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

INTRODUCTION

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. Although there are multiple biochemical and pathophysiological consequences of oxidative stress, there are special considerations for the development of acute organ injury such as lipid peroxidation, activation of redox sensitive intracellular signalling pathways, genetic response to oxidative stress leading to adaptive survival or injury/death, 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. 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, 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.

Role of ischemia-reperfusion injury

Normal antioxidant defence mechanisms function to limit oxidative injury during times of health. During ischemia, there is a build up of substances 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.

Role of oxidative stress

Oxidative stress has been defined as a disturbance in the equilibrium status of pro-oxidant/anti-oxidant systems in intact cells. This implies that cells have intact 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. Evidence for the role of a variety of chemicals called radicals (may be a small gas molecule or a large biomolecule) in these processes has led to interest in the reactions of partially reduced oxygen products.

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.

Liver injury

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. IR injury of liver is a clinically significant manifestation of several hepatic surgical procedures. 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.

Attenuation of liver injury by N-acetylcysteine

NAC increases glutathione levels in hepatocytes and in turn limits the production of ROS which cause hepatocellular injury. 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.

Lung injury

The lung, because of its interface with the environment, is a major target organ for injury by exogenous oxidants 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.

Attenuation of lung injury by carbon monoxide

CO forms 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. 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.

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. 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 whether by thrombolysis or invasive procedures, does not guarantee the survival of ischemic cells. 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.

Attenuation of cardiac injury by glutathione S-transferase

Glutathione plays several essential roles in the protection of the cell. As well as secondary oxidant and xenobiotic detoxification, GST's are thought to be involved in modulation of cell proliferation and apoptotic signalling pathways. Significant variations in GST, 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 and cardiovascular diseases.

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.

SETTINGS

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

Under general anaesthesia, 3 groups of mongrel dogs (n = 5 per group) were subjected to (1) 60-min hepatic ischemia, (2) same ischemia preceded by intravenous administration of 150 mg kg(-1) NAC, and (3) three episodes of IPC (10-min ischemia followed by 10-min reperfusion) prior to same ischemia. Hepatic reperfusion was maintained for a further 180 min, with hemodynamic and hepatic function parameters monitored throughout. We evaluated the possibility that repeated ischemic preconditioning or N-acetylcysteine (NAC) could prevent ischemia-reperfusion injury as determined by indocyanine green plasma disappearance rate (ICG-PDR) or has favourable hemodynamic effects during reperfusion in an in vivo canine liver model. Plasma disappearance rate of indocyanine green and serum levels of aspartate transferase and alanine transferase showed no significant differences between groups. Although liver injury was obvious, reflected by hemodynamic, blood gas, and liver function tests, NAC and IPC failed to prevent decay in hepatic function in this canine model. The cardiac index and the intrathoracic blood volume index were significantly higher in the preconditioning group compared to the controls throughout the study period. Repeated ischemic preconditioning improved hemodynamic parameters, whereas we were unable to find any significant differences between the groups regarding N-acetylcysteine. 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.

II. EFFECTS OF INHALED CARBON MONOXIDE ON ACUTE LUNG INJURY IN MICE

Anaesthetized C57BL/6 mice were ventilated with oxygen in the presence or absence of CO (500 parts per million) for 1 h before lung injury was induced by lipopolysaccharide (LPS) or oleic acid (OA) administration. Ventilation was then continued with the same gases for a further 2-3 h, with hemodynamic and respiratory parameters monitored throughout. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are major causes of morbidity and mortality in the intensive care unit, but despite continuing research few effective therapies have been identified. In recent years, inhaled carbon monoxide (CO) has been reported to have cytoprotective effects in several animal models of tissue injury. We therefore evaluated the effects of inhaled CO in three different in vivo mouse models of ALI. Intratracheal LPS administration induced lung injury with alveolar inflammation (increased lavage fluid neutrophils, total protein, and cytokines). In contrast, intravenous LPS induced a predominantly vascular lung injury, with increased plasma TNF and increased neutrophil activation (surface Mac-1 upregulation and L-selectin shedding) and sequestration within the pulmonary vasculature. Intravenous OA produced deteriorations in lung function, reflected by changes in respiratory mechanics and blood gases and lavage fluid neutrophil accumulation. Although lung injury was achieved, addition of CO to the inspired gas did not produce significant changes in the measured physiological or immunological parameters in the mouse models used in this study. 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.

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

Primary culture of neonatal rat cardiomyocytes was prepared and cells obtained from ventricular myocytes of 2-4 day-old Wistar rats using collagenase 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. After a concentration of 150 μM and a treatment time of 5 hours, viability of cardiomyocytes was determined by colorimetric MTT assay to measure the absolute number of living cells in different groups. Ratio of apoptosis was evaluated after double staining with FITC-labelled annexin V and propidium iodide using flow cytometry. 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. EA elevated the ratio of apoptotic cells during H_2O_2 treatment and increased the number of necrotic cells during reperfusion. 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. Interestingly, EA raised the amount of necrotic cells (annexin V negative and PI positive) during IR with a decreased number of living cells. JNK activation increased markedly upon administration of EA to cardiac myocytes. IR caused noticeable increase in JNK activation. Both H_2O_2 incubation and IR resulted in significant increase of p38 MAP kinase activation. H_2O_2 treatment resulted in more pronounced decrease of Akt phosphorylation when GST was inhibited by EA. ERK phophorylation increased in GST inhibited groups (incubated with EA) either treated with H_2O_2 or exposed to IR. 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.

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, 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 GST model proves its essence for survival of cardiomyocytes under stress conditions and serves as a future model for in vivo experiments exploring GST activity.

PUBLICATIONS

Papers related to this thesis:

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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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8.3 Presentations:

1) National Congress for Hungarian Society of Anaesthesiology, MAITT 2010 Eger, Hungary.

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2) National Congress for Hungarian Society of Anaesthesiology, MAITT 2009 Balatonfured, Hungary.

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Ghosh S, Szakmány T, Baumann J, Roth E, Bogar L: Hemodynamic effects of N-acetylcysteine and repeated normothermic ischaemic preconditioning after liver ischaemia-reperfusion injury.

7) International Symposium for Myocardial Cytoprotection, 2003, Pécs, Hungary.

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