingen, USA) was added to the tubes as described by manufacturers. The samples were immediately measured by BD FacsCalibur flow cytometer (BD Biosciences, USA) and analysed with cell quest software. Cells in each category are expressed as percentage of the total number of stained cells counted.

5.2.4 Statistics

All data are presented as mean ± standard error of the mean (S.E.M). Differences between groups were assessed with one-way ANOVA followed by Neuman–Keul's post hoc analysis. A P-value of less than 0.05 was considered significant.

5.3 Results

MTT assay was performed to measure the absolute number of living cells in different groups. The amount of living cells was taken to 100 %. EA alone reduced the ratio of living cells to 43,41±11,15% measured by MTT assay. Both IR and H_2O_2 alone caused marked reduction in amount of living cells. The effect of cell death was significantly stronger upon administration of EA in groups treated with H_2O_2 or exposed to IR (Figure 14). EA elevated the ratio of apoptotic cells during H_2O_2 treatment and increased the number of necrotic cells during reperfusion. (Figure 16, 17).

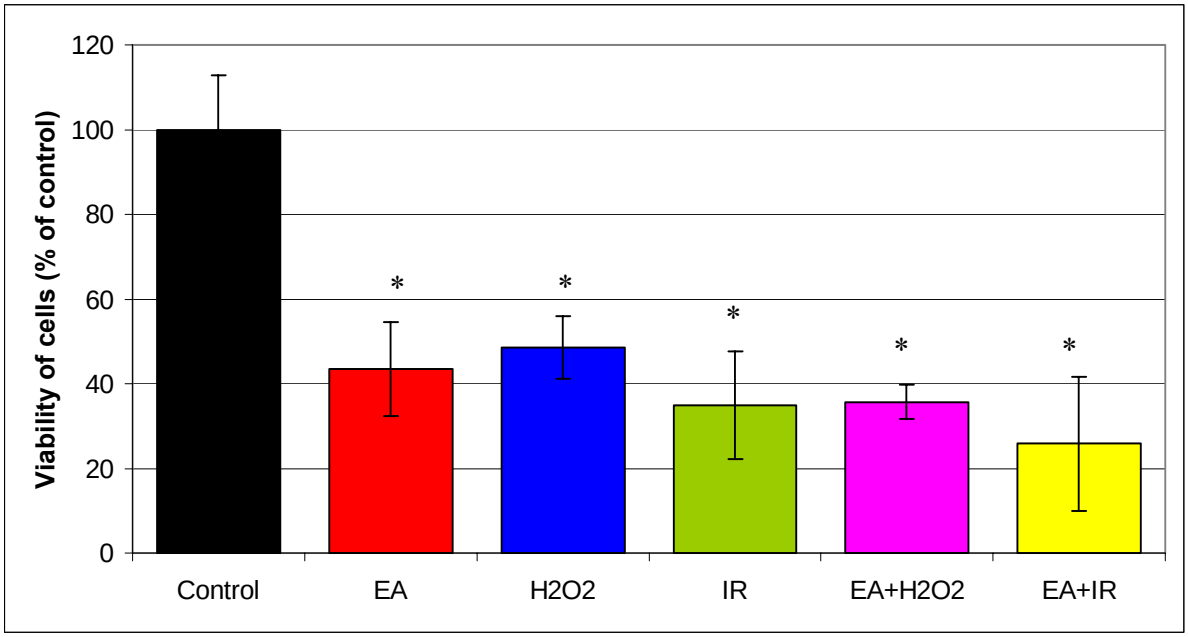


Figure 14: *Viability of cells with MTT assay. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.*

The control group had 85.7±1.94 % of intact, living (annexin V and PI negative) cells and 4.6±0.82 % of cells in early phase of apoptosis (annexin V positive and PI negative) (Figure 15, 16). EA administration decreased the number of living cells and increased the percentage of apoptotic cells. A significant increase of apoptotic cells was observed in both the H_2O_2 treated and IR groups with a lower number of living cells (Figure 15, 16). When EA was added in groups treated with the quantity of apoptotic cells was further increased with reduced amount of living cells. Interestingly, EA raised the amount of necrotic cells (annexin V negative and PI positive) during IR with a decreased number of living cells.

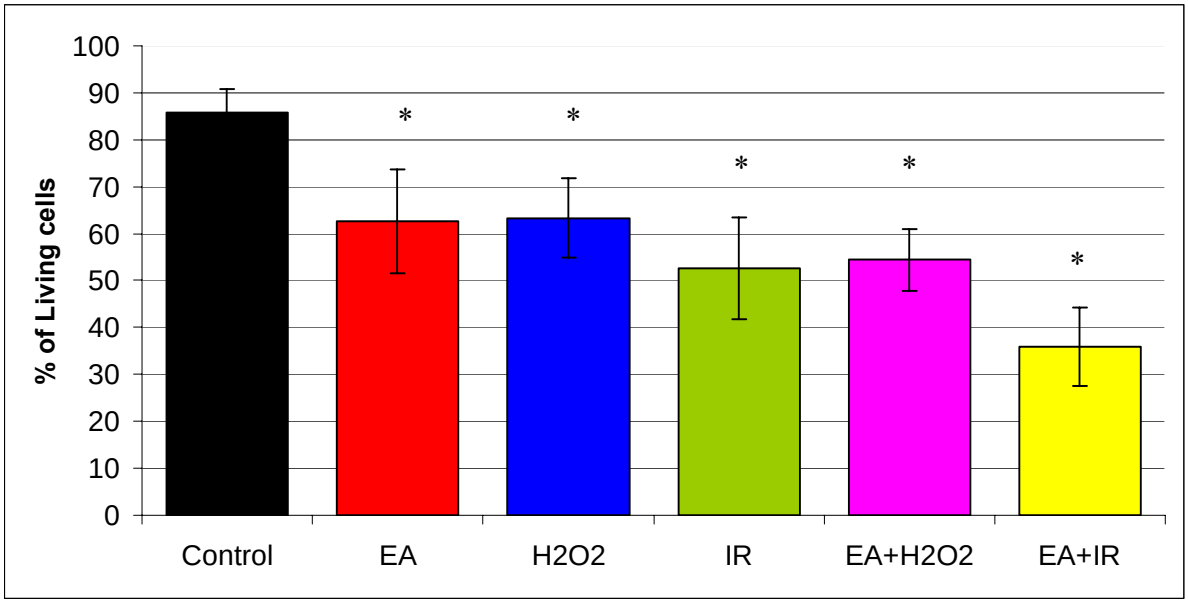


***Figure 15*: Comparison of living cells with the graphs demonstrating the mean percentage of living cells as determined by flow cytometry following annexin V and propidium iodide double-staining. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.**

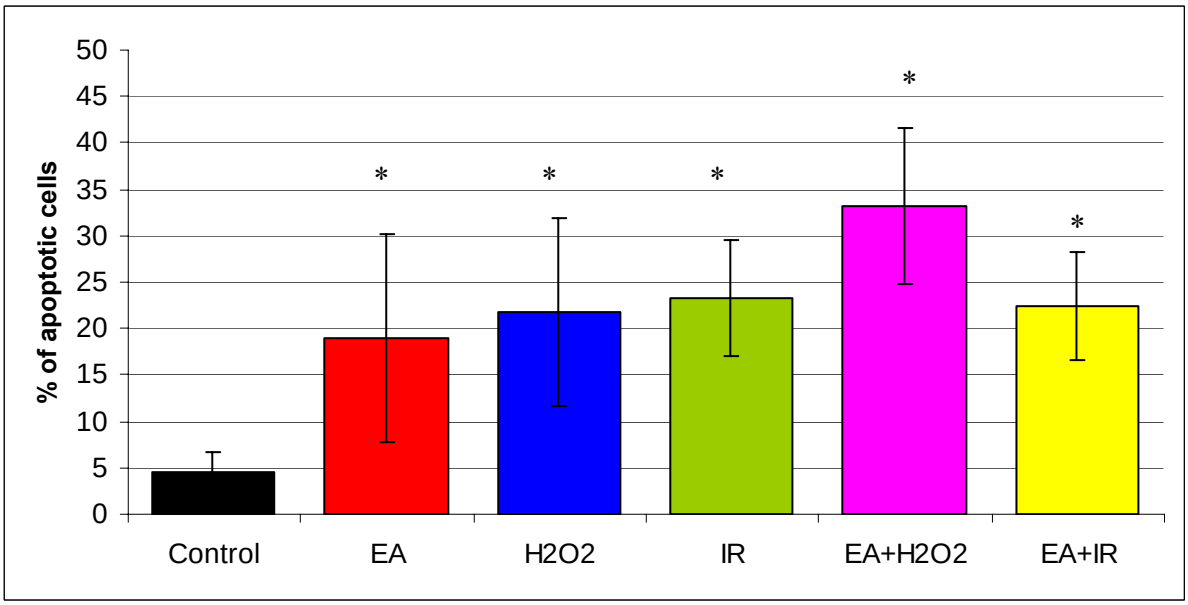


***Figure 16*: Comparison of apoptotic cells with the graphs demonstrating the mean percentage of living cells as determined by flow cytometry following annexin V and propidium iodide double-staining. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.**

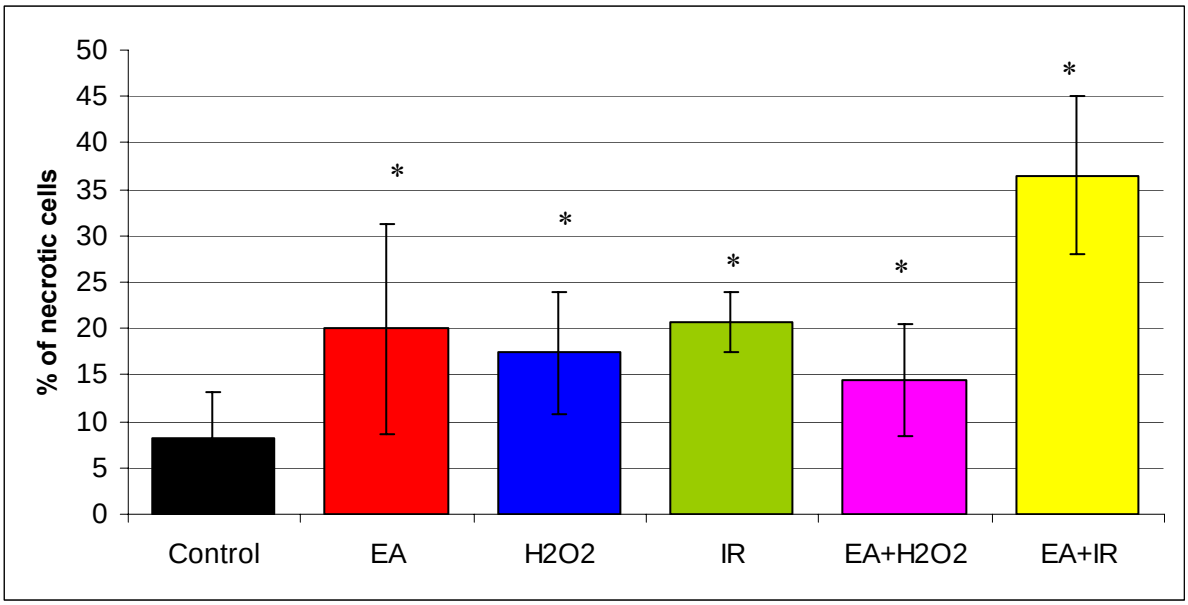


Figure 17**:** *Comparison of living necrotic cells with the graphs demonstrating the mean percentage of living cells as determined by flow cytometry following annexin V and propidium iodide double-staining. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.*

JNK activation increased markedly upon administration of EA to cardiac myocytes. H_2O_2 treatment raised the level of activated JNK, nonetheless this difference was not significant. IR caused noticeable increase in JNK activation. On the other hand, EA could augment the activation of JNK significantly both when cells were co-treated with H_2O_2 or when cells were exposed to IR (Figure 18).

Inhibition of GST led to significant increase in p38 activation related to non- treated cells. Both H_2O_2 incubation and IR resulted in significant increase of p38 MAP kinase activation. EA administration during IR increased p38 activity to 357.57 ± 5.39 % of control values. Likewise, when cells were incubated with H_2O_2 together with EA the level of phosphorilated p38 increased markedly, however this difference was not statistically significant compared to group treated with H_2O_2 alone (Figure 19).

Both administration of EA and H_2O_2 and IR caused non-significant reduction of Akt activity. On the other hand, H_2O_2 treatment resulted in more pronounced decrease (40.49±

5.68%) of Akt phosphorylation when GST was inhibited by EA (Figure 20). There was no significant difference between groups as evaluated by ANOVA.

ERK phophorylation increased in GST inhibited groups (incubated with EA) either treated with H_2O_2 or exposed to IR without any statistical significant difference (218.12 ±4.28% and 308.21± 8.55% respectively). ANOVA analysis failed to evaluate significant difference between groups. Moreover, analysis of difference using Student 's t test between group receiving IR and group incubated with EA during IR, revealed statistically significant divergence (Figure 21).

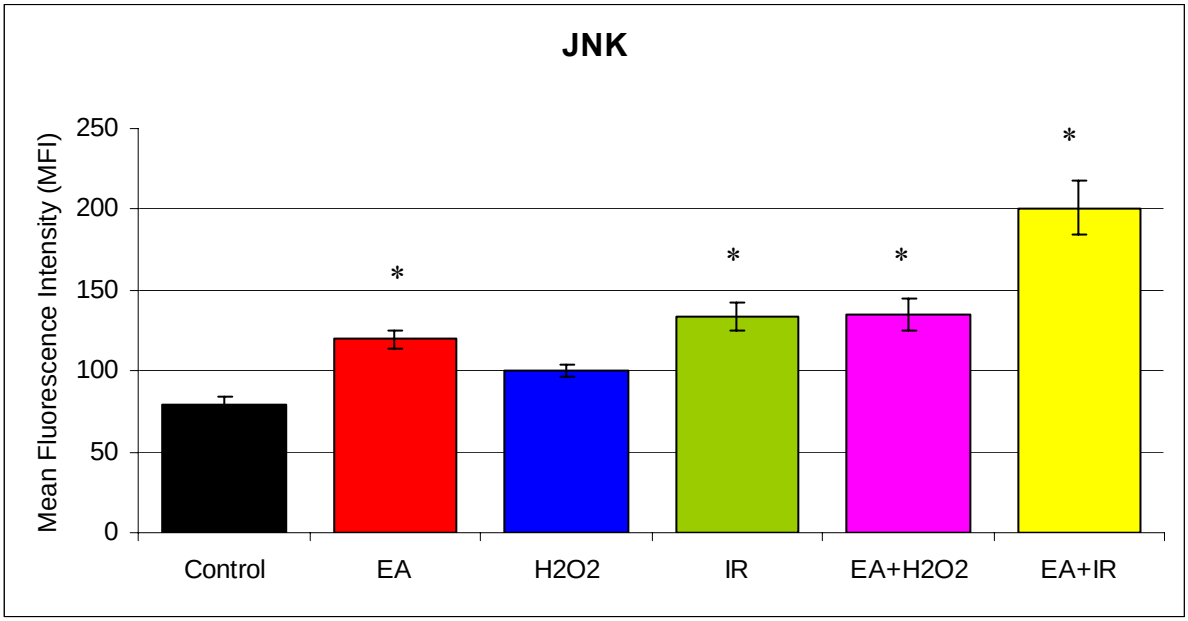


Figure 18**:** *Effects of Ethacrynic Acid (EA) on the activation of JNK in cardiomyocytes. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.*

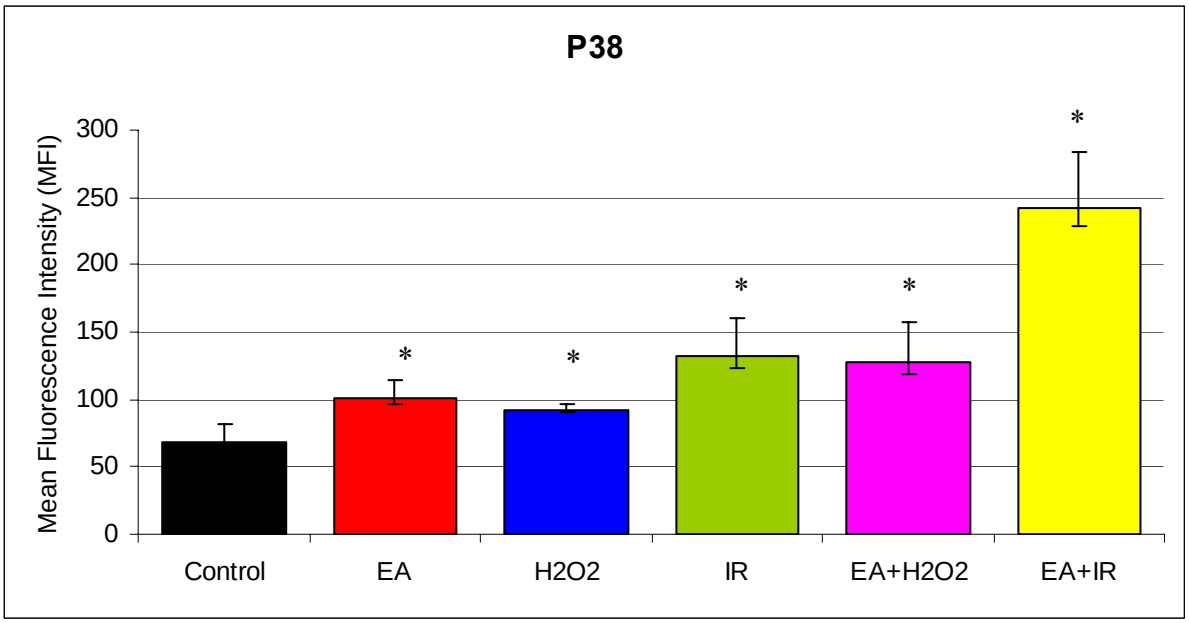


Figure 19**:** *Increase in p38 activation seen with introduction of EA. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.*

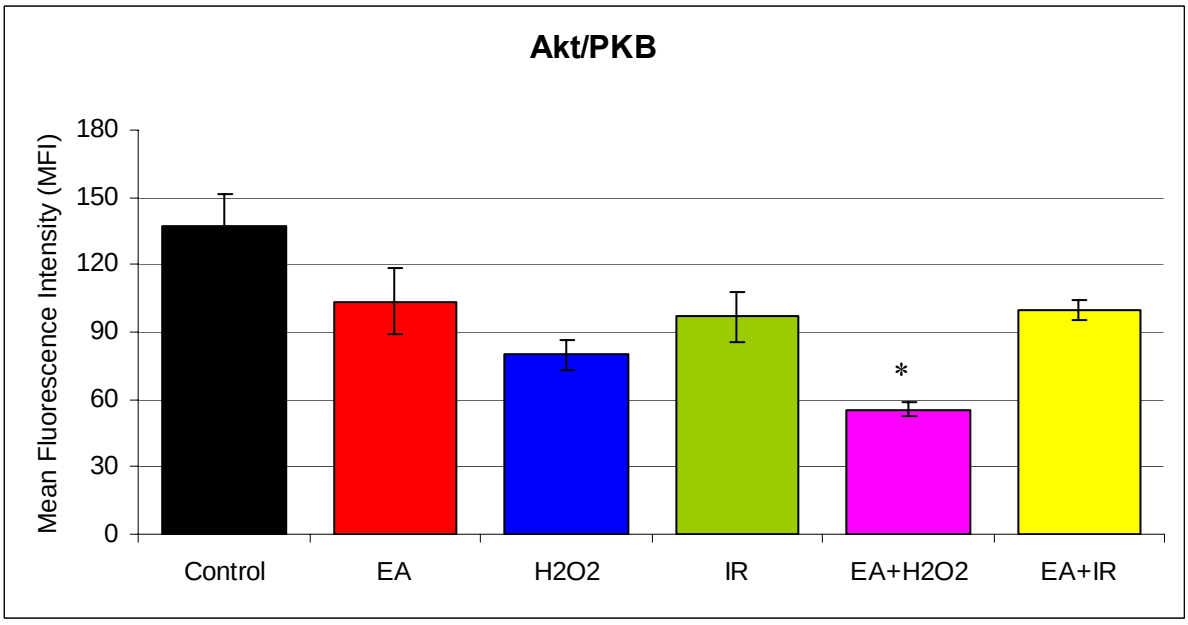


Figure 20: *Administration of Ethacrynic Acid (EA) caused reduction of Akt activity. Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.*

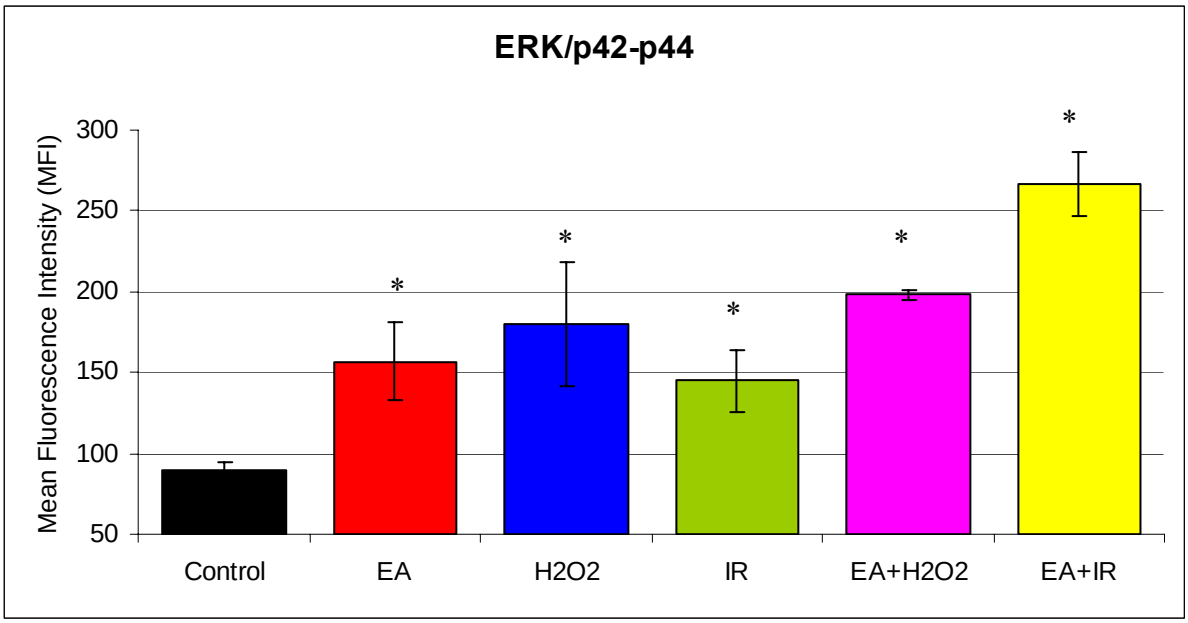


Figure 21*: *ERK phosphorylation increase after incubation with Ethacrynic Acid (EA). Cells were exposed to Ethacrynic Acid (EA) alone, H_2O_2 alone, Ischemic buffer (IR) alone, EA with H_2O_2 and EA with IR compared to controls. Data are presented as mean ± S.E.M. *p<0.05 vs untreated controls.

5.4 Discussion

GST acts as regulator of MAP kinase pathways. For example, GST has been shown to be an endogenous inhibitor of JNK via protein-protein interaction thus influencing cellular stress response and apoptosis [95]. JNK has been implicated in apoptotic signalling and mediates cytotoxicity in various conditions including IR and oxidative, nitrosative stress [96, 97]. In normal cells JNK activity is maintained on low level through interaction with GST [98]. Upon exposure to oxidative or nitrosative stress GST and JNK dissociated thus activating JNK furthermore GST underwent oligomerisation. Likewise the thereby liberated JNK regain its activity by phosphorylation during oxidative or nitrosative stress and further phosphorylate c-Jun, the downstream effector of JNK [99]. Moreover, a number of phosphatases are subjected to interaction with GST and glutathionylation thus regulating phosphorylation state of signalling pathways [100]. It has been already described that GST knockout mice have high basal JNK activity furthermore treatment of cells with potent GST inhibitor causes activation of JNK [101, 102]. We have found that pharmacological inhibition

of GST augments JNK activity by itself. This could be explained by elimination of sequestration of JNK within a protein complex with GST and inhibition of S-glutathionylation. On the other hand effective inhibition of GST may cause oxidative injury also in otherwise unstressed cells due to hindered elimination of trivially developing oxidant and toxic materials. This may occur as a result of JNK phosphorylation.

The same paradigm seems to hold for additional kinases, like thioredoxin or ASK1 influencing cellular stress response and cell fate decision [103, 104]. Like JNK, the activity of ASK1 is also reduced by protein sequestration, under unstressed conditions [105]. Oxidative stress however triggers dissociation of thioredoxin: ASK1 complex and further catalyses the dissociation of GST: ASK1 leading to ASK1 activation. Liberalization of thioredoxin causes p38 activation [106]. On the other hand ASK triggers apoptogenic kinase cascade leading to phophorylation of JNK and p38-MAP kinase, moreover it regulates dynamic balance between apoptotic (JNK, p38) and survival (Akt, ERK) pathways [107, 108]. Recently a novel role of GST has been described, since it has been identified that GST also heterodimerizes with tumour necrosis factor receptor associated factor 2 (TRAF2) thus reducing activation of both JNK and p38 [109].

Signalling pathway through p38 MAPK is activated by oxidative stress and is tied to cellular damage and mediation stress response, cytokine production. We found that oxidative injury and IR cause noticeable induction of p38 activity in cardiomyocytes, which was further increased by administration of EA. Our results about p38 activation upon GST inhibition can be explained by above described, ASK1-mediated processes.

According to our results ERK activated upon GST inhibition in presence of H_2O_2 or during reperfusion. The ERK activity of GST inhibited cells receiving IR exceeded the phospho-ERK level of cells undergone IR alone. These findings may represent the association between ERK and GST. It has been already described that immortalized fibroblast isolated

from GST π genotype expressed significantly elevated activity of ERK. Moreover treatment with potent GST inhibitor resulted in activation of ERK [110]. And vice versa the transcriptional induction of GST gene is orchestrated by signalling pathways, such as ERK, which might be deteriorated by GST inhibition [111].

Although similar relationship between the synthesis of GST and Akt is well investigated, the effect of GST inhibition on Akt mediated cellular survival has not been fully described [112, 113]. Akt activity reduced significantly in the group receiving GST inhibitor EA when compared to control values. Our results failed to prove any further association between GST inhibition and Akt activity. The hindered antioxidant, antitoxic defence of cells treated with EA furthermore the activation of ASK1 may explicate the results described by us.

Overall, our findings support our original hypothesis and suggest that GST activity is required for survival of cultured cardiomyocytes under stress conditions. However, future studies are needed i) to compliment these pharmacological studies, ii) to better define the biochemistry involved and iii) to explore the *in vivo* relevance of GST inhibition.

Chapter 6

NOVEL FINDINGS

1) In a clinically relevant canine model of hepatic ischemia-reperfusion injury, haemodynamic and metabolic analysis along with ICG spectrophotometry we found that NAC did not influence haemodynamic state or liver function.
2) A novel method of IPC introduced by our group revealed higher ICG-PDR, CI and ITBVI.
3) In a clinically relevant *in vivo* mouse model of ALI/ARDS, the effects of inhaled CO revealed that CO did not affect respiratory or haemodynamic parameters even with significant COHb levels.
4) Our innovative 3 mouse models of acute injury is a setting for more prolonged exposure to CO facilitating investigation of subacute to chronic lung inflammation.
5) In an *in vitro* model of cardiac ischemia-reperfusion to identify the effect of GST inhibition, when cells are exposed to various stress components EA reduced the ratio of living cells with MTT assay and FACS, caused a stronger effect of cell death when compared to treatment by H_2O_2 or exposure to IR and elevated the ratio of apoptotic cells during H_2O_2 treatment.
6) Our model of GST activity proves its essence for survival of cardiomyocytes under stress conditions and serves as a future model for in vivo experiments exploring GST activity.

Chapter 7

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Chapter 8

PUBLICATIONS by author

8.1 Papers related to this thesis:

1) **Ghosh S,** Gal J, Marczin N. Carbon Monoxide: endogenous mediator, potential diagnostic and therapeutic target for lung disease: a focus on critical illness. *Ann Med.* 2010; 42: 1-12. **IF: 5.435**

2) *Baumann J[†], ***Ghosh S**, Szakmany T, Jancso G, Ferencz A, Roth E, Bogar L. Short Term Effects of N-Acetylcysteine and Ischemic Preconditioning in a Canine Model of Hepatic Ischemia-Reperfusion Injury. *Eur Sur Res.* 2008; 41: 226-230. **IF: 1.327**

   * *Equal contribution*

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- Isaac Newton (Principia: Vol. 1 The Motion of Bodies)

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