INVESTIGATING THE CYTOPROTECTIVE MECHANISMS OF VITAMINS B6 AND B1 AGAINST ENDOGENOUS TOXIN-INDUCED OXIDATIVE STRESS

By

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Abstract

Investigating the cytoprotective mechanisms of vitamins B6 and B1 against endogenous toxin-induced oxidative stress
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Recent epidemiological evidence suggests that many chronic health disorders in the developed world are associated with endogenous toxins formed from the Western diet. The Western diet, which encompasses calorie dense foods, processed foods and increased quantities of red meat, can cause intracellular oxidative stress through increased formation of reactive oxygen species (ROS) and reactive carbonyl species (RCS). A number of micronutrients have been investigated for their protective capacity in *in vitro* and *in vivo* models of oxidative stress. This thesis investigated the cytotoxic targets of Fenton-mediated ROS and RCS and the subsequent protective mechanisms of vitamins B1 (thiamin) or B6 (pyridoxal, pyridoxamine or pyridoxine) in an isolated rat hepatocyte model. The approach was to use an "accelerated cytotoxicity mechanism screening" technique (ACMS) to develop an *in vitro* cell system that mimicked *in vivo* tissue cytotoxicity. Using this technique, we investigated the protective mechanisms of vitamins B1 and/or B6 against the cytotoxic effects of two endogenous toxins associated with the Western diet: 1) RCS, as exemplified by glyoxal, a glucose/fructose autoxidation product and 2) biological ROS induced by exogenous iron. Firstly, we developed an understanding of the sequence of events contributing to glyoxal-induced oxidative stress, with a focus on protein carbonylation. Next, we determined the mechanisms by which carbonyl scavenging drugs (vitamin B6 included) protected against the intracellular targets of glyoxal-induced toxicity. Our results suggested that the agents used were cytoprotective by multiple mechanisms and glyoxal trapping was only observed when the agents were administered at concentrations equal to

glyoxal. We also evaluated the protective capacity of vitamins B1 and B6 against iron-catalyzed cytotoxicity and found that hepatocytes could be rescued from protein and DNA damage when vitamins B1 or B6 were added up to one hour after treatment with iron. The vitamins also varied in their primary mechanisms of protection. Our improved understanding of Western diet-derived endogenous toxins enabled us to identify and prioritize the specific inhibitory mechanisms of vitamins B1 or B6. The ability to delay, inhibit or reverse toxicity using multi-functional B1 or B6 vitamins could prove useful as therapy to minimize oxidative stress in diet-induced chronic conditions.

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List of Abbreviations

ACF	Aberrant crypt foci
ACMS	Accelerated cytotoxic mechanism screening
ADP	Adenosine diphosphate
AGE	Advanced glycation end product
$ALDH_2$	Aldehyde dehydrogenase 2
ALE	Advanced lipid/lipoxidation end product
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
B1 (vitamin)	Thiamin or thiamin diphosphate/pyrophosphate
B6 (vitamin)	Pyridoxal, pyridoxamine, pyridoxine or pyridoxal phosphate
BHA	Butylated hydroxyanisole
t-BuOOH	tert-Butyl hydroperoxide
BCKDH	Branched-chain ketoacid dehydrogenase
BMI	Body mass index
BSA	Bovine serum albumin
CRC	Colorectal cancer
CoA	Coenzyme A
Cu	Copper
Cu, Zn-SOD	Copper/zinc superoxide dismutase
CYP450	Cytochrome P450
DCF	2',7'-Dichlorofluorescein

DCFD	2',7'-Dichlorofluorescein diacetate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNPH	2',4'-Dinitrophenylhydrazine
E1	Pyruvate dehydrogenase
E2	Dihydrolipoyl transacetylase
E3	Dihydrolipoyl dehydrogenase
EC-SOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
Fe, Fe^{2+}, Fe^{3+}	Iron, ferrous iron, ferric iron
FAD	Flavin adenine dinucleotide
FADH	Reduced flavin adenine dinucleotide
FI	Fluorescence intensity
FMN	Flavin mononucleotide
FOX 1	Ferrous oxidation of xylenol orange
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Glutathione disulfide (oxidized glutathione)
H_2O_2	Hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HFCS	High-fructose corn syrup
4-HNE	4-Hydroxy-2-nonenal
$HO_2^{\bullet}$	Hydroperoxyl radical

HOCl	Hypochlorous acid
HPLC	High pressure liquid chromatography
8-HO-dG	8-Hydroxy 2'-deoxyguanosine
8-HQ	8-Hydroxyquinoline
IL-1 (-6)	Interleukin
α-KGDH	alpha-Ketoglutarate dehydrogenase
LD50/LC50	Dose/concentration of a compound that produces 50% death of the population (e.g., cells) under study
LDL	Low density lipoprotein
LMP	Low melting-point
LPO	Lipid peroxidation
MDA	Malondialdehyde
MMO	Monooxygenase system
MMP	Mitochondiral membrane potential
Mn-SOD	Manganese superoxide dismutase
n	Number of observations
NAC	n-Acetyl-cysteine
NAD	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCRF	National Cancer Research Foundation
NIH	National Institute of Health
$NO^{\bullet}$	Nitric oxide

NOX	NADPH oxidase
NOS	Nitric oxide synthase
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide anion
O_3	Ozone
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OP	*ortho*-Phenanthroline
OTM	Olive tail moment
p	Probability, represents statistical significance
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase
PIH	Pyridoxal isonicotinoyl hydrazone
PLP	Pyridoxal phosphate
PPP	Pentose phosphate pathway
PUFA	Polyunsaturated fatty acids
RIPA	Radioimmunoprecipitation buffer
RCS	Reactive carbonyl species
RDA	Recommended dietary allowance
RNS	Reactive nitrogen species
$RO^{\bullet}$	Alkoxyl radical
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis
SDS	Sodium dodecyl sulfate

SEM/SE	Standard error of mean
SOD	Superoxide dismutase
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
TEMPOL	4-Hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl
TK	Transketolase
TMP	Thiamin monophosphate
TNF-α	Tumor necrosis factor-α
TPP	Thiamin diphosphate/pyrophosphate
UV	Ultraviolet
WCRF	World Cancer Research Fund
WHO	World Health Organization
XO	Xanthine oxidase

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Appendices

List of publications of the author relevant to thesis

1. Depeint F, Bruce W.R., Shangari N, **Mehta R**, and O'Brien P.J., Mitochondrial function and toxicity: Role of B vitamins on the one-carbon transfer pathways, *Chemico-Biological Interactions*, 2006, 163 (1-2), 113-132.

   A portion of this review was used with permission from the publisher in the introduction (Chapter 1). My contribution to this review was with editing the manuscript and providing my results on vitamin B6 protection in hepatocytes.

2. Depeint F, Bruce W.R., Shangari N, **Mehta R**, and O'Brien P.J., Mitochondrial function and toxicity: Role of the B vitamin family on mitochondrial energy metabolism, *Chemico-Biological Interactions*, 2006, 163 (1-2), 94-112.

   A portion of this review was used with permission from the publisher in the introduction (Chapter 1). My contribution to this review was with editing the manuscript and providing my results on vitamin B1 protection in hepatocytes.

3. **Mehta R**, O'Brien P.J., Therapeutic intracellular targets for preventing carbonyl cell death with B vitamins or drugs, *Enzymology and Molecular Biology of Carbonyl Metabolism*, 2007, 13th Ed, 113-120, Weiner H, Maser E, Lindahl R, Plapp B (Eds).

   My contribution to this study was through carrying out all of the experiments, performing all of the analysis and writing the manuscript with assistance from Dr. O`Brien. This manuscript has been cited in this thesis, but has not been used in its entirety in the thesis.

4. Shangari N, **Mehta R,** O'Brien P.J., Hepatocyte susceptibility to glyoxal is dependent on cell thiamin content, *Chemico-Biological Interactions*, 2007, 165(2), 146-154.

   My contribution to this study was through assisting with some of the experiments and editing the manuscript.

5. Depeint F, Bruce, W.R., Lee O, **Mehta R,** O'Brien P.J., Micronutrients that decrease endogenous toxins, a strategy for disease prevention, *Micronutrients and health research advances*, 2008, 315, 147-179, Columbus F (Ed), Nova Science Publishers.

   My contribution to this book chapter was with providing my results on micronutrient protection in our in vitro hepatocyte model (Table 2, 3) and drawing a figure on the possible protective roles of B vitamins against endogenous toxins (Figure 5).

6. ***Mehta R**, Shangari N, O'Brien P.J., Preventing cell death induced by carbonyl stress, oxidative stress or mitochondrial toxins with vitamin B anti AGE agents, *Molecular Nutrition and Food Research,* 2008, 52(3), 379-385.

This work has been reproduced with permission from Molecular Nutrition and Food Research (Chapter 2). My contribution to this study was through conducting all of the research, completing the analysis and writing the manuscript.

7. ***Mehta R**, Wong L, O'Brien P.J., Cytoprotective mechanisms of carbonyl scavenging drugs in isolated rat hepatocytes, *Chemico-Biological* Interactions, 2009, 178(1-3), 317-323.

   This work has been reproduced with permission from Chemico-Biological Interactions (Chapter 3). My contribution to this study was through carrying out the majority of the research, completing all of the analysis and writing the manuscript.

8. Lee O, Bruce W.R., Dong Q, Bruce J, **Mehta R**, O'Brien P.J., Fructose and carbonyl metabolites as endogenous toxins, *Chemico-Biological Interactions*, 2009, 178(1-3), 332-339.

   My contribution to this study was through editing the manuscript and carrying out complementary research that aided in formulating the study hypotheses.

9. Feng C.Y., Wong S, Dong Q, Bruce J, **Mehta R**, Bruce W.R., O'Brien P.J. Hepatocyte inflammation model for cytotoxicity research: fructose as a source of endogenous toxins, *Archives of Physiology and Biochemistry*, 2009, 115(2), 105-111.

   My contribution to this study was through editing the manuscript and carrying out complementary research that aided in formulating the study hypotheses.

10. O'Brien P.J., Feng C.Y., Lee O, **Mehta R,** Bruce J, Bruce W.R., Fructose-derived endogenous toxins, *Endogenous Toxins,* 2010, 1, 173-212, O'Brien P.J., Bruce W.R., (Eds), Wiley-VCH.

    My contribution to this book chapter was through assisting with some of the experiments.

11. ***Mehta R,** Dedina L, O'Brien P.J., Rescuing hepatocytes from iron-catalyzed oxidative stress with vitamin B1 and B6, *Toxicology in Vitro*, 2011, In Press.

    This work has been reproduced for use as Chapter 4. My contribution to this study was through carrying out the majority of the research, completing all of the analysis and writing the manuscript.

*References that are used as Chapters in the thesis.

Preface

Diet and Obesity in the Western World

According to the World Health Organization (WHO), over one billion adults globally are overweight, of whom at least one third are obese (Morrill and Chinn, 2004; WHO, 2005; WHO, 2010). Obesity is also a growing epidemic in developed countries (Figure 1): Rates for obesity have increased over three-fold since 1980 in North America, the United Kingdom, Eastern Europe, the Middle East, the Pacific Islands, Australasia and China (WHO, 2010). Obesity poses a major risk for serious chronic conditions that decrease the overall quality of life, and is now associated with a spectrum of metabolic disorders including glucose intolerance, insulin resistance, type 2 diabetes, hypertension, atherosclerosis, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), gallbladder disease, and some forms of cancer (colorectal (CRC), breast, endometrial and kidney) (Eckel *et al.*, 2005; National Institute of Health, 2008; Ogden *et al.*, 2007; World Cancer Research Fund, 2007). The obesity epidemic also has significant economic consequences. The WHO estimates that in many developed countries, obesity now accounts for 2-7% of all health care spending (WHO, 2010). The social costs of obesity along with the costs of attempts to prevent or treat obesity are also high. In 2002, the direct medical costs associated with obesity were estimated at more than $92 billion, and in 1989 Americans spent more than $30 billion on weight loss programs and products (Ogden *et al.*, 2007). What has led to the increased prevalence of obesity in the developed world? What are its side effects and how can they be prevented?

*Prevalence of adult obesity (%)

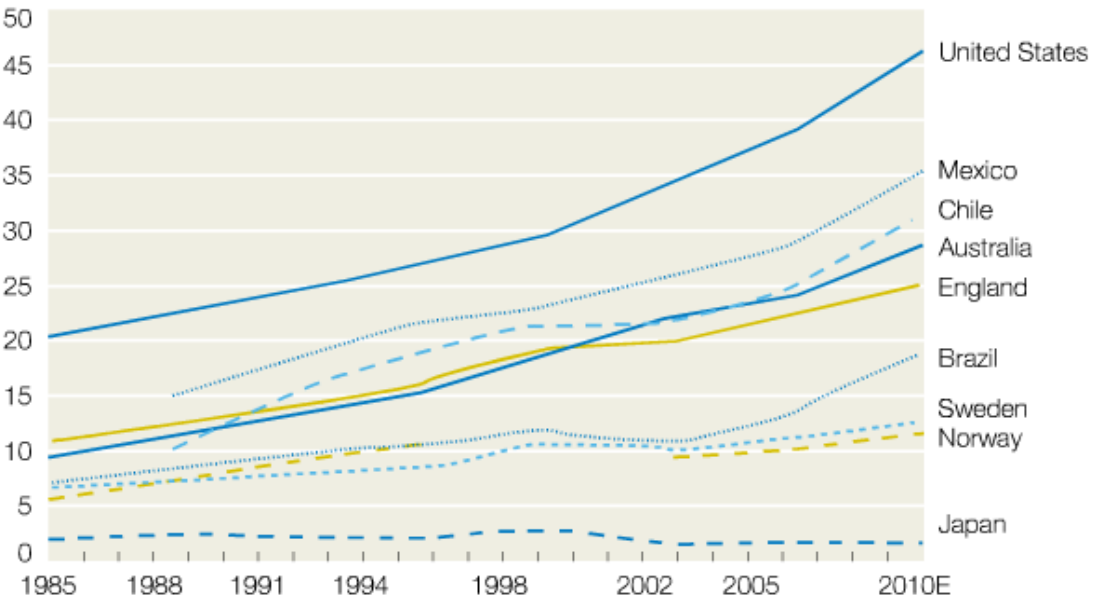

Figure 1: The prevalence of obesity is rising around the world. The figure compares the obesity rates amongst a range of countries from 1985 to 2010 (estimate, E). *Obesity in adults is defined as a body mass index (BMI) of 30 or higher for people aged 16 or older [Reproduced from (WHO, 2005)].

Obesity is often described as a simple issue of body weight; however, the health risk factors associated with obesity may not be captured adequately by simple measurements of body weight. Rather, obesity is a multi-factorial condition that reflects a state of excess adipose tissue distribution that in turn reflects a combination of individual behaviours, genetic factors, physiological status and environmental and social influences (Morrill and Chinn, 2004). The economic growth and urbanization of populations in the developed world have led to a nutritional transition that has given rise to unhealthy dietary habits and sedentary ways of life. The Western dietary patterns pose a major risk factor for obesity and obesity-related chronic diseases as they consist of more energy-dense, nutrient poor foods combined with reduced physical activity (WHO, 2010). The Western diet is characterized by high intakes of sugar

(fructose), saturated fat, red-meat and refined or processed foods (Babio *et al.*, 2010; Bermudez and Gao, 2010; Cordain *et al.*, 2005). Increased consumption of these micronutrient deficient, dietary components can lead to the formation and accumulation of endogenous toxins which can cause intracellular oxidative stress and subsequently lead to obesity and chronic metabolic disease (Depeint *et al.*, 2008).

Chapter 1. General Introduction

This introduction covers four major topics that form the basis of this thesis. The introduction begins with a discussion on oxidative stress, with emphasis on the formation of mediators of oxidative stress including reactive oxygen species (ROS), lipid peroxidation, protein carbonylation and DNA damage. The detoxification of ROS is also discussed in this section. The introduction then describes two endogenous toxins formed as a result of the Western Diet: Reactive carbonyl species (RCS), in particular glyoxal derived from the autoxidation of dietary sugars, and biological ROS derived from dietary iron. The endogenous formation and detoxification pathways of these reactive species as well as their connection to oxidative stress and chronic disease are also explained. Discussion follows regarding the role of vitamins B1 and B6 on cell function and their possible role in the prevention of oxidative stress and mitochondrial toxicity. A brief overview of other micronutrients is also covered in this section. The final part of this chapter describes the *in-vitro* screening system developed by our group: "Accelerated Cytotoxicity Mechanism Screening" (ACMS). The ACMS technique allows for the study of drug/xenobiotic molecular cytotoxic mechanisms in freshly isolated hepatocytes from male Sprague-Dawley rats. Chapter 1 concludes with the thesis problem formulation, hypotheses and objectives, followed by a note on the relevance of the thesis chapters.

1.1 Oxidative Stress

The field of oxidative stress has been generating a large amount of interest because of its established role as a contributor to disease (Perez-Matute *et al.*, 2009). Oxidative stress results from the increased production of reactive oxygen species (ROS) beyond the antioxidant capacity of the cell. ROS can be subdivided into one-electron (radical) and two-electron (non-radical) oxidants. Examples of ROS include superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen

peroxide (H_2O_2) (Valko *et al.*, 2006). Low concentrations of ROS are necessary for normal cell redox status, cellular function and intracellular signaling. However at high concentrations and limited antioxidant defenses, ROS can be important mediators of damage to cell structures, including lipids, proteins and DNA (Perez-Matute *et al.*, 2009; Valko *et al.*, 2006). Chronic conditions associated with increased oxidative stress include obesity, type 2 diabetes, atherosclerosis, Alzheimer's disease, liver disease, rheumatoid arthritis, ischemia-reperfusion injury and cancer (Perez-Matute *et al.*, 2009).

1.2 Chemistry of ROS

ROS are reactive chemical entities comprising two major groups: Free radicals ($O_2^{\bullet-}$ and $OH^{\bullet}$) and non-radical oxidizing derivatives formed from oxygen (H_2O_2). Oxygen readily reacts to form these partially reduced species, which are generally short-lived and highly reactive. The reactivity of free radicals is a consequence of the presence of unpaired electrons which renders them unstable.

$$O_2 \xrightarrow{e^-} O_2^{\bullet-} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH^{\bullet} \xrightarrow{e^-} H_2O$$

Figure 1.1 The sequential univalent pathway of oxygen reduction. Superoxide anion, $O_2^{\bullet-}$; hydrogen peroxide, H_2O_2; hydroxyl radical, $OH^{\bullet}$ (Paravicini and Touyz, 2008).

$O_2^{\bullet-}$ is the first intermediate in the sequential reduction of O_2 and is formed through one electron reduction (Paravicini and Touyz, 2008). $O_2^{\bullet-}$ is unique in that it can lead to the formation of many other reactive species, including $OH^{\bullet}$, H_2O_2 and the intermediate

hydroperoxyl radical ($HO_2^{\bullet}$) by protonation of $O_2^{\bullet-}$, making it a much stronger oxidative species than $O_2^{\bullet-}$ itself (Yu, 1994). It is short-lived in biological systems as it is rapidly reduced to H_2O_2 by superoxide dismutase (SOD) (Valko *et al.*, 2006). Furthermore, its charge makes it unable to cross cell membranes (Yu, 1994).

H_2O_2 is less reactive, more stable and has a longer half-life than the free radicals. The biological importance of H_2O_2 arises in part from its ability to easily diffuse within and between cells (Paravicini and Touyz, 2008). Because of the participation of H_2O_2 in the production of $OH^{\bullet}$, the importance of its role has been placed on the initiation of free radical cytotoxicity (Yu, 1994).

The $OH^{\bullet}$ is considered the most potent oxidant in biological systems (Yu, 1994). Because of its high reactivity and hence short half-life, it reacts very close to its site of formation and can cause oxidative damage by reacting with adjacent lipids, proteins or nucleic acids. The majority of $OH^{\bullet}$ produced *in vivo* comes from the metal-catalyzed breakdown of H_2O_2 (e.g., iron (Fe) or copper (Cu)). Evidence suggests that an excess of $O_2^{\bullet-}$ under conditions of stress may trigger the release of unbound Fe^{2+} from Fe-containing molecules. The "free" Fe^{2+} may then react with H_2O_2 and generate $OH^{\bullet}$ in the Fenton reaction (Equation 1). $OH^{\bullet}$ was previously suggested to be generated by the reaction of $O_2^{\bullet-}$ and H_2O_2 in the Haber-Weiss reaction (Equation 3) (Valko *et al.*, 2006), but the reaction was recognized to be thermodynamically unfavourable and the use of transition metals to serve as catalysts was suggested. The iron-catalyzed Haber-Weiss reaction which makes use of Fenton chemistry is considered to be the main mechanism by which $OH^{\bullet}$ is generated in biological systems (Kehrer, 2000).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$ (1)

(Fenton reaction)

$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$ (2)

Net reaction:

$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$ (3)

(Haber-Weiss reaction)

Additional radicals derived from oxygen that can be formed in biological systems include the peroxyl radical ($ROO^{\bullet}$), alkoxyl radical ($RO^{\bullet}$) and hydroperoxyl radical ($HO_2^{\bullet}$). Non-radical derivatives also derived from oxygen include hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1O_2$). The hydroperoxyl radical ($HO_2^{\bullet}$) is the conjugate acid of $O_2^{\bullet-}$ and well known for its involvement in lipid peroxidation in human disease and toxicology (Valko *et al.*, 2006).

Reactive nitrogen species (RNS) contain an atom of nitrogen in their molecules and are also biologically important reactive substances that are derived from nitric oxide ($NO^{\bullet}$) (Pourova *et al.*, 2010). $NO^{\bullet}$ is an abundant reactive radical that is produced by nitric oxide synthase (NOS) during metabolism of arginine to citrulline in biological tissues. It is important as a major signalling molecule in neurons and the immune system (Pourova *et al.*, 2010; Valko *et al.*, 2006). However, the toxicity of $NO^{\bullet}$ is linked to its ability to react with $O_2^{\bullet-}$ to form the toxic anion peroxynitrite ($ONOO^-$). $ONOO^-$ can cause DNA fragmentation, lipid oxidation and can alter protein function by forming protein adducts through nitrosylation reactions (Valko *et al.*, 2006).

1.3 Sources of ROS

ROS are produced from both exogenous and endogenous sources. Exogenous sources include microbes, environmental carcinogens, dietary factors, various xenobiotics, metal ions, ultraviolet

light and ionizing radiation (Lim *et al.*, 2010; Valko *et al.*, 2006). Endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, NADPH oxidases and xanthine oxidases (Perez-Matute *et al.*, 2009). An overview of inflammation as an exogenous source of ROS is provided below, followed by a description of the endogenous sources of ROS.

1.3.1 Inflammation

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection, toxic compounds and tissue injury. A controlled inflammatory response is generally regarded as being safe, as it provides protection against infection. But it can also be damaging if imbalance occurs (Medzhitov, 2008). The inflammatory response in general consists of four components: 1) The inflammatory inducers (e.g. microbes, toxic compounds); 2) The sensors that detect and kill them by releasing ROS (e.g. macrophages); 3) The inflammatory mediators induced by the sensors (e.g. cytokines—tumour necrosis factor-α (TNF-α), interleukin-1 (IL) or IL-6); 4) The target tissues (e.g. liver) that are affected by the inflammatory mediators. An excessive inflammatory response is detrimental to tissue function, often resulting in tissue damage (Medzhitov, 2010).

A growing number of chronic inflammatory responses have been described where the initiating trigger and subsequent mechanisms involved are not clearly defined. These responses do not seem to be caused by the classic instigators of inflammation, infection and injury, but rather seem to be associated with the homeostatic imbalance and malfunctioning of the target tissue. These inflammatory conditions are of particular interest because they accompany many chronic diseases which are prevalent in the Western world, including obesity, type 2 diabetes and cancer (Medzhitov, 2008).

The endogenous sources of ROS are described below.

1.3.2 Mitochondrial ROS

The production of $O_2^{\bullet-}$ occurs mostly within the mitochondria of a cell. Mitochondria consume 90% of the body's oxygen (O_2) by oxidative phosphorylation for energy production and cellular respiration (Valko *et al.*, 2004). Oxidative phosphorylation uses controlled oxidation of NADH and $FADH_2$ to generate an energy potential for protons across the inner mitochondrial membrane, which is used to phosphorylate adenosine diphosphate (ADP). Oxygen occupies the final position in the electron transport chain. Along the electron transport chain, electrons derived from the oxidation of NADH or $FADH_2$ can "leak" and directly react with oxygen to produce $O_2^{\bullet-}$ (Ma, 2010; Valko *et al.*, 2006). Free radical production occurs primarily at complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase), with the latter being the major site of ROS production (Ma, 2010). About 1-3% of the oxygen molecules in the mitochondria are converted into $O_2^{\bullet-}$ (Valko *et al.*, 2004).

1.3.3 Cytochrome P450 enzymes

The induction of cytochrome P450 (P450) enzymes also results in the production of ROS, mainly $O_2^{\bullet-}$ and H_2O_2, following the breakdown or uncoupling of the P450 catalytic cycle (Zangar *et al.*, 2004). P450 enzymes are the terminal oxidases located within the membrane-bound microsomal monooxygenase system (MMO), which is localized in the endoplasmic reticulum of most animal tissues. The main role of P450 is the detoxification of a variety of exogenous and some endogenous compounds into less toxic products (Valko *et al.*, 2004; Zangar *et al.*, 2004). P450 utilizes oxygen for the oxidation of foreign compounds in the detoxification process, and performs hydroxylation reactions to remove or inactivate toxic compounds in the

body. During both reactions, electrons may be leaked onto oxygen molecules, resulting in the formation of $O_2^{\bullet-}$(Valko *et al.*, 2004). The oxygenation of foreign compounds in the MMO system plays an important role in drug hepatotoxicity, carcinogenesis and the pathogenesis of many diseases (Zangar *et al.*, 2004).

1.3.4 Peroxisomes

Peroxisomes are essential organelles that harbor more than 100 enzymes and play a key role in the production and utilization of ROS. Their main function is the β-oxidation of fatty acids, including long-chain, branched-chain and polyunsaturated fatty acids, dicarboxylic acids and a side chain of bile acid precursors (Antonenkov *et al.*, 2010). The peroxisomal β-oxidation enzyme system is similar to that of the mitochondria in that it degrades fatty acids by two carbon units (acetate) in each cycle. However, the peroxisomal respiratory chain, unlike that of the mitochondria, is not coupled to oxidative phosphorylation and therefore does not produce ATP. The O_2 consumed in peroxisomes is mostly converted to H_2O_2 rather than to $O_2^{\bullet-}$, as seen in the mitochondrial transport chain. Peroxisomes are also home to many antioxidant enzymes, including catalase (described in the next section), which provide protection against ROS through detoxification at the site of ROS formation. Diminished detoxification capacity of antioxidants results in oxidative stress, and can lead to protein, lipid and DNA damage (Singh, 1996).

1.3.5 NADPH oxidases

The family of NADPH oxidases (gene coded as NOX) is a group of plasma membrane-associated enzymes that have been implicated as a major source of ROS generation (Nauseef, 2008; Perez-Matute *et al.*, 2009). They share the capacity to transport electrons across the plasma membrane and generate $O_2^{\bullet-}$ and other downstream ROS. Phagocytic NADPH oxidase is one of

the most thoroughly studied of the family and is located on the cell membrane of polymorphonuclear cells, macrophages and endothelial cells (Perez-Matute *et al.*, 2009). When a phagocytic cell is exposed to foreign compounds such as microbes or cytokines (e.g. TNF-α, IL-1 or IL-6), the defense enzyme undergoes a series of reactions called the "respiratory burst" that enable the cell to provide oxidizing agents to destroy such compounds (Ma, 2010; Valko *et al.*, 2004; Valko *et al.*, 2006). Specifically, NADPH oxidase becomes activated and retrieves cytoplasmic NADPH to reduce cytochrome b_{558} which catalyzes the NADPH-dependent reduction of O_2 to $O_2^{\bullet-}$. Phagocytic myeloperoxidase then catalyzes the formation of hypochlorous acid, the strongest physiological oxidant and antimicrobial agent (Hampton *et al.*, 1998). Oxidative conditions activated by inflammation are therefore associated with an excessive stimulation of NADPH oxidase by cytokines and other factors, resulting in increased ROS production (Waris and Ahsan, 2006).

1.3.6 Xanthine oxidases

Xanthine oxidase (XO) is a highly versatile enzyme that is widely distributed among mammalian tissues (Valko *et al.*, 2006). XO is an important source of ROS under a variety of conditions, such as proteolysis, heating and ischemia (Schrader and Fahimi, 2006). It is a member of a group of enzymes known as molybdenum iron-sulfur flavin hydroxylases and is well known for its role in the terminal oxidation of purines. In the purine degradation pathway, XO catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid. O_2 is reduced in both steps of the reaction, forming $O_2^{\bullet-}$ in the first step and H_2O_2 in the second (Valko *et al.*, 2006). Under ischemic conditions, xanthine dehydrogenase is converted to XO by proteolysis and oxidation, leading to the formation of $O_2^{\bullet-}$ (Yu, 1994).

The major endogenous and exogenous sources of ROS have been summarized in Figure 1.2:

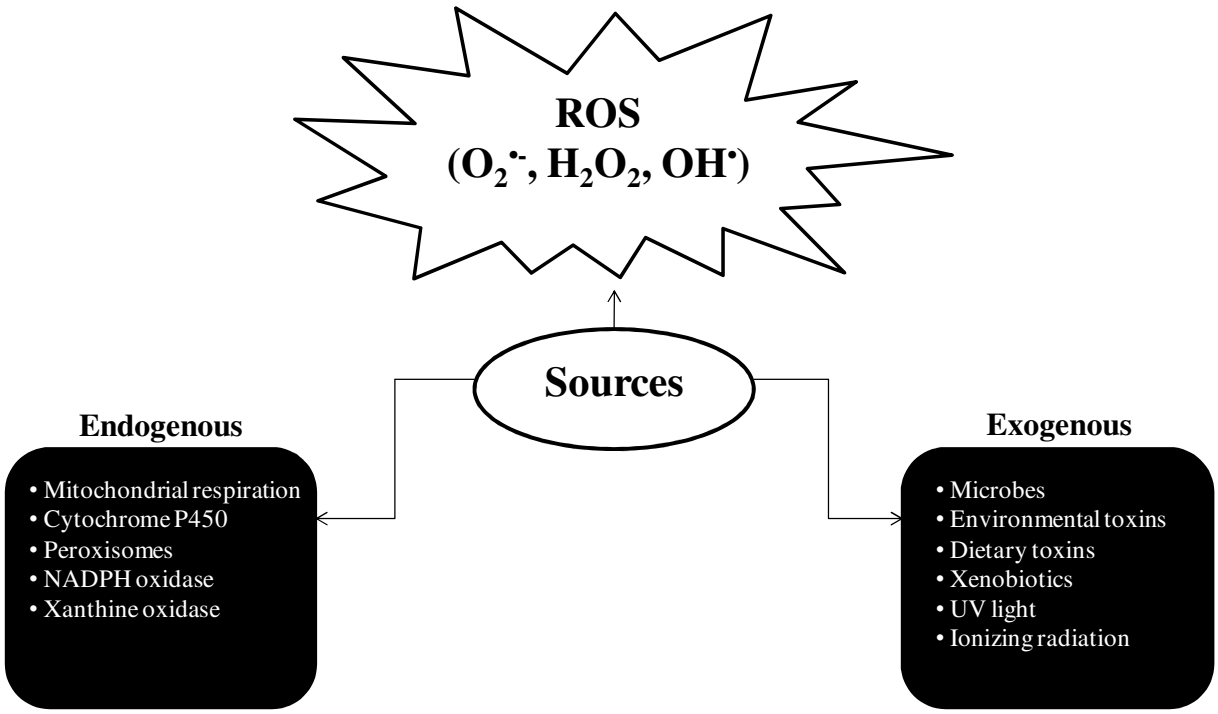

Figure 1.2 Endogenous and exogenous sources of ROS production. Superoxide anion, $O_2^{\bullet-}$; hydrogen peroxide, H_2O_2; hydroxyl radical, $OH^{\bullet}$.

1.4 Detoxification of ROS

Cells contain a variety of defense mechanisms against oxidative stress which include preventative, repair, physical and antioxidant (Valko *et al.*, 2007). The effect of ROS is specifically balanced by the intracellular action of enzymatic and non-enzymatic antioxidants which are strategically localized within cells to provide maximum protection. The main antioxidant defense enzymes responsible for protection against oxidative stress are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Non-enzymatic antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamins E), thiol antioxidants (glutathione (GSH), lipoic acid), uric acid, carotenoids and flavonoids (Yu, 1994). These antioxidants are

extremely important as they represent the direct removal of ROS thus providing maximal protection at biological sites (Valko *et al.*, 2006). The main non-enzymatic antioxidants are summarized in Table 1.

1.4.1 Superoxide dismutase (SOD)

SOD is one of the most effective intracellular enzymatic antioxidants that converts $O_2^{\bullet-}$ to H_2O_2 as shown in Equation 4. Three forms of SOD enzymes exist in humans: cytosolic copper-zinc-SOD (Cu, Zn-SOD), mitochondrial manganese-SOD (Mn-SOD) and extracellular SOD (EC-SOD), which also contains a Cu-Zn group (Valko *et al.*, 2006; Yu, 1994). In rat hepatocytes, ~70% of SOD is found in the cytoplasm (Yu, 1994).

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \qquad (4)$$

1.4.2 Catalase

Catalase is located primarily in the peroxisomes and catalyzes the conversion of H_2O_2 to water as shown in Equation 5. Catalase has one of the highest turnover rates amongst the enzymes: One molecule of catalase can complete the reaction below up to 6 million times in one minute (Valko *et al.*, 2006).

$$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2 \qquad (5)$$

1.4.3 Glutathione peroxidase (GPx)

Glutathione (GSH) metabolism is one of the most essential antioxidant defense mechanisms in cells. There are two forms of GPx: Selenium-independent (glutathione-*S*-transferase) and selenium-dependent (GPx). GPx acts in conjunction with GSH, which is found

in high concentrations within cells. GPx catalyzes the reduction of H_2O_2 and organic hydroperoxides while simultaneously oxidizing GSH as shown in Equations 6-7 (Valko *et al.*, 2006).

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG \text{ (glutathione disulfide)} + 2H_2O \quad (6)$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + H_2O + ROH \quad (7)$$

Table 1.1 Major non-enzymatic antioxidant components

Non-enzymatic antioxidants	Structure	Tissue site	Actions
Vitamin C	Water-soluble vitamin	Wide distribution in intracellular and extracellular fluids	• Scavenges $O_2^{\bullet-}$ and $OH^{\bullet}$ • Neutralizes oxidants from stimulated neutrophils • Contributes to regeneration of Vitamin E
GSH	Tripeptide	Largely intracellular	• Substrate in GSH redox cycle • Reacts with $O_2^{\bullet-}$, $OH^{\bullet}$ and organic free radicals
α-Lipoic acid	Water- and fat-soluble disulphide derivative	Distributed in cell membranes and cytosol	• Scavenges ROS • Regenerates antioxidants involving Vitamins E, C and GSH • Chelates transition metals (Fe and Cu) • Repairs oxidized proteins
Cysteine	Amino acid	Wide distribution	• Reduces various organic compounds by donating electrons from sulfhydyl groups
Uric acid	Oxidized purine base	Wide distribution	• Scavenges $O_2^{\bullet-}$, $OH^{\bullet}$ and peroxyl radicals • Prevents oxidation of Vitamin C • Binds transition metals
Vitamin E	Fat-soluble vitamin	Lipid membranes, extracellular fluids	• Converts $O_2^{\bullet-}$, $OH^{\bullet}$ and lipid peroxyl radicals to less reactive forms • Breaks lipid peroxidation chain reactions
β-carotene	Metabolic precursor to Vitamin A	Membranes of tissues	• Scavenges $O_2^{\bullet-}$ and reacts directly with peroxyl radicals
Flavonoid	Polyphenolic compound	Wide distribution	• Scavenges free radicals and lipid peroxyl radicals (donates electron) • Chelates transition metals (Fe and Cu)

Glutathione, GSH; superoxide anion, $O_2^{\bullet-}$; hydroxyl radical, $OH^{\bullet}$ [Adapted from (Valko *et al.*, 2006; Yu, 1994)].

1.5 Cellular damage by ROS

An imbalance between ROS and antioxidants resulting from the increased production of ROS and/or the reduction in the amount of antioxidants generates a state of oxidative stress in the cell (Ma, 2010). As mentioned earlier, oxidative stress is capable of causing damage to various cell constituents, such as lipids, proteins and DNA, leading to aging (Squier, 2001), non-alcoholic fatty liver disease (NAFLD) (Lewis and Mohanty, 2010), carcinogenesis (Kawanishi *et al.*, 2001) and many other diseases. This next section describes the intracellular targets of oxidative damage.

1.5.1 Lipid peroxidation

Lipids have a critical structural and functional role in membranes. Any disruption of this role can lead to oxidative damage. Since free radicals are usually generated near membranes, lipid peroxidation (LPO) is often the first reaction to occur (Toyokuni, 1998) and consists of three steps as shown in Figure 1.3: initiation, propagation and termination (Goetz and Luch, 2008). The double bonds found in polyunsaturated fatty acids (PUFAs) are the targets for free radical attack (1). The free-radical mediated abstraction of a hydrogen atom from one of the double bonds yields a carbon-centered lipid radical species (2) that can readily interact with O_2. The resulting lipid peroxyl radical (3) can abstract a hydrogen atom from another fatty acid yielding another radical and a lipid hydroperoxide (4) thereby establishing a chain reaction (Kehrer, 2000). LPO can be terminated by chain-breaking antioxidants, such as Vitamin E (Mattson, 2009).

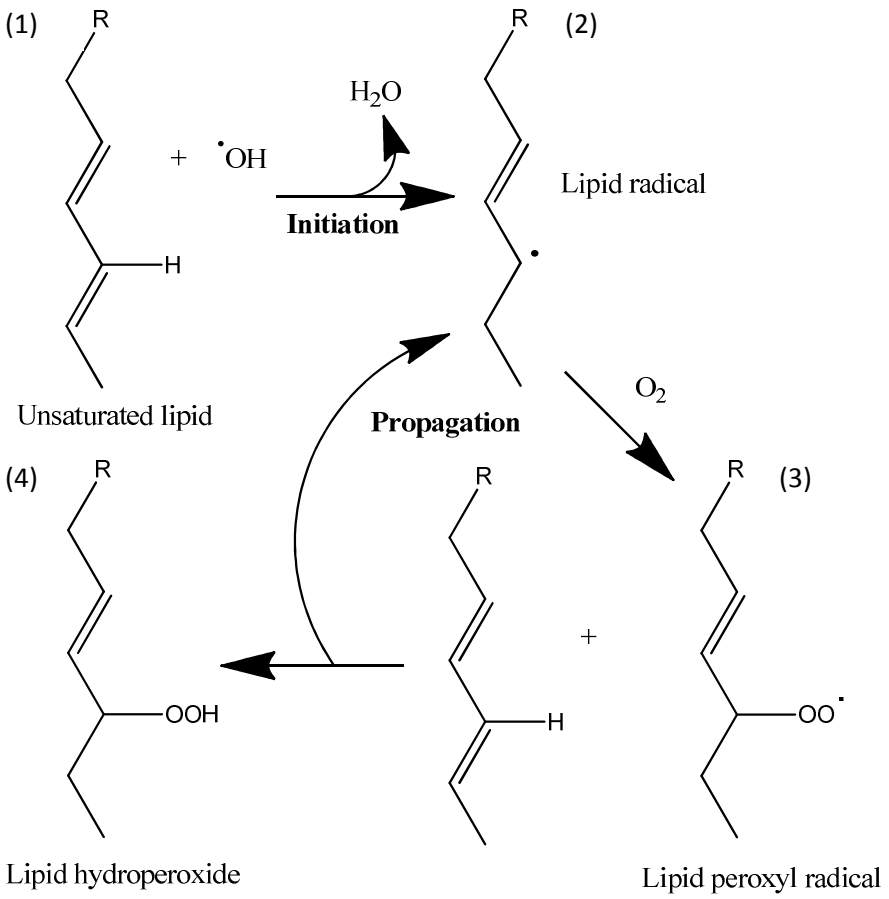

Figure 1.3 The process of lipid peroxidation. See text for details. Adapted from (Clark, 2008).

By-products of LPO include reactive carbonyl species (RCS), such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), glyoxal and methylglyoxal (Negre-Salvayre *et al.*, 2008). The RCS can react with proteins (Negre-Salvayre *et al.*, 2008) and DNA (Nair *et al.*, 2007), resulting in further oxidative damage. Ionic channels may also become affected during LPO and the lipid bilayer itself may become more permeable thereby disrupting ion homeostasis (Kehrer, 2000).

1.5.2 Protein carbonylation/Protein oxidation

Under oxidative stress, proteins may be modified either indirectly by RCS formed by the autoxidation of lipids or carboyhydrates or directly by ROS with the eventual formation of oxidized amino acids.

Reaction of proteins with LPO-derived RCS, known as protein carbonylation, results in the formation of adducts known as advanced lipoxidation end products (ALEs) which induce protein damage, thereby modifying cellular responses (Negre-Salvayre *et al.*, 2008). As shown in Figure 1.4, RCS such as HNE can react by Michael addition reactions with amino groups of proteins eventually resulting in the formation of specific ring structures (Toyokuni, 1998). RCS may also react with protein amino groups to form reversible Schiff base adducts, which can be converted to covalently bound Amadori products. Amadori products can undergo multiple dehydration and rearrangements to form irreversible ALEs (O'Brien *et al.*, 2005). Protein carbonyls are elevated in many pathological diseases including diabetes, NAFLD, cancer, Alzheimer's and Parkinson's disease. Protein carbonylation is accelerated during diabetes as a result of the increased formation of RCS through glycation reactions arising from increased levels of carbohydrates, such as fructose and glucose. The accumulation of protein-glycation products results in the formation of advanced glycation end products (AGEs) which like ALEs, cause irreversible oxidative damage to proteins (O'Brien *et al.*, 2005).

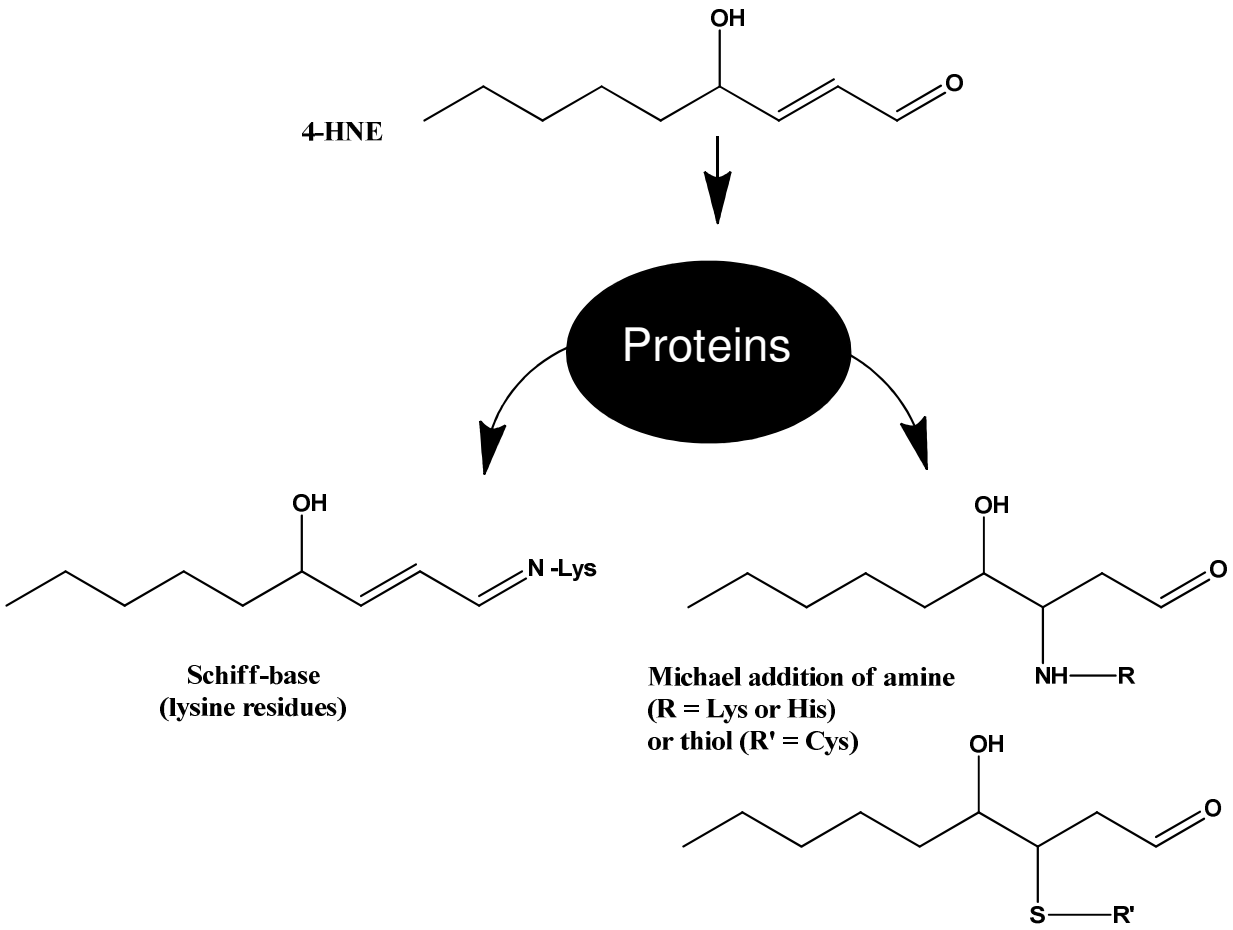

Figure 1.4 Schematic reaction of 4-HNE with proteins leading to the formation of 4-HNE-protein adducts. Schiff base formation on primary amine can lead to the formation of more complex cross-linked compounds. Similarly, Michael addition of 4-HNE on amino groups can lead to cyclization and hemi-acetal or hemi-thioacetal formation. Modified from (Negre-Salvayre *et al.*, 2008).

Protein amino acids, such as cysteine, lysine, arginine and histidine, may also be oxidized directly by ROS ($OH^{\bullet}$) to form many different kinds of inter- and intra-protein cross-linkages (Goetz and Luch, 2008; O'Brien *et al.*, 2005; Valko *et al.*, 2006). Examples include: (i) the addition of lysine side chains to the carbonyl group of an oxidized protein; (ii) the interaction of two carbon-centered radicals obtained from the abstraction of hydrogens from the polypeptide backbone ($OH^{\bullet}$ -mediated); (iii) the oxidation of cysteine sulphydryl groups to form disulphide crosslinks (-S-S-); (iv) the oxidation of tyrosine to form –tyr-tyr- crosslinks (Valko *et al.*, 2006).

The carbonyl content of proteins is therefore an index of the amount of oxidative protein damage attributable to either direct attack of free radicals or the modification of proteins by oxidation products of PUFAs or carbohydrates. Oxidized proteins that are functionally and structurally modified may be degraded or may be prone to reversible reaction, but some can gradually accumulate with time and thereby contribute to the oxidative damage associated with aging and various metabolic and neurodegenerative diseases (Kehrer, 2000; Valko *et al.*, 2006).

1.5.3 DNA oxidation

RCS-DNA interaction can damage DNA by directly oxidizing DNA bases or by forming exocyclic-adducts with DNA bases that induce base-pair substitution mutations (Nair *et al.*, 2007). Reaction of MDA with DNA bases G, A and C results in the formation of mutagenic MDA-deoxyguanosine adducts (M_1G-, M_1A, M_1C-deoxyribose, respectively) (Toyokuni, 1998; Valko *et al.*, 2006). Other promutagenic adducts formed by reaction of LPO products with DNA include etheno- and propano-adducts (Goetz and Luch, 2008; Valko *et al.*, 2006). These adducts including those formed by MDA-DNA reaction, are considerably influenced by diet and lifestyle factors (Goetz and Luch, 2008).

DNA damage by $OH^{\bullet}$ results in single- or double-strand breaks, base modifications and DNA-DNA or DNA-protein cross-links (Toyokuni, 1998). The $OH^{\bullet}$ is known to react with all components of the DNA molecule, damaging the deoxyribose backbone and both purine and pyrimidine bases (Valko *et al.*, 2006). 8-Hydroxy 2'-deoxyguanosine (8-HO-dG) (Figure 1.5) represents the most extensively studied oxidative DNA modification because it is easily formed due to the sensitivity of guanine to oxidation and it is both mutagenic and carcinogenic (Goetz and Luch, 2008; Valko *et al.*, 2006). It is a good biomarker for DNA-oxidative injury and a

potential biomarker of carcinogenesis (Valko *et al.*, 2006). Although DNA oxidation results in impaired DNA function, numerous enzymes exist which may repair or remove damaged DNA. However, when DNA damage occurs at critical sites or when repair processes are interrupted by $OH^{\bullet}$, oxidized purines or pyrimidines can cause functional problems, leading to cell death (Kehrer, 2000). Despite both being susceptible to oxidative damage, mitochondrial DNA may be more prone to oxidative stress than nuclear DNA due to (i) its proximity to the major source of cellular ROS formation and (ii) its limited DNA repair capacity (Ma, 2010).

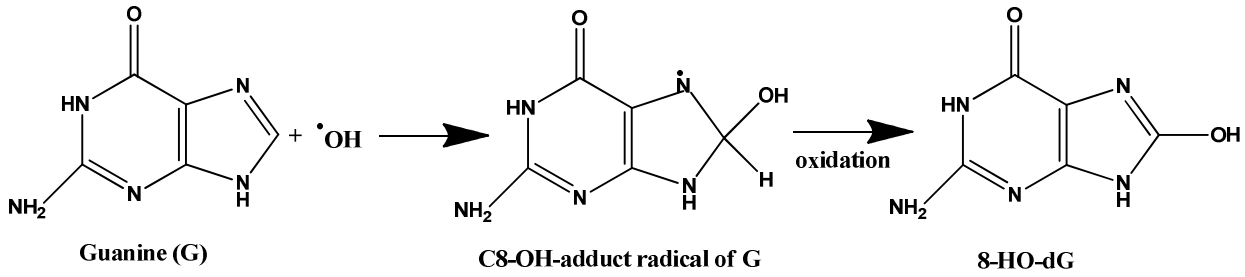

Figure 1.5 Reaction of guanine with hydroxyl radical. The hydroxyl radical ($OH^{\bullet}$) reacts with the double bond of guanine through an addition reaction. Modified from (Valko *et al.*, 2006).

ROS- and RCS-derived protein and DNA adducts can be used as biomarkers to measure oxidative stress and predict targets for prevention (Chapters 3 and 4). The next section discusses the formation, detoxification and toxicity of two Western diet-derived endogenous toxins: glyoxal (RCS) and iron (biological ROS) that are implicated in chronic metabolic disease.

1.6 Glyoxal and endogenous sources

Glyoxal (Figure 1.6) is a reactive dicarbonyl (also known as α-oxoaldehyde) that can be formed endogenously by numerous non-enzymatic sources, including lipid peroxidation

(previously discussed), degradation of glycolytic intermediates, glycated proteins and DNA oxidation.

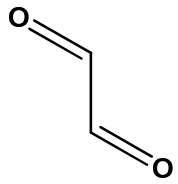

Figure 1.6 Structure of glyoxal

Under physiological conditions, the dicarbonyl glyoxal is formed through the autoxidation of glycolytic intermediates, such as glucose, fructose and glycolaldehyde, and is also formed by the metal-catalyzed autoxidation of ascorbate. Glyoxal is also a major DNA oxidative degradation product that is formed from the ROS-mediated oxidation of deoxyribose, resulting in a strand break. The glyoxal released is capable of forming adducts with neighbouring DNA guanines (O'Brien *et al.*, 2005). The endogenous formation of glyoxal is of added concern because of its propensity to react with proteins to form AGEs or ALEs. Elevated levels of AGEs and ALEs are associated with aging and the long-term complications of diabetes, NASH and atherosclerosis (Giacco, 2010; Thornalley, 2007).

1.6.1 Protein glycation and AGE formation

Protein glycation constitutes the non-enzymatic reaction of reducing sugars (e.g. fructose, glucose) or dicarbonyls (e.g. glyoxal) with protein amino groups to form covalent bonds in what is known as the Maillard reaction (Shangari *et al.*, 2003). The Maillard reaction is initiated by the condensation of a protein amino group (e.g. arginine) with the carbonyl group of a reduced sugar or dicarbonyl (Shangari *et al.*, 2003). The electron-withdrawing effects of the carbonyl group render the carbon-oxygen group electrophilic and favour the Michael addition reaction and/or

nucleophilic reactions with protein amino groups (O'Brien *et al.*, 2005). The resulting Schiff base undergoes rearrangement to form stable ketoamines known as Amadori products. The Amadori products are rapidly subjected to dehydration, cyclization, oxidation and further rearrangement to form AGEs (Shangari *et al.*, 2003) that cause protein cross-linking and tissue deterioration (O'Brien *et al.*, 2005). Once formed, AGEs cannot be removed from proteins unless the protein is digested (O'Brien *et al.*, 2005). Glyoxal is not only a potent glycating agent of proteins, but also of DNA. Glyoxal glycates arginine groups of proteins forming mainly hydroimidazolone AGE derivatives, (Thornalley, 2007) and reaction of glyoxal with lysine groups results in the formation of the AGE adduct *N*-(carboxymethyl)lysine (Shangari *et al.*, 2003). With DNA, glyoxal reacts with deoxyguanosine to form imidazopurinone and *N2*-carboxyalkyl AGE derivatives. Glycation reactions involving glyoxal are implicated in diabetes, liver disease, Alzheimer's disease, arthritis and aging (Thornalley, 2007). The reducing sugars fructose and glucose can participate in the Maillard reaction and may become autoxidized to glyoxal in the Amadori stage or Heyns stage (in the case of fructose), eventually forming AGE derivatives as shown in Figure 1.7 (Schalkwijk *et al.*, 2004; Suarez *et al.*, 1989).

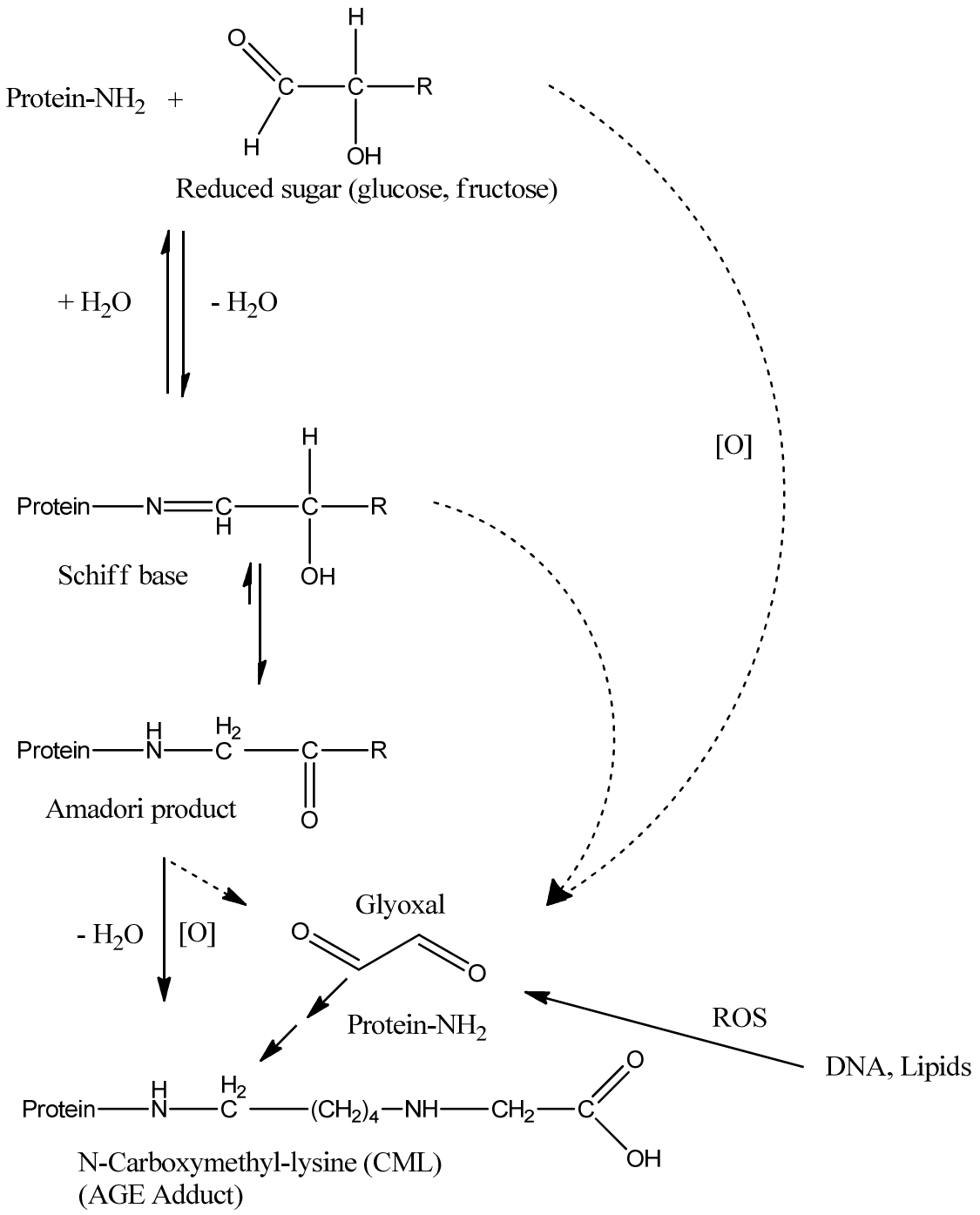

Figure 1.7 Formation of endogenous glyoxal from glycated proteins. The first step in the Maillard reaction (reaction between reducing sugars and amino groups of proteins, referred to as glycation) results in the formation of reversible Schiff base adducts. The next step involves the mostly irreversible conversion to covalently bound Amadori rearrangement products. These Amadori products can be rearranged to form AGEs through glycoxidation reactions accelerated by oxygen and transition metals. In addition, reducing sugars can be autoxidzed to glyoxal, while Schiff base and Amadori products can undergo multiple dehydrations and rearrangements, resulting in the degradation of proteins to release glyoxal or other RCS. Glyoxal can then react

with other protein amine groups, resulting in the formation of AGE adducts (e.g. CML). The metal-catalyzed oxidation of lipids and DNA also results in the formation of glyoxal. Modified from (O'Brien *et al.*, 2005).

1.6.2 Glyoxal metabolism and metabolizing enzymes

Glyoxal accumulation is of concern because it is a reactive electrophile that can form adducts with cellular protein amines or thiols and can therefore cause toxicity. As previously described, glyoxal can participate in glycation reactions causing subsequent AGE formation. Glyoxal can also participate in lipoxidation reactions when it is generated as a product of LPO, resulting in the formation of ALEs. The protein adducts may be reversible in the early stages, but upon degradation to AGEs and ALEs, they can covalently cross-link proteins that accumulate with aging and chronic metabolic disease and increasingly disrupt protein and cellular function (O'Brien *et al.*, 2005).

The ability of cells to metabolize and detoxify glyoxal depends on the cellular content of glyoxal-metabolizing enzymes. The metabolizing enzyme groups responsible for detoxifying glyoxal (and methylglyoxal) are the glyoxalase pathway (glyoxalase I, II and GSH) and the aldo-keto reductases which are required in order to maintain the glycation balance under physiological conditions. Glyoxal is primarily metabolized by forming a conjugate with GSH, which is then isomerized by glyoxalase I (cytosol) and II (mitochondria). The glyoxalase pathway, however, is inhibited by GSH oxidation/depletion, which occurs during conditions of oxidative stress and results in the accumulation of glyoxal and/or methylglyoxal. The reaction of glyoxal with protein amino groups can also cause the inactivation of various enzymes containing target amino groups (e.g. arginine), such as GSH reductase, GSH peroxidase, glyceraldehyde-3 phosphate

dehydrogenase (GAPDH) and other glycolytic enzymes, resulting in an increase of tissue glyoxals (O'Brien *et al.*, 2005).

1.6.3 Dietary factors resulting in the formation of glyoxal

Of particular importance to this thesis and the focus of this section is the formation of the endogenous toxin glyoxal as a result of the increased dietary intake of the reducing sugar fructose in the Western diet. Increased dietary fructose consumption has been linked to obesity, diabetes and NAFLD (Gaby, 2005) presumably as a result of its oxidative degradation to glyoxal (Feng *et al.*, 2009; Lee *et al.*, 2009; Manini *et al.*, 2006).

Dietary intake of fructose, often resulting from high-fructose corn syrup (HFCS), has grown dramatically in the Western world over the last three decades. The impact of fructose was insignificant several years ago when the main source was from fruits and vegetables. During these times, dietary fructose consumption averaged a mere 15 g/day and was accompanied by other nutrients embedded within the whole foods. Overconsumption of fructose is now a result of the extensive use of HFCS in a wide variety of packaged products, such as soft drinks, juices, breads, soups, baked goods, canned fruits and condiments such as jam, jellies, ketchup, dressings and marinades (Gaby, 2005).

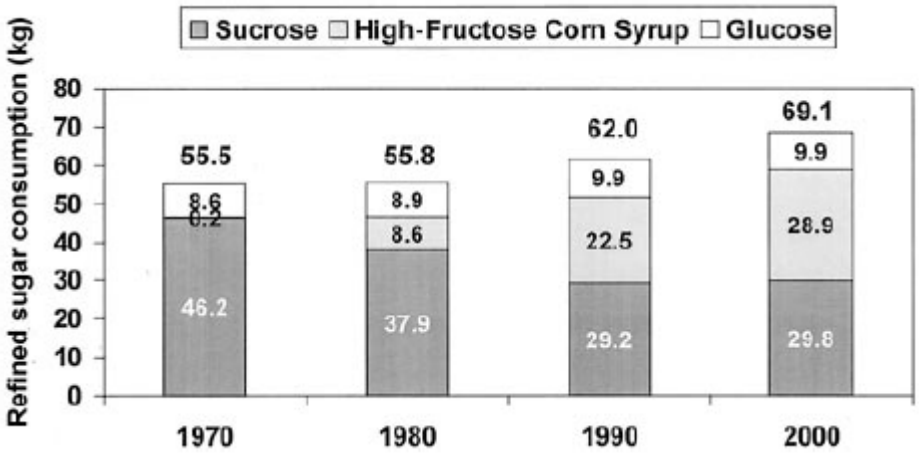

Figure 1.8. Consumption of refined sugars (kg) in the USA per capita from 1970-2000. High-fructose corn syrup (HFCS) intake increased significantly over the last 30 years, and continues to rise and replace other sweeteners as a cost-effective alternative in pre-packaged products [Adapted from (Cordain *et al.*, 2005)]. The first box (top of the column) represents glucose, followed by HFCS and lastly, sucrose (bottom of column).

Moderate fructose consumption was previously regarded as a relatively safe form of sugar for diabetics because it does not adversely affect the glycemic index in the short-term (Gaby, 2005). However, the overconsumption of fructose, in contrast to glucose, has been associated with deleterious effects that directly affect human health, such as hepatic lipogenesis, contributing to obesity and fatty liver (Samuel, 2011). Increased fructose consumption results in elevated tissue concentrations of fructose and its highly reactive metabolites, which can in turn potentiate the Maillard reaction and generate AGEs at a much faster rate than glucose (Gaby, 2005; Schalkwijk *et al.*, 2004). Despite this fact, the link between fructose and its downstream effects are still not completely understood.

Recent evidence by our group suggests that Fenton-mediated oxidation of fructose or other intermediates of the glycolytic pathway yields carbonyl metabolites including glyoxal (Feng *et al.*, 2009). An illustration is provided in Figure 1.9 which summarizes the ROS-

mediated oxidation of fructose or fructose metabolites. Manini et al. also found that fructose or its metabolites glyceraldehyde and dihydroxyacetone underwent autoxidation to glyoxal in 15 minutes using a cell free Fenton-type system (Manini *et al.*, 2006). Furthermore, fructose may also cause ROS formation by fructose-induced activation of NADPH oxidase or inactivation of antioxidant enzymes catalase and SOD (Schalkwijk *et al.*, 2004), which may in turn oxidize fructose metabolites to glyoxal. Fructose or fructose metabolite toxicity therefore requires Fenton-mediated oxidative stress or an inflammatory response before cytotoxicity can occur.

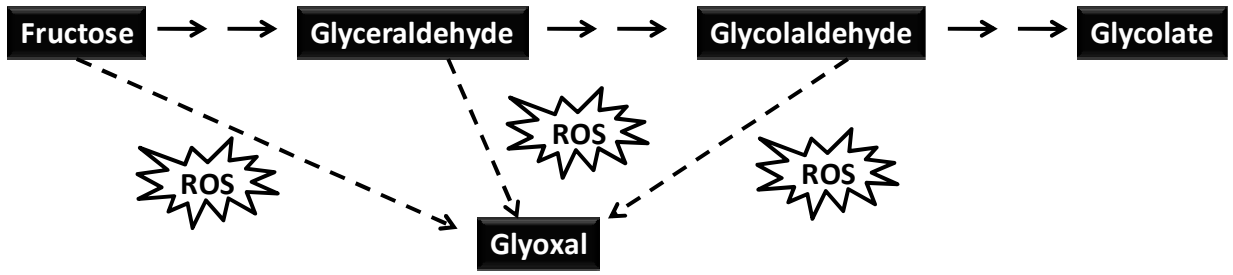

Figure 1.9 ROS-mediated oxidation of fructose or fructose metabolites to glyoxal. Fructose metabolism (in the fructose-glycolate pathway) bypasses the regulatory enzymatic pathway and enters glycolysis unregulated via glyceraldehyde and dihydroxyacetone phosphate (not shown). Glyceraldehyde can undergo various enzymatic oxidation and reduction steps to form glycolaldehyde (the most genotoxic fructose metabolite formed from glycolysis) and finally glycolate. Fructose and fructose metabolites—glyceraldehyde and glycolaldehyde have been suggested to form glyoxal via Fenton-mediated oxidation or inflammatory processes (Feng *et al.*, 2009; Lee *et al.*, 2009).

As previously described, the generation of reactive $OH^{\bullet}$ is catalyzed by transition metals, such as Fe, which can be released from Fe-containing molecules under conditions of stress and become free to react with H_2O_2 in the Fenton reaction (Kehrer, 2000). However, an increased concentration of free Fe may also become available from the diet and is of particular concern in the pathogenesis of certain cancers, including colorectal (CRC) (Corpet *et al.*, 2010). The next section therefore describes the formation of endogenous ROS from dietary intake of Fe.

1.7 Iron overload and health implications

Fe is a vital mineral for all living cells and organisms. Dietary Fe is necessary to build the body's oxygen carriers (blood haemoglobin and muscle myoglobin) and important enzymes such as those within the electron transport chain that make ATP, carriers such as cytochrome *c* and enzymes such as catalase and xanthine oxidase. Fe deficiency resulting in anemia remains a widespread public health risk, notably in premenopausal women and in developing countries. However, Fe overload from the Western diet also poses a health hazard and increases the risk of several chronic diseases that are prevalent in developed countries, including liver disease and CRC. Excess Fe accumulates in tissues and organs and disrupts their normal function through the process of oxidative stress toxicity, resulting from either long-term excess intake of red meat, hereditary hemochromatosis, or multiple blood transfusions. The pro-oxidant effects of Fe may be attributed to its ability to catalyze the oxidation of ROS ($OH^{\bullet}$, $O_2^{\bullet -}$), proteins, lipids, sugars, nucleic acids, AGEs and ALEs (Corpet *et al.*, 2010).

Individuals without any evidence of Fe overload disorder might have excess Fe body stores that are associated with the Western diet (Corpet *et al.*, 2010)**.** Red and processed meat consumption have been associated with an increased risk of CRC, heart disease, rheumatoid arthritis, type 2 diabetes and Alzheimer's disease (Tappel, 2007). The health risk of Fe in red meat, and not plant-based Fe-rich foods, results from evidence suggesting that heme Fe from meat is much better absorbed than non-heme Fe present in plant foods (WCRF, 2007). This concept explains why the increased intake of Fe from plant sources in the Eastern diet is not associated with a higher likelihood of chronic disease. Heme Fe is also 10-100 times more effective than inorganic Fe at oxidizing diet-derived lipid hydroperoxides. Hemoproteins found in red meat are thus potent inducers of LPO (Corpet *et al.*, 2010). Dietary Fe may therefore

contribute to chronic disease risk through the production of ROS. The toxic effects of Fe-catalyzed oxidative stress on proteins, lipids and DNA were described earlier in this introduction.

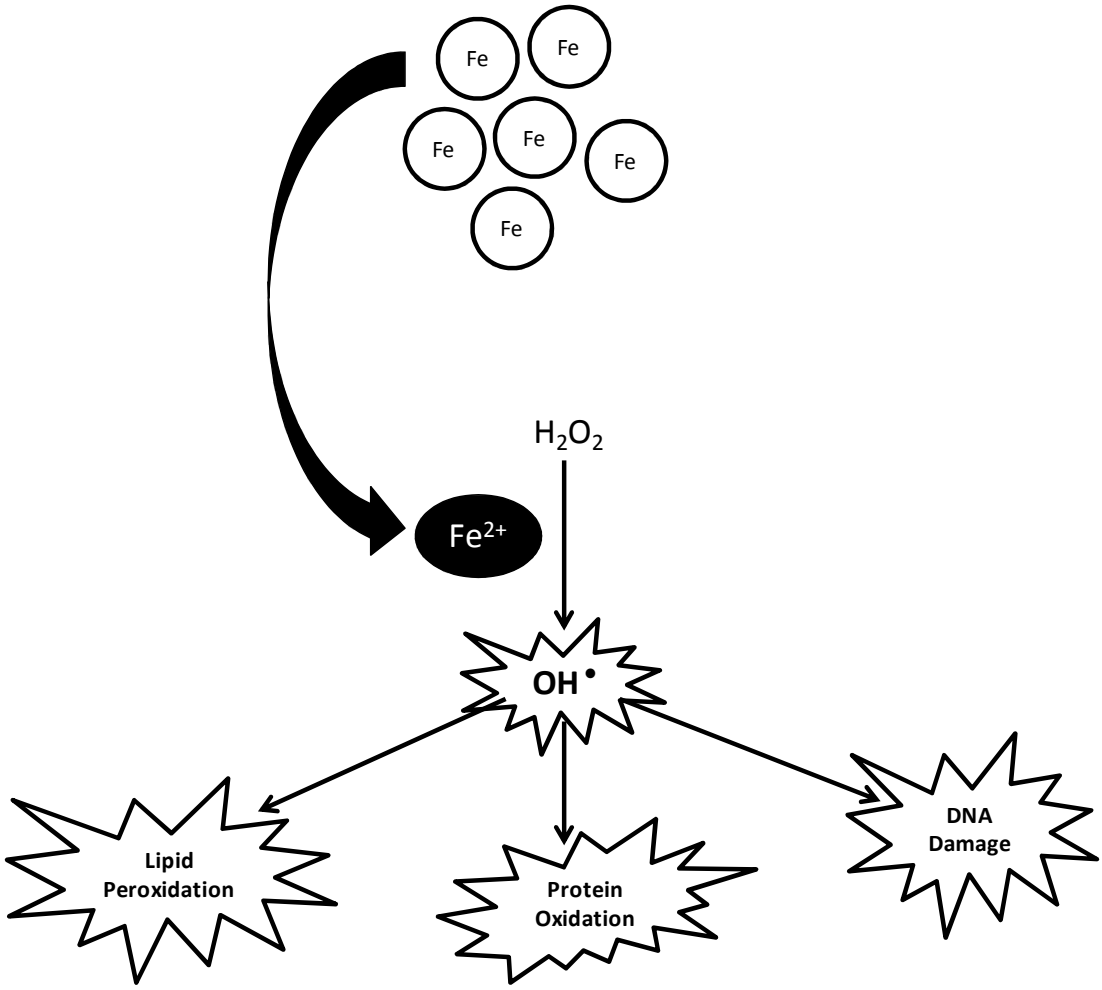

Figure 1.10 Formation of deleterious hydroxyl radicals via dietary intake of iron. This figure illustrates the delivery of ferrous iron from excess consumption of red meat (heme iron) in the Western diet. Iron can participate in the Fenton reaction, generating hydroxyl radicals which can damage lipids, proteins or DNA.

1.8 Micronutrients for the intervention of oxidative stress-induced chronic diseases

A broad range of nutritional interventions have the capacity to mitigate oxidative stress that has arisen as either a primary or secondary outcome. A number of micronutrients (vitamins,

minerals, plant-derived antioxidants) have been investigated for their ability to prevent the cytotoxic effects of endogenous toxins in *in vitro* and *in vivo* models of oxidative stress. Such micronutrients include vitamins A, C, E, lipoic acid, calcium, phenolic compounds such as polyphenols (found in green tea, curcumin, resveratrol) and flavanoids (genistein from soy) and isothiocyanates in cruciferous vegetables and garlic (Khor *et al.*, 2010). The study of dietary patterns in relation to chronic disease has also been investigated in cohort and population-based case-control epidemiological studies (Miller *et al.*, 2010). Although research is being done, insufficient evidence exists and long-term implications remain unresolved. Continued research efforts are therefore needed to evaluate the cumulative and interactive effects of numerous dietary exposures on chronic metabolic disease and cancer risk and to lend credibility to these potentially valuable naturally driven interactions.

The next section discusses the various roles played by vitamins B1 and B6 in the body followed by a review of their proposed protective mechanisms.

1.8.1 Thiamin (Vitamin B1)

Thiamin is active in the form of thiamin diphosphate (TPP). As a cofactor, TPP is essential to the activity of cytosolic transketolase (TK) and the mitochondrial dehydrogenases pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and branched-chain ketoacid dehydrogenase (BCKDH). Vitamin B1 was among the first vitamins discovered and was identified as the dietary factor that was missing in polished rice and responsible for the disease Beriberi. Since then, several additional conditions resulting from thiamin deficiency have been observed. Most recently, a link was established between thiamin deficiency and CRC (Bruce *et al.*, 2003).

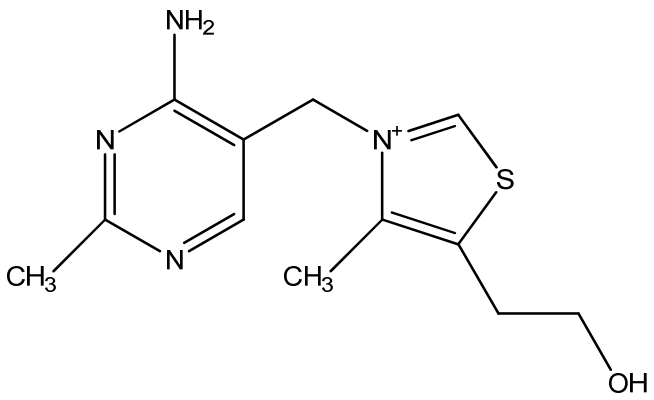

Figure 1.11 Chemical structure of thiamin

1.8.1.1 Thiamin delivery from the diet to the mitochondria

Thiamin (adult RDA of approximately 1 mg/day) is found in raw foods, such as cereals, green vegetables, nuts and egg yolk. Little is found in refined foods, such as sugar, as well as in foods heated for a long time or at high temperatures. Many processed products (e.g. flour, breads, cereals) have been fortified with thiamin since the early 1940s. Thiamin uptake into cells is enhanced by thiamin deficiency and is decreased by diabetes, ethanol and age. Chronic alcohol consumption is the most common cause of acute thiamin deficiency in affluent societies (Ball, 2004; Machlin *et al.*, 1991).

Vitamin B1 is present in the body as thiamin or its phosphorylated forms, thiamin monophosphate (TMP) or TPP. The total thiamin in the body is only about 30 mg (Ariaey-Nejad *et al.*, 1970), with 40% in the muscle and stores also in the brain, heart, liver and kidney. Thiamin absorption from the diet takes place primarily in the proximal small intestine and at low concentrations, requires saturable high affinity, low capacity transporters. TMP is taken up by cells via a thiamin transporter protein and once inside, thiamin is rapidly phosphorylated to TPP. The reaction is catalyzed by thiamin diphosphokinase located in the cytosol. TPP is rapidly taken

up by mitochondria by the TPP/thiamin antiporter system (Barile *et al.*, 1990; Song and Singleton, 2002). In the mitochondrial matrix, where TPP is mostly bound to enzymes, the intracellular TPP concentration is estimated to be 30 μM with only 2 μM unbound (Bettendorff *et al.*, 1996). Mitochondrial TPP can undergo hydrolysis by thiamin pyrophosphatase to form TMP. TMP can efflux the mitochondria and can then be hydrolyzed by a cytosolic TMP phosphohydrolase to form thiamin (Barile *et al.*, 1990).

1.8.1.2 Essential role of thiamin in mitochondrial and cellular function

TPP is involved in dehydrogenase reactions as a cofactor in three mitochondrial enzyme complexes: pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase (α-KGDH) and branched-chain ketoacid dehydrogenase (BCKDH). These enzymes are central to mitochondrial energy production as they catalyze the oxidative decarboxylation of α-ketoacids to release CO_2. The ketoacids include pyruvate, the isocitrate metabolite, α-ketoglutarate and the branched-chain amino acid metabolites, ketoisocaproate, ketomethylvalerate and ketoisovalerate. These ketoacids are crucial for mitochondrial energy production through their role in the tricarboxylic (TCA) cycle: Pyruvate, the endproduct of glycolysis, forms acetyl-CoA, the entry point of the TCA cycle. The third step of the TCA cycle involves α-ketoglutarate metabolism to succinyl-CoA. As part of amino acid catabolism, α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate are transformed by BCKDH into isovaleryl-CoA, α-methylbutyryl-CoA and isobutyryl-CoA, respectively. Isovaleryl-CoA and isobutyryl-CoA can then form succinyl-CoA (Depeint *et al.*, 2006b). The three enzyme complexes have similar structures and mechanisms. Figure 1.12 shows the details of the five-step process of pyruvate dehydrogenase complex activity.

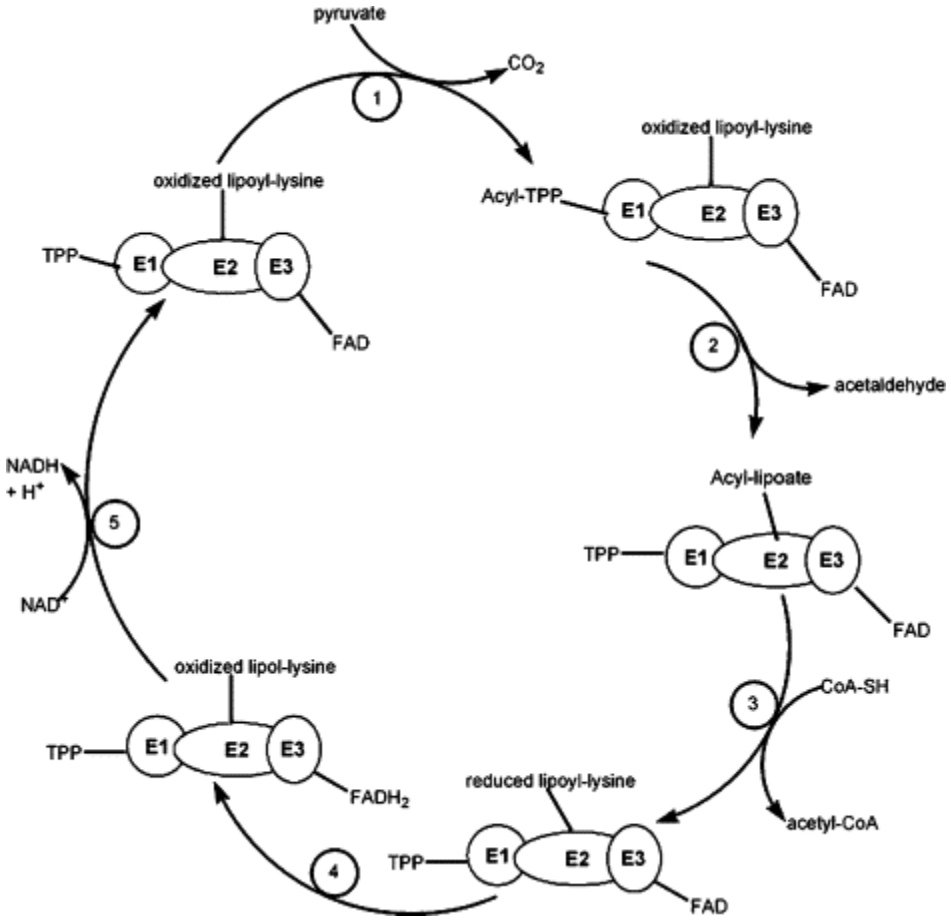

Figure 1.12 Dehydrogenase enzyme complex. The oxidative decarboxylation of pyruvate (in this example) is catalyzed by a multi-enzyme complex containing: (E1) pyruvate dehydrogenase, (E2) dihydrolipoyl transacetylase and (E3) dihydrolipoyl dehydrogenase. The cycle follows five steps: (1) decarboxylation of pyruvate is catalyzed by E1 (cofactor: TPP), (2) acetaldehyde reacts with the oxidized lipoyllysine, (3) acetaldehyde is transformed into acetyl-CoA by E2 (cofactor: lipoic acid), (4) FAD (cofactor) oxidizes lipoyllysine catalyzed by E3, (5) NAD^+ (cofactor) reduces $FADH_2$ back to FAD. It is important to note the role not only of TPP (E1), but also CoA (E2), FAD and NAD^+ (E3) for both catalytic activity and regeneration of the enzyme.

TPP is also a cofactor of TK, a reversible cytosolic enzyme that catalyzes the first and last step of the pentose phosphate pathway (PPP). The PPP plays a major role in cellular function in the production of NADPH for maintaining cellular redox, GSH levels and protein sulphydryl groups, as well as fatty acid synthesis. The PPP also supplies ribose for nucleic acid synthesis. In this enzymatic system, TPP serves as a cofactor that helps transfer a glycolaldehyde from a

ketose donor to an aldose acceptor. The thiazole ring of TPP does this by forming a complex with the xylulose phosphate substrate, releasing glyceraldehyde-3-phosphate and forming a thiamin/glycolaldehyde complex. The glycolaldehyde is then transferred to ribose phosphate to form sedoheptulose phosphate (Depeint *et al.*, 2006b).

1.8.1.3 Prevention of oxidative stress by thiamin

Thiamin demonstrates antioxidant activity. At 100 μM it prevents microsomal LPO. In the process, thiamin is oxidized to form thiochrome (by tricyclic thiamin formation) and thiamin disulfide (by opening of the thiazole ring). This antioxidant effect probably involves the successive transfer of protons from the pyrimidine NH_2 group and thiazole ring (Lukienko *et al.*, 2000).

Thiamin may also be important in preventing diabetes complications. Thiamin therapy has been shown to counter the development of streptozotocin-induced diabetes in rats (Babaei-Jadidi *et al.*, 2004) as well as complications, such as dyslipidemia, atherosclerosis or nephropathy in rodent models. TPP was found to be better than the anti-diabetic agent aminoguanidine at preventing the non-enzymatic glycation of proteins by glucose. The mechanism is unknown but is likely to involve the amine group of TPP forming Schiff bases with the carbonyls of open-chain sugars, dicarbonyl fragments, Amadori products or with post-Amadori intermediates, thus preventing AGE formation (Booth *et al.*, 1997).

1.8.2 Vitamin B6

The term “vitamin B6” covers the three vitamers: pyridoxal (Figure 1.13a), pyridoxamine (Figure 1.13b) and pyridoxine (Figure 1.13c). Pyridoxine, pyridoxal and pyridoxal phosphate (PLP) are the major dietary forms of the vitamin. Pyridoxine was first identified in 1938. The

catalytically active aldehyde (pyridoxal) or amine (pyridoxamine) and their phosphates were discovered in the early 1940s. Vitamin B6 dependency was first recognized in 1954 with a recessively inherited condition in which children deficient in B6 developed epileptic seizures beginning early in life (Baxter, 2003). Clinical evidence of vitamin B6 deficiency is not common because of its widespread availability in foods, but conditions such as anemia, renal failure, dermatitis and epilepsy have been linked with deficiency of the vitamin. An association between colon cancer and B6 deficiency has also been noted most recently (Eussen *et al.*, 2010; Larsson *et al.*, 2010). Approximately 10% of the US population consumes less than half of the RDA (adult RDA ~ 1 mg/day) for B6 and could be at an increased risk of cancer and neural decay (Ames *et al.*, 2005).

(a) Pyridoxal (b) Pyridoxamine (c) Pyridoxine

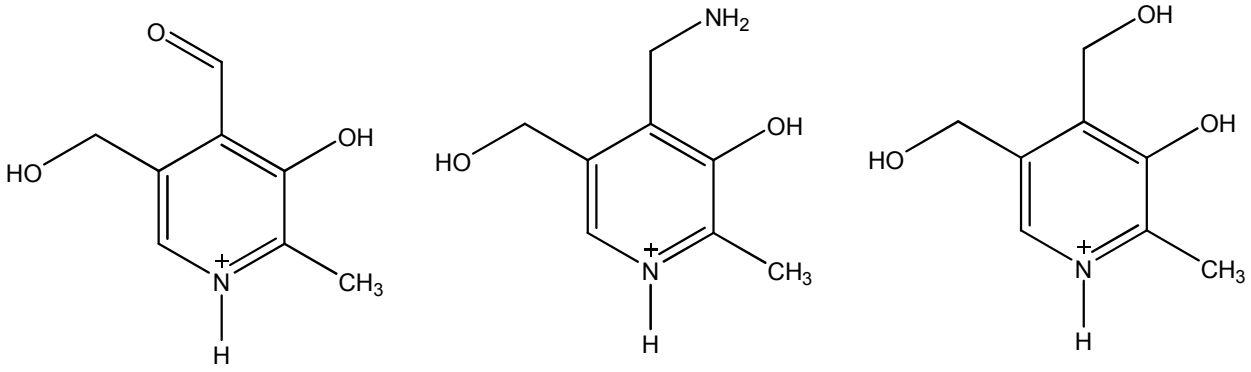

Figure 1.13 The three vitamers that are collectively known as "Vitamin B6": Pyridoxal (a), pyridoxamine (b) and pyridoxine (c).

1.8.2.1 Vitamin B6 delivery from the diet to the mitochondria

Vitamin B6 is synthesized by plants, and products that are particularly rich in B6 include vegetables, whole grain cereals and nuts. An increased risk of B6 deficiency can be observed in advanced age, severe malnutrition, excessive alcohol intake and dialysis. Thermal losses of B6 in food processing and preservation vary from 49% to 77% in canned foods or infant formula presumably because of the reaction of protein lysine groups with pyridoxal at 37°C to form Schiff bases which rearrange to form pyridoxylidenelysine (Huang *et al.*, 2001; Young and Blass, 1982). Furthermore, the Schiff base hydrazone products formed with hydrazine carbonyl reagents or drugs (e.g. isoniazid, semicarbazide, hydrazine and hydroxylamine) may compete with B6 for the B6 enzyme binding site and therefore inhibit pyridoxal phosphate (PLP) coenzyme formation (Bhagavan and Brin, 1983).

Vitamin B6 is absorbed from the diet mainly by simple diffusion across the enterocyte brush-border of the jejunum. Enterocyte and other cell plasma membranes are not permeable to PLP, and PLP needs to be dephosphorylated by intestinal phosphatases prior to being taken up by enterocytes (Wilson and Davis, 1983). The three B6 vitamers are then phosphorylated (i.e. metabolically trapped) in the enterocyte but require dephosphorylation by plasma membrane phosphatases (similar to alkaline phophatases) before being released into the portal vein for transport to the liver. About 10% of the total body pool of PLP is in the liver and about 20% of cellular B6 is located in the liver mitochondrial matrix as PLP. Pyridoxal, pyridoxamine and pyridoxine are taken up rapidly by hepatocytes and accumulate by kinase-catalyzed phosphorylation or metabolic trapping (Figure 1.14, Reaction 1). Kinases first form pyridoxine-5′-phosphate and pyridoxamine-5′-phosphate. These are oxidized at the 4′ position by a cytosolic pyridoxine/pyridoxamine phosphate oxidase (Reaction 2) that requires flavin mononucleotide

(FMN) and O_2. The PLP formed is then transferred to PLP-dependent apoenzymes (Cheung *et al.*, 2003; Merrill, Jr. *et al.*, 1984).

Mitochondria have no kinase or pyridoxine/pyridoxamine-P oxidase activities so PLP is taken up by liver mitochondria by passive diffusion facilitated by protein binding, which acts as a store (Lui *et al.*, 1982). Mitochondria may also take up pyridoxamine-P by passive diffusion as mitochondria contain similar amounts (45% each) of pyridoxal-P and pyridoxamine-P, whereas pyridoxine-P only accounts for 2% of the B6 vitaminers (Lui *et al.*, 1981). PLP can be released by hepatocytes into the plasma as an albumin complex. Similarly, it can bind avidly to other plasma proteins such as transferrin or α-glycoproteins through the formation of Schiff bases. Any unbound pyridoxine or pyridoxal in hepatocytes is oxidatively inactivated by aldehyde oxidase/ALDH2 to form 4-pyridoxic acid which is released into the plasma and excreted in the urine (Merrill, Jr. *et al.*, 1984).

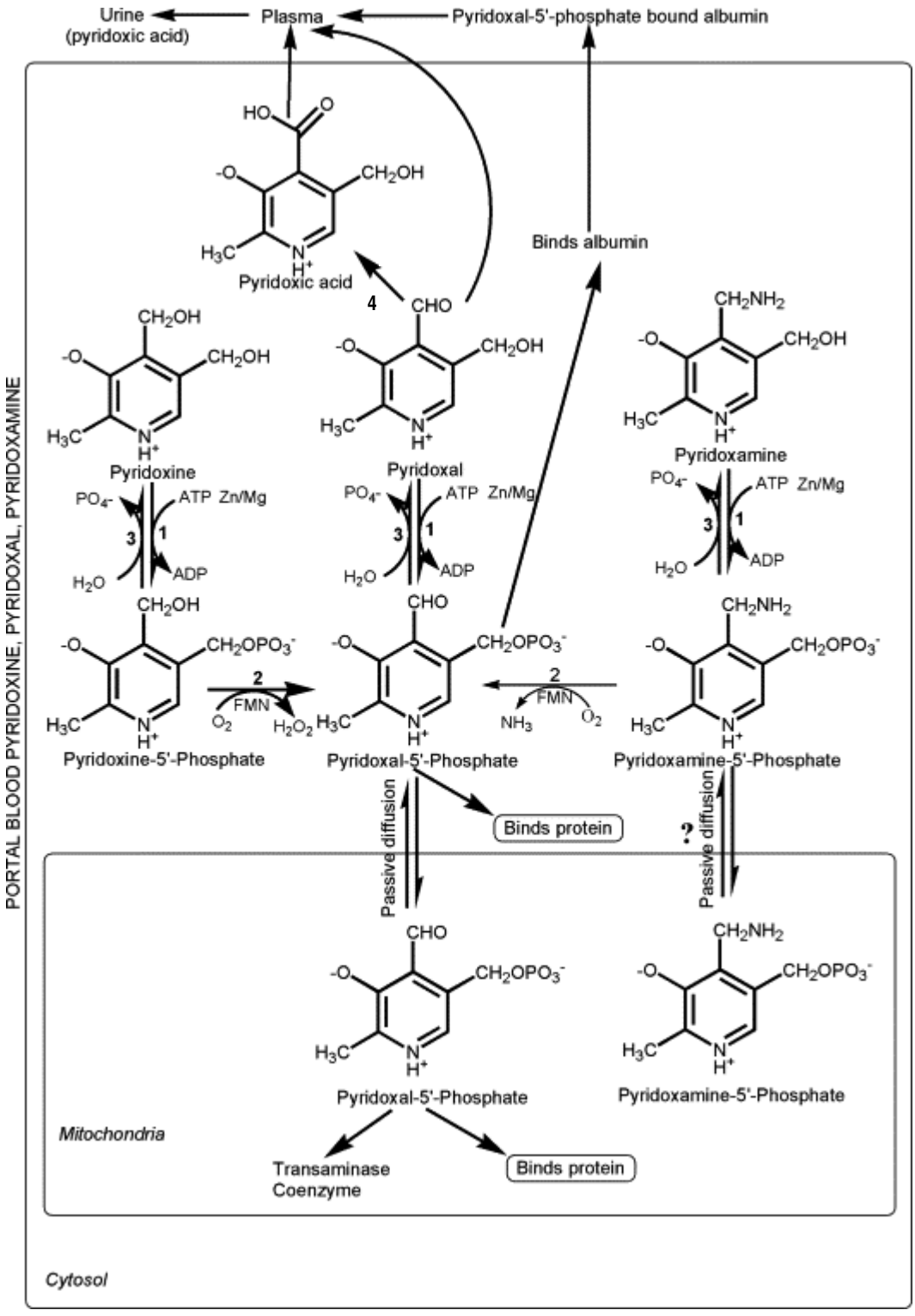

Figure 1.14 Metabolism of B6 vitamers. Metabolic conversion of B6 vitamers catalyzed by: (1) pyridoxal kinase, (2) PLP oxidase, (3) B6 vitamin kinase conversion can be reversed by phosphatases, (4) any unbound pyridoxal is oxidized by aldehyde oxidase/aldehyde

dehydrogenase 2 (ALDH2) to form pyridoxic acid which is released into the plasma and excreted in the urine. See text for a detailed description.

1.8.2.2 Essential role of vitamin B6 in mitochondrial and cellular function

Vitamin B6 is involved in aminotransferase reactions. Mitochondrial function is more dependent on PLP than other subcellular organelles for a number of reasons. PLP functions as a coenzyme for transaminases that participate in the catabolism of all amino acids by the urea cycle of the mitochondria. Accordingly the human requirement for pyridoxine increases as protein intake increases. The chemical basis for PLP catalysis is to enable its carbonyl group to first form a Schiff base with the amine component of the amino acid substrate. The protonated form of PLP then acts as an electrophilic catalyst before the Schiff base is cleaved.

Transaminases can utilize PLP by either forming α-oxo acids from α-amino acids or primary amines via decarboxylase catalytic action. Transaminases may also participate in the malate aspartate shuttle which enables the mitochondria to oxidize NADH formed by glycolysis. Lastly, transaminases can link amino acid metabolism to energy production through two reactions: First, alanine can form pyruvate, which feeds into the glycolysis pathway, and secondly, glutamate can form α-ketoglutarate, feeding directly into the TCA cycle.

Vitamin B6 is also involved in decarboxylation reactions. PLP is a coenzyme for aminolevulinate synthase located in the mitochondrial matrix, which catalyzes the initial and rate limiting step for heme synthesis from glycine and succinyl-CoA. Furthermore, vitamin B6 is a cofactor for many enzymes involved in side-chain cleavage reactions and can thus play a role in a large number of reactions including cysteine, glycine and taurine formation, heme formation and cytochrome *c* synthesis, therefore having an inhibitory impact on the mitochondrial

respiratory chain at complex III during B6 deficiency. A specific example includes the involvement of vitamin B6 in the regeneration of tetrahydrofolate into the active methyl-bearing form in the methionine cycle and in glutathione biosynthesis from homocysteine (Depeint *et al.*, 2006a).

80% of PLP in the body is stored in the muscle where it is attached to the phosphorylase enzyme. It is released during food deprivation, but is not released during marginal pyridoxine deficiency. PLP binds to albumin for storage, thus conditions associated with low albumin levels are also likely to show decreased pyridoxal activity (Chiang *et al.*, 2003).

1.8.2.3 Prevention of oxidative stress by vitamin B6

Vitamin B6 can have antioxidant activity. Pyridoxamine was shown to prevent superoxide radical formation during glucose autoxidation (Jain and Lim, 2001), as well as inhibit H_2O_2-induced mitochondrial toxicity in monocytes (Kannan and Jain, 2004). Vitamin B6 can also have transition metal chelating activity as pyridoxine prevented chromium-induced kidney oxidative stress toxicity in rodents (Anand, 2005), while pyridoxal induced iron excretion in rats (Johnson *et al.*, 1982). Supplementary pyridoxine was also shown to decrease the formation of colon tumors in mice given repeated injections of azoxymethane (Matsubara *et al.*, 2003), but the protective mechanism of action was not elucidated.

Additional evidence suggests that vitamin B6 may have anti-diabetic properties. Pyridoxine therapy was first described when vitamin supplementation was used to delay peripheral neuropathy in diabetic patients, presumed to be exposed to increased oxidative stress (Cohen *et al.*, 1984). Pyridoxamine also prevented the development of retinopathy and nephropathy in streptozotocin-induced diabetic rats (Alderson *et al.*, 2004; Stitt *et al.*, 2002) as

well as the non-enzymatic oxidative glycation of proteins by glucose. The inhibitory effect of pyridoxamine has been attributed to the formation of a Schiff base linkage of its amine group with the carbonyls of open-chain sugars, RCS (e.g. glyoxal and methylglyoxal), Amadori products and post-Amadori intermediates (Booth *et al.*, 1996; Voziyan *et al.*, 2002).

There may be safety risks associated with chronic excessive pyridoxine administration. Multivitamin supplements typically provide about 4 mg/day of B6. Symptoms of peripheral sensory neuropathy attributed to B6 toxicity have been observed with chronic dosing at or above 50 mg/day for 6 to 60 months with full recovery after cessation of the treatment (Dalton and Dalton, 1987).

1.9 Accelerated cytotoxic mechanism screening (ACMS) with hepatocytes

The ACMS method determines the cytotoxic effectiveness of xenobiotics when incubated for 2 h with hepatocytes freshly isolated from male Sprague Dawley rats. The assumption with ACMS is that the *in vitro* cytotoxicity of the xenobiotic predicts its hepatotoxicity *in vivo* occurs at 6-48 h. ACMS is useful for understanding the cytotoxic mechanisms of toxins or reactive metabolites, and for identifying the hepatocyte metabolizing enzymes that both form and detoxify the metabolites (Chan *et al.*, 2007).

The procedures established are as follows:

1. Determine the concentration of xenobiotics required to induce a 50% loss of membrane integrity (LD50) of freshly isolated hepatocytes in 2 h using the trypan blue exclusion assay. A major assumption with ACMS is that high dose/short time (*in vitro*) exposure simulates low dose/long time (*in vivo*) exposure. With 24 halobenzenes, it was found that the relative

LD50 concentrations required to cause 50% cytotoxicity in 2 h with hepatocytes isolated from phenobarbital-induced Sprague Dawley rats correlated with *in vivo* hepatotoxicity at 24 h (Chan *et al.*, 2007). Moreover, using this technique, the molecular hepatotoxic mechanisms found *in vitro* for seven classes of xenobiotics/drugs were found to be similar to the rat hepatotoxic mechanisms reported *in vivo* (O'Brien *et al.*, 2004).

2. Identify the major xenobiotic metabolic pathways by determining the effects of inhibiting, activating or inducing metabolizing enzymes on cytotoxicity induced by the toxin. ACMS assumes that the metabolic pathways determined at the xenobiotic concentrations *in vitro* in 2 h are similar to those *in vivo* (Chan *et al.*, 2007; O'Brien *et al.*, 2004).

3. Determine the molecular cytotoxic mechanisms of xenobiotics by: 1. Investigating the changes in bioenergetics (mitochondrial membrane potential, respiration, ATP, glycogen depletion), oxidative stress (ROS generation, lipid peroxidation, GSH/GSSG, lactate/pyruvate ratio) and electrophile stress (protein/DNA adducts, GSH conjugates); 2. Using therapeutic agents as antidotes to prevent xenobiotic-induced cytotoxicity. If cytotoxicity was caused by oxidative stress, then oxidative stress should precede cytotoxicity and the therapeutic agents should prevent or delay cytotoxicity. If the reverse should occur then any oxidative stress likely resulted from cytotoxicity. If cytotoxicity was caused by mitochondrial toxicity, then glycolytic substrates should protect and restore the mitochondrial membrane potential (Chan *et al.*, 2007; O'Brien *et al.*, 2004).

Using ACMS, we developed an *in vitro* cell system associated with specific endogenous toxins and investigated the protective mechanisms of specific micronutrients against the cytotoxic endpoints identified.

1.10 Problem Formulation

The origin of chronic diseases is not well understood. It is clear that systemic chronic inflammation occurs in a wide variety of diseases common to the Western world, but much less is known about the specific causes and mechanisms of the inflammatory conditions (Medzhitov, 2008).

Oxidative damage by ROS is implicated in the pathogenesis of a number of chronic diseases including NASH and cancer (Kawanishi *et al.*, 2001; Lewis and Mohanty, 2010). ROS formed by exogenous and/or endogenous sources can attack various substrates in the body including lipids, proteins or nucleic acids. Oxidation of these substrates can contribute to the development of chronic disease if not balanced by the inhibitory effects of intracellular antioxidants and detoxifying enzymes (Ma, 2010).

Previously published work by our group has shown that fructose and some of its metabolites are toxic under conditions of chronic inflammation and oxidative stress, as characterized by non-toxic levels of H_2O_2 and Fenton chemistry (Feng *et al.*, 2009; Lee *et al.*, 2009). ROS generated during these conditions is suggested to oxidize fructose or its metabolites to the RCS glyoxal, a potent glycating agent of proteins and DNA. The resulting AGE derivatives formed downstream have been implicated in a host of chronic diseases, including

diabetes, NASH, Alzheimer's disease, arthritis and age-related conditions such as macular degeneration (Glenn and Stitt, 2009; Thornalley, 2007).

It is generally accepted that nutrition and related factors such as obesity, alcohol intake and physical activity, play an important role in the etiology of chronic disease and cancer in the developed world. A large amount of research on the role of nutrients in the prevention of chronic diseases has also accumulated over the past several decades. However, despite this effort, scientific evidence on the relationship between chronic metabolic diseases and the diet remains insufficient, thereby hindering the establishment of solid conclusions.

As a means to help fill the knowledge gaps, the application of oxidative stress biomarkers *in vitro* represents a useful method for elucidating the protective mechanisms of micronutrients (Mayne, 2003). Using this method, one could propose that the inhibition of an oxidative stress biomarker by a specific vitamin intervention is predictive of its therapeutic effect against chronic diseases characterized by similar targets of oxidative stress.

1.11 Objectives and Hypotheses

Naturally occurring compounds are hypothesized to prevent oxidative stress-induced diseases by protecting cells or tissues from the deleterious effects of endogenous toxins. As these micronutrient compounds are an integral part of our diet, they are often regarded as safe for long-term consumption. Micronutrients with potent cytoprotective effects against oxidative stress are therefore suggested as a plausible approach for clinical interventions of chronic metabolic diseases that are induced by the Western diet.

In the following work, we set out to understand the connection between micronutrients, biomarkers of oxidative stress and chronic metabolic disease. In order to do this, we identified the cytotoxic mechanisms of the Western diet derived-endogenous toxins of which we hypothesized played an important role in the development of chronic disease. Specifically, we demonstrated oxidative damage induced by glyoxal (a fructose oxidation product) and Fenton-mediated ROS (catalyzed by excess iron), and we measured lipid peroxidation, protein carbonylation and DNA damage-induced by the two endogenous toxins. We therefore studied the individual toxic components derived by the Western diet in order to specifically elucidate the protective mechanisms of the micronutrients investigated.

Hypothesis 1: *The endogenous toxins, glyoxal and Fenton-mediated ROS, can cause oxidative damage to hepatocytes by a) oxidizing lipids, generating further RCS; b) forming protein carbonyls; c) oxidizing DNA. The increased damage by ROS and RCS can overcome intracellular antioxidant defense and subsequently lead to cytotoxicity.*

We chose to study the two B vitamins, B1 and B6, which have been suggested to play a fundamental role in the prevention of oxidative damage in chronic conditions but whose mechanisms of cytoprotection are only partially understood. We investigated the protective ability of both vitamins against a range of toxins in order to develop a preliminary understanding of their cytoprotective mechanisms (Chapter 2). This screening method provided us with an initial understanding of the roles of vitamins B1 and B6 in our hepatocyte model. Our next objective was to examine more closely the specific protective capabilities of vitamins B1 or B6 against oxidative damage induced by diet-derived endogenous toxins.

Hypothesis 2: *B1 or B6 vitamins are multifunctional agents which can delay, prevent or rescue hepatocytes from oxidative damage induced by RCS (as exemplified by glyoxal) or Fenton-mediated ROS.*

1.12 Organization of thesis chapters

As discussed previously, the Western diet can trigger intracellular oxidative stress through increased ROS and RCS formation which can consequently result in cytotoxicity and chronic metabolic disease. A number of natural agents have been investigated for their role in preventing oxidative stress in *in vitro* and *in vivo* animal models.

In the following chapters, the *in vitro* cytotoxic mechanisms of the endogenous toxins and the cytoprotective mechanisms of therapeutic agents were investigated using the ACMS method. In Chapter 2, the ACMS technique was used to determine the molecular mechanisms of protection of vitamins B1 and B6 against hepatocyte oxidative stress cytotoxicity induced by RCS (glyoxal, acrolein), ROS (copper, hydroperoxide) and mitochondrial toxins (cyanide, copper). In Chapter 3, attention was focused on the mechanisms of protection of a group of known carbonyl trapping agents on the cytotoxic intracellular targets of glyoxal. The aim of Chapter 4 was to determine the targets of iron-mediated ROS damage in hepatocytes and to subsequently uncover the ability and extent of vitamins B1 and B6 to reverse or inhibit oxidative damage. Finally, Chapter 5 summarizes the main findings of this thesis and outlines future areas of research.

***Chapter 2*.** Preventing cell death induced by carbonyl stress, oxidative stress or mitochondrial toxins with vitamin B anti AGE agents

Rhea Mehta, Nandita Shagari and Peter J. O'Brien

2.1 Abstract

Carbonyls generated by the autoxidation of carbohydrates or lipid peroxidation have been implicated in advanced glycation end product (AGE) formation in tissues adversely affected by diabetes complications. Tissue AGEs and associated pathology have been decreased by vitamins B1 and B6 in trials involving diabetic animal models. In order to understand the molecular cytoprotective mechanisms involved, the effects of B1 and B6 vitamers against cytotoxicity induced by AGE/advanced lipoxidation end product (ALE) carbonyl precursors (glyoxal/acrolein) have been compared with cytotoxicity induced by oxidative stress (hydroperoxide) or mitochondrial toxins (cyanide/copper). Thiamin was found to be best at preventing cell death induced by carbonyl stress and mitochondrial toxins but not oxidative stress cytotoxicity induced by hydroperoxide, suggesting that thiamin pyrophosphate (TPP) restored pyruvate (PDH) and α-ketoglutarate dehydrogenases (α-KGDH) inhibited by mitochondrial toxicity. However, the B6 vitamers were most effective at preventing oxidative stress or lipid peroxidation-mediated cytotoxicity, suggesting that pyridoxal or pyridoxal phosphate (PLP) were behaving as antioxidants or transition metal chelators. A therapeutic vitamin cocktail could provide maximal prevention against carbonyl stress toxicity associated with diabetic complications.

2.2 Introduction

Reactive dicarbonyls formed endogenously by the autoxidation of carbohydrates, nucleic acids and unsaturated lipids can bind covalently to proteins and undergo further oxidation to form advanced glycation end-products (AGEs) and advanced lipid end products (ALEs). The prolonged dyslipidemia and hyperglycemia in diabetes increases AGEs which contribute to the development of diabetes complications (O'Brien *et al.*, 2005; Thomas *et al.*, 2005).

High-dose vitamin B1 (thiamin) therapy prevents dyslipidemia, incipient nephropathy, mild thiamin deficiency and the increases in plasma AGEs in streptozotocin-induced diabetic rats (Babaei-Jadidi *et al.*, 2003; Karachalias *et al.*, 2010). Benfotiamine, a thiamin monophosphate analogue, has also been shown to be effective at alleviating diabetic polyneuropathy and other microvascular complications in animal studies and clinical trials (Haupt *et al.*, 2005; Karachalias *et al.*, 2010). A clinical trial is presently in progress to evaluate the effect of benfotiamine on the change in excretion of urinary albumin and β-microglobulin in patients with type 2 diabetes (Turgut and Bolton, 2010). Research conducted by our group in partially thiamin deficient rats also determined an increase in plasma AGE levels, providing further indication of the interrelationship between thiamin and AGE formation (Shangari *et al.*, 2005).

Another vitamin involved in the attenuation of AGE-related complications is pyridoxamine. Pyridoxamine, a B6 vitamer, has been an effective AGE inhibitor in various biological systems and prevents incipient diabetic nephropathy or retinopathy in streptozotocin-induced diabetic rats (Babaei-Jadidi *et al.*, 2003; Stitt *et al.*, 2002). Pyridoxamine also prevents the renal and vascular pathology and the increases in skin collagen AGE/ALEs and hyperlipidemia in the Zucker obese non-diabetic rat model (Alderson *et al.*, 2003). Results from several preclinical studies conclude

that oral pyridoxamine has efficacy in preserving kidney function in rat models for type 1 and type 2 diabetes. Phase 2 clinical studies have also been conducted to establish the safety and tolerability of pyridoxamine in type 1 and type 2 diabetic patients with proteinuria (Turgut and Bolton, 2010).

The therapeutic mechanisms of thiamin and pyridoxamine are not fully understood. However, the following mechanisms (Depeint *et al.*, 2006a; Depeint *et al.*, 2006b) have been proposed: 1) Thiamin is converted in the cell to thiamin pyrophosphate (TPP), the coenzyme for transketolase (TK), a rate limiting step of the pentose phosphate pathway. TK activation could decrease the accumulation of glyceraldehyde-3-phosphate and fructose-6-phosphate during glycolysis and thereby prevent methylglyoxal formation (Babaei-Jadidi *et al.*, 2003; Karachalias *et al.*, 2005). B6 vitamers are also coenzymes required by transaminases for mitochondrial function, but their contribution to xenobiotic cytotoxicity requires further investigation. 2) Pyridoxamine, pyridoxal phosphate (PLP) and to a lesser extent TPP were much more effective than pyridoxal or thiamin as "late" AGE inhibitors when ribose was incubated with ribonuclease A for days. This effect of vitamin B1 and B6 derivatives was attributed to the inhibition of post-Amadori formation of AGEs (Booth *et al.*, 1997). Pyridoxamine was later shown to trap the dicarbonyl AGE precursors glyoxal and methylglyoxal and the adducts formed were identified (Nagaraj *et al.*, 2002; Voziyan *et al.*, 2002). However, the mechanisms underlying the inhibition of AGE formation by thiamin or TPP remain to be investigated. 3) Pyridoxamine can also trap lipid hydroperoxide intermediates *in vivo* to form amide adducts that are excreted in the urine (Metz *et al.*, 2003). 4) Thiamin has antioxidant and reactive oxygen species (ROS) scavenging activity that results in thiamin oxidation (Lukienko *et al.*, 2000). Pyridoxamine or PLP also prevents H_2O_2 cytotoxicity (Kannan and Jain, 2004). Pyridoxamine chelates transition metals

copper (Cu) and iron (Fe) (Gustafson and Martell, 1957) and it also inhibits Cu-catalyzed AGE formation (Price *et al.*, 2001). The Cu chelator trientine has also been effective at preventing diabetic neuropathy and diabetic heart disease (Brewer, 2005). It would be useful to prioritize these molecular cytoprotective mechanisms that have been suggested for thiamin and B6 vitamers in order to develop second generation AGE inhibitors.

We hypothesize that the cytoprotective mechanisms of vitamin B1 or B6 vitamers against carbonyl stress, oxidative stress or mitochondrial toxicity depend on the molecular cytotoxic mechanism involved. In the following, we determined the relative effectiveness of the suggested B vitamins at preventing: 1) Carbonyl stress cytotoxicity induced by dicarbonyl AGE precursors or acrolein (an ALE precursor); 2) Oxidative stress cytotoxicity induced by hydroperoxide; 3) Bioenergetic stress using mitochondrial toxins e.g. cyanide or copper. The therapeutic effectiveness of the vitamins was correlated using biomarkers for ROS generation and lipid peroxidation.

2.3 Materials and methods

2.3.1 Chemicals

Cupric sulfate, potassium cyanide, acrolein, glyoxal, tertiary-butyl hydroperoxide, thiamin, thiamin pyrophosphate, pyridoxal 5'-phosphate, pyridoxal, pyridoxine, pyridoxamine, dichlorofluorescin diacetate (DCFD), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Type II Collagenase was purchased from Worthington (Lakewood, NJ).

2.3.2 Animal treatment and hepatocyte preparation

Male Sprague-Dawley rats weighing 275-300 g (Charles River Laboratories, MA, USA) were housed in ventilated plastic cages over PWI 8-16 hardwood bedding. There were 12 air changes per hour, 12 h light photoperiod (lights on at 08:00 h) and an environmental temperature of 21-23˚C with a 50-60% relative humidity. The animals were fed with a normal standard chow diet and water ad libitum. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldéus and coworkers (Moldeus *et al.*, 1978). Isolated hepatocytes (10^6 cells/ml) (10 ml) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid *(*HEPES) in continually rotating 50 ml round-bottomed flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37˚C for 30 min (Moldeus *et al.*, 1978).

2.3.3 Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by the trypan blue (0.1% w/v) exclusion test (Moridani *et al.*, 2003). Hepatocyte viability was determined every 30 min during the 3 h incubation and the cells were at least 80-90% viable before use. Cupric sulfate, cyanide, acrolein, glyoxal and tertiary-butyl hydroperoxide stock solutions were prepared immediately prior to use and were made in double distilled water, dimethylsulfoxide (DMSO) or methanol. Toxin concentrations were used that caused approximately 50% cytotoxic death at 2 h. Toxins were added to the hepatocytes either directly after the addition of vitamins or after the hepatocytes had been pre-incubated with vitamins. The pre-incubation time is specified in each table and mostly reflects differences in vitamer transportation and metabolic activation (Depeint *et al.*, 2006a; Depeint *et al.*, 2006b). The minimal concentrations of vitamins that prevented cytotoxicity in 2 h were determined for

each of the different toxic systems and ranged from 1-10 mM. At these concentrations, the vitamins alone were not cytotoxic.

2.3.4 Determination of reactive oxygen species

Hepatocyte ROS generation induced by the toxins was determined by adding dichlorofluorescin diacetate (DCFD) to the hepatocyte incubate. DCFD penetrates hepatocytes and is hydrolyzed to form non-fluorescent dichlorofluorescin. Dichlorofluorescin then reacts with ROS to form the highly fluorescent dichlorofluorescein and effluxes the cell. 1 ml samples were withdrawn at 30 and 90 min after incubation with copper, cyanide and glyoxal. These samples were then centrifuged for 1 min at 50 x *g*. The cells were resuspended in H_2O and 1.6 µM DCFD was added (Possel *et al.*, 1997). The cells were allowed to incubate at 37˚C for 10 min. The fluorescence intensity of ROS product was measured using a Shimadzu RF5000U fluorescence spectrophotometer at $\lambda_{excitation}$ = 490 nm and $\lambda_{emission}$ = 520 nm.

2.3.5 Lipid peroxidation assay

Acrolein and tertiary-butyl hydroperoxide reacted with thiobarbituric acid (TBA) (0.8% w/v) to form pink products that absorbed at 532 nm. Lipid peroxidation was therefore assayed by measuring thiobarbituric acid reactive substances (TBARS) mostly formed from malondialdehyde, a lipid hydroperoxide decomposition product. 1 ml samples were withdrawn at 30 and 90 min from the hepatocyte flasks. 250 µl of trichloroacetic acid (TCA) (70% w/v) were added to the samples to stop the reaction and lyse the cells. The formation of lipid peroxidation products was determined by adding 1 ml of TBA (0.8% w/v) to the samples. The samples were then incubated in a boiling water bath for 20 min. The samples were cooled on ice for 5 min, and then centrifuged at high speed for 5 min. The supernatant was measured at 532 nm to detect the

amount of TBARS formed during the decomposition of lipid hydroperoxides, using a Pharmacia Biotech Ultrospec 1000 (Smith *et al.*, 1982). Results were expressed as μM concentration of malondialdehyde ($\varepsilon = 1.56 \times 10^5$ $mol^{-1} \bullet cm^{-1}$). None of the vitamins at the concentrations used reacted with TBA or absorbed at 532 nm.

2.3.6 Microsomal preparation

Adult male Sprague-Dawley rats (250-300 g) were anesthetized by sodium pentobarbital (60 mg/kg body). Livers were removed under sterile conditions and perfused with KCl solution (1.18% w/v, 4˚C). Hepatic microsomes were prepared by differential centrifugation as described by Dallner et al (Dallner, 1978). The microsomal pellet was suspended and homogenized in sterile potassium phosphate buffer with KCl solution [50 mM KH_2PO_4 and 0.23% (w/v) KCl, pH 7.4] before storage at -70˚C. Microsomal protein was determined by the method of Joly et al (Joly *et al.*, 1975).

2.3.7 Determination of microsomal lipid peroxidation

Microsomal lipid peroxidation was determined by measuring the amount of thiobarbituric reactive substances (TBARS) at 532 nm, formed during the decomposition of lipid hydroperoxides using a Pharmacia Biotech Ultrospec 1000. Briefly, the reaction mixture contained 1 ml of 0.1 M potassium phosphate buffer (pH 7.4), microsomes (1 mg/ml protein), 500 μM tertiary-butyl hydroperoxide, and B vitamins. All of the test tubes containing the reactions mixture were incubated at 37˚C in a water bath with agitation. At 90 min, each test tube was treated with TCA (70% w/v) and TBA (0.8% w/v) as previously described. The suspension was then boiled for 20 min and read at 532 nm using a Pharmacia Biotech Ultrospec 1000 (Smith

et al., 1982). Results were expressed as μM concentration of malondialdehyde (ε = 1.56 x 10^5 mol^{-1}•cm^{-1}).

2.3.8 Statistical analysis

Statistical analysis was performed by one-way ANOVA (analysis of variance) test, and significance was assessed by employing Tukey's post-hoc test. Results were presented as the mean±standard error (S.E.) from there separate experiments, and a probability of less than 0.05 was considered significant.

2.4 Results and Discussion

2.4.1 Carbonyl stress induced cytotoxicity:

Glyoxal cytotoxicity and ROS formation

Previous research from our laboratory showed that glyoxal added to hepatocytes caused cell death after lipid peroxidation, ROS formation and mitochondrial toxicity. Inhibiting lipid peroxidation with the antioxidant butylated hydroxyanisole (BHA) also prevented glyoxal-induced cytotoxicity (Shangari and O'Brien, 2004). As shown in Table 2.1, the vitamins (3mM) PLP, pyridoxal and thiamin showed the greatest amount of protection against glyoxal-induced cytotoxicity whereas TPP, pyridoxamine and pyridoxine were less effective at preventing cytotoxicity. Glyoxal-induced ROS generation, however, was best inhibited by pyridoxamine, which was more similar to that found for preventing copper-induced ROS formation than glyoxal-induced cytotoxicity. This suggests that the ROS scavenging activity of the vitamers does not fully explain their cytoprotective ability. The ROS scavenging activity of pyridoxamine has been attributed to its 3-hydroxyl ring substituent (Voziyan and Hudson, 2005). Thiamin

protection has been attributed to its intracellular conversion to TPP which is an essential coenzyme for transketolase, a rate limiting enzyme of the pentose phosphate pathway that supplies NADPH. Glyoxal is reductively detoxified by aldehyde reductase and NADPH (Babaei-Jadidi *et al.*, 2004; Pourahmad *et al.*, 2001). Furthermore TPP is a coenzyme for alpha-ketoglutarate dehydrogenase (α-KGDH) that is inactivated by mitochondrial toxins including glyoxals (Ostrovtsova, 1998; Sheline and Choi, 2004) or ROS formed by glyoxal (Shangari and O'Brien, 2004). Cytoprotection by thiamin rather than TPP is likely because hepatocytes take up thiamin (but not TPP) via a thiamin transporter THTR (Depeint *et al.*, 2006b; Said *et al.*, 2002).

Pyridoxal diffuses rapidly into hepatocytes whereas PLP is first converted to pyridoxal by a plasma membrane phosphatase when it enters the cell (Depeint *et al.*, 2006a). The cytoprotection by PLP could be related to the prevention of AGE formation by PLP when ribonuclease A was incubated with ribose for days (Booth *et al.*, 1997). This was attributed to PLP forming Schiff base adducts with protein amino groups which limited the amount of amino groups available for AGE formation by ribose glycation products (Booth *et al.*, 1997). Pyridoxamine was less effective than PLP or pyridoxal in preventing glyoxal cytotoxicity even though its primary amine group can slowly form a hemiaminal adduct with glyoxal ($t_{1/2}$ = 0.94 h) at high concentrations (10mM) (Nagaraj *et al.*, 2002).

Table 2.1 Glyoxal cytotoxicity and ROS formation

Toxin and B Vitamin Treatment	Percent Cytotoxicity (trypan blue uptake)			ROS Formation (FI units)
	60 min	120 min	180 min	90 min
Control	18 ± 3	22 ± 3	25 ± 4	98 ± 5
+Glyoxal 5mM	42 ± 4[a]	58 ± 7[a]	100[a]	240 ± 12[a]
+Thiamin 3mM	23 ± 2[b]	31 ± 3[b]	83 ± 5[b]	126 ± 10[b]
+Thiamin Pyrophosphate 3mM	18 ± 1[b]	41 ± 3[b]	91 ± 6[b]	133 ± 13[b]
+Pyridoxal Phosphate 3mM*	19 ± 2[b]	31 ± 3[b]	66 ± 3[b,c]	140 ± 11[b]
+Pyridoxal 3mM	22 ± 2[b]	26 ± 2[b]	73 ± 4[b,c]	131 ± 12[b]
+Pyridoxine 3mM	33 ± 3[b]	41 ± 5[b]	92 ± 7	145 ± 7[b]
+Pyridoxamine 3mM	29 ± 2[b]	36 ± 3[b]	90 ± 5[b]	111 ± 11[b,d]

Please refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. Briefly, isolated rat hepatocytes (10^6 cells/ml) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. ROS was determined by measuring DCFD oxidation which was expressed as fluorescence intensity (FI) units. λ_{ex}=490, λ_{em}=520. [a]significant as compared to control ($P < 0.05$), [b]significant as compared to Glyoxal 5mM ($P < 0.05$), [c]significant as compared to thiamin, TPP, pyridoxine and pyridoxamine ($P < 0.05$), [d]significant as compared to PLP and pyridoxine ($P < 0.05$). *pre-incubation for 1 hour.

Acrolein cytotoxicity and lipid peroxidation

The addition of acrolein to hepatocytes caused iron release and lipid peroxidation. Cytotoxicity was prevented by the iron chelator desferrioxamine but was only partly decreased by antioxidants (Silva and O'Brien, 1989). Lipid peroxidation and cytotoxicity were best inhibited by thiamin > pyridoxal, and to a lesser degree by pyridoxamine and PLP. However, TPP and pyridoxine were not protective (Table 2.2).

Thiamin was the best at preventing death induced by acrolein likely because it is a coenzyme for pyruvate dehydrogenase (PDH) and α-KGDH, which were inhibited when acrolein was incubated with mitochondria (Pocernich and Butterfield, 2003; Sun *et al.*, 2006). TPP, on the other hand, was not effective suggesting that TPP was taken up poorly by hepatocytes. The difference in therapeutic activity between pyridoxal and PLP was also likely a result of the poor uptake of PLP into hepatocytes (Depeint *et al.*, 2006a). However, protection by both pyridoxal and PLP was probably attributed to their ability to chelate iron. Acrolein causes the release of iron from hemoproteins in intact cells which results in ROS formation and lipid peroxidation (Ciccoli *et al.*, 1994; Silva and O'Brien, 1989). Pyridoxal complexes iron *in vivo* as ^{59}Fe excretion in the rat increased when pyridoxal was given intravenously at a dose of 150 mg/kg at 6 h after the i.v. administration of ^{59}Fe-ferritin (Johnson *et al.*, 1982). Pyridoxamine and pyridoxine were found to be poor acrolein scavengers (Burcham *et al.*, 2002).

Table 2.2 Acrolein cytotoxicity and lipid peroxidation

Toxin and B Vitamin Treatment	Percent Cytotoxicity (trypan blue uptake)			Lipid Peroxidation
	60 min	120 min	180 min	90 min
Control	18 ± 2	19 ± 2	20 ± 2	0.24 ± 0.01
+Acrolein 150µM	24 ± 1[a]	51 ± 1[a]	100[a]	2.71 ± 0.24[a]
+Thiamin 3mM*	14 ± 2[b]	32 ± 2[b]	34 ± 2[b,c]	0.35 ± 0.03[b,c]
+Thiamin Pyrophosphate 3mM	23 ± 2	46 ± 2[b]	100	2.78 ± 0.22[b]
+Pyridoxal Phosphate 3mM	23 ± 2	43 ± 2[b]	71 ± 6[b]	1.22 ± 0.08[b]
+Pyridoxal 3mM	17 ± 1[b]	32 ± 1[b]	58 ± 5[b,d]	0.77 ± 0.06[b,d]
+Pyridoxine 3mM	16 ± 1[b]	32 ± 1[b]	96 ± 7	2.47 ± 0.12[b]
+Pyridoxamine 3mM	16 ± 1[b]	41 ± 2[b]	79 ± 6[b]	1.53 ± 0.09[b]

Please refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. Briefly, isolated rat hepatocytes (10^6 cells/ml) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. Lipid peroxidation was determined by measuring thiobarbituric acid reactive metabolites as µM concentration of malondialdehyde ($\varepsilon = 1.56 \times 10^5$ $mol^{-1} \bullet cm^{-1}$). [a]significant as compared to control ($P < 0.05$), [b]significant as compared to Acrolein 150µM ($P < 0.05$), [c]significant as compared to TPP, PLP, pyridoxal, pyridoxine and pyridoxamine ($P < 0.05$), [d]significant as compared to TPP, pyridoxine and pyridoxamine ($P < 0.05$). *pre-incubation for 1 hour.

2.4.2 Oxidative stress cytotoxicity:

Tertiary-butyl hydroperoxide cytotoxicity and lipid peroxidation

The treatment of cells with hydroperoxides represents a common model for studying the molecular mechanisms of oxidative stress cytotoxicity. Tertiary-butyl hydroperoxide (tBuOOH) induced hepatic lipid peroxidation, DNA damage and toxicity *in vivo* which was prevented by

antioxidants or desferrioxamine, a ferric iron chelator. Similar results were also obtained with isolated hepatocytes (Hwang *et al.*, 2005). As shown in Table 2.3, the B6 vitamers were best at inhibiting hydroperoxide-induced cytotoxicity and lipid peroxidation. Protection by pyridoxine and pyridoxamine was greatest when the vitamins were incubated with hepatocytes prior to the addition of tBuOOH, which was probably attributed to the time required for the metabolic activation of the agents (Depeint *et al.*, 2006a). The inhibition of tBuOOH-induced cytotoxicity and lipid peroxidation by the B6 vitamers suggests they are effective antioxidants and/or iron chelators.

Contrary to the therapeutic activity of the B6 vitamins, tBuOOH-induced cytotoxicity and lipid peroxidation were unaffected by TPP and thiamin. The lack of protection by vitamin B1 indicates that in the hydroperoxide oxidative stress model, α-KGDH and PDH were not inhibited and thiamin was a poor antioxidant. This also suggests that thiamin may confer greater scavenging ability for other forms of ROS not generated in the hydroperoxide model.

Table 2.3 Tertiary-butyl hydroperoxide cytotoxicity and lipid peroxidation

Toxin and B Vitamin Treatment	Percent Cytotoxicity (trypan blue uptake)			Hepatocyte Lipid Peroxidation	Microsome Lipid Peroxidation
	60 min	120 min	180 min	90 min	90 min
Control	16 ± 1	22 ± 2	24 ± 3	0.35 ± 0.03	0.34 ± 0.02
+tert-butyl hydroperoxide 500µM	43 ± 5[a]	63 ± 8[a]	79 ± 7[a]	2.58 ± 0.15[a]	4.15 ± 0.25[a]
+Thiamin 3mM*	50 ± 7	72 ± 7	82 ± 8	2.69 ± 0.16	3.50 ± 0.21[b]
+Thiamin 3mM	45 ± 3	73 ± 4	95 ± 6[b]	2.06 ± 0.18 [b]	3.24 ± 0.19[b]
+Thiamin Pyrophosphate 3mM	61 ± 7[b]	76 ± 7	84 ± 5	2.91 ± 0.17[b]	4.18 ± 0.33
+Pyridoxal Phosphate 3mM	27 ± 2[b]	44 ± 3[b]	46 ± 3[b,c]	0.96 ± 0.09[b,c]	2.34 ± 0.14[b,c]
+Pyridoxal 3mM	20 ± 2[b]	36 ± 3[b]	39 ± 3[b,c]	0.38 ± 0.03[b,c]	1.99 ± 0.12[b,c]
+Pyridoxine 3mM*	14 ± 2[b]	21 ± 2[b]	29 ± 3[b,c]	0.92 ± 0.07[b,c]	2.86 ± 0.17[b,c]
+Pyridoxine 3mM	24 ± 2 [b]	47 ± 4 [b]	54 ± 4 [b,c]	1.64 ± 0.09[b]	3.01 ± 0.18[b]
+Pyridoxamine 3mM**	31 ± 3[b]	55 ± 6	70 ± 6[c]	0.92 ± 0.04[b,c]	3.28 ± 0.20[b]
+Pyridoxamine 3mM	28 ± 2[b]	54 ± 4	84 ± 5	1.32 ± 0.08[b,c]	3.30 ± 0.18[b]

Please refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. Briefly, isolated rat hepatocytes (10^6 cells/ml) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. Lipid peroxidation was determined for hepatocytes and microsomes by measuring thiobarbituric acid reactive metabolites as µM concentration of malondialdehyde ($\varepsilon = 1.56 \times 10^5\ mol^{-1} \bullet cm^{-1}$). [a]significant as compared to control ($P < 0.05$), [b]significant as compared to tBuOOH 500µM ($P < 0.05$), [c]significant as compared to thiamin and TPP ($P < 0.05$). *pre-incubation for 30 min, **pre-incubation for 1 hour.

2.4.3 Mitochondrial toxin induced cytotoxicity

Cyanide cytotoxicity and ROS formation

Cyanide is a cytochrome oxidase inhibitor, which when added to hepatocytes prevented mitochondrial NADH oxidation thereby causing reductive stress and iron release as the hepatocyte NADH:NAD^+ ratio was increased (Niknahad *et al.*, 1995). Research in our laboratory also showed that cyanide added to hepatocytes caused rapid ROS formation (Siraki *et al.*, 2002), and ROS scavengers or desferrioxamine prevented cytotoxicity (Niknahad *et al.*, 1995). As shown in Table 2.4, cyanide-induced toxicity was inhibited by thiamin > pyridoxamine and pyridoxine. Cytotoxicity and ROS generation were not prevented by TPP, pyridoxal and PLP in this hepatocyte mitochondrial toxicity model.

Cytoprotection by thiamin was likely a result of its mitochondrial α-KGDH and pyruvate dehdrogenase (PDH) coenzyme activity. In mixed neuronal cells, thiamin partly restored α-KGDH activity that was inhibited by ROS and subsequently normalized cellular levels of ATP (Sheline *et al.*, 2004). Protection by pyridoxamine could probably be attributed to its ability to scavenge ROS (Jain and Lim, 2001; Kannan and Jain, 2004).

Table 2.4 Cyanide cytotoxicity and ROS formation

Toxin and B Vitamin Treatment	Percent Cytotoxicity (trypan blue uptake)			ROS Formation (FI units)
	60 min	120 min	180 min	90 min
Control	19 ± 2	23 ± 2	25 ± 2	103 ± 7
+Cyanide 1.5mM	45 ± 4[a]	65 ± 8[a]	100[a]	161 ± 10[a]
+Thiamin 3mM*	34 ± 2[b]	36 ± 3[b]	51 ± 4[b,c]	132 ± 7[b,e]
+Thiamin 3mM	30 ± 3[b]	55 ± 4	100	154 ± 9[b]
+Thiamin Pyrophosphate 3mM	37 ± 3[b]	55 ± 5	93 ± 8[b]	155 ± 13[b]
+Pyridoxal Phosphate 3mM	46 ± 6[b]	61 ± 6	100	170 ± 12[b]
+Pyridoxal 3mM	40 ± 3[b]	51 ± 5	100	151 ± 12[b]
+Pyridoxine 3mM*	37 ± 3[b]	49 ± 4[b]	69 ± 5[b,d]	138 ± 12[b,e]
+Pyridoxamine 3mM*	32 ± 2[b]	44 ± 4[b]	62 ± 5[b,d]	130 ± 8[b,e]

Please refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. Briefly, isolated rat hepatocytes (10^6 cells/ml) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. ROS was determined by measuring DCFD oxidation which was expressed as fluorescence intensity (FI) units. λ_{ex}=490, λ_{em}=520. [a]significant as compared to control ($P < 0.05$), [b]significant as compared to Cyanide 1.5mM ($P < 0.05$), [c]significant as compared to TPP, PLP, pyridoxal, pyridoxine and pyridoxamine ($P < 0.05$), [d]significant as compared to TPP, PLP and pyridoxal ($P < 0.05$), [e]significant as compared to PLP ($P < 0.05$). *pre-incubation for 5 min.

Copper cytotoxicity and ROS formation

Copper overload in rats induced hepatotoxicity, lipid peroxidation and mitochondrial dysfunction (Mehta *et al.*, 2006). Research previously conducted in our laboratory showed that the addition of cupric sulfate to hepatocytes caused ROS formation before cytotoxicity ensued (Pourahmad *et al.*, 2001). As shown in Table 2.5, the following order of vitamer cytoprotection

was observed: PLP, pyridoxamine > thiamin, TPP. Pyridoxal and pyridoxine were not protective in this system.

Thiamin protected against copper-induced toxicity likely by restoring mitochondrial α-KGDH and PDH enzyme activity. As further support, thiamin restored α-KGDH activity and prevented cytotoxicity towards mixed neuronal/glial cells incubated with copper (Sheline *et al.*, 2004). The estimated effectiveness reported for the B_6 vitamer inhibition of copper-catalyzed ascorbic acid (expressed as IC_{50}) was 1.0 mM pyridoxamine > 3.6 mM pyridoxine > 5.0 mM pyridoxal (Price *et al.*, 2001). Pyridoxamine also complexes copper more readily than pyridoxal (El-Ezaby and El-Shatti, 1979). Negligible activity by pyridoxine could be a result of the inhibition of pyridoxine phosphate oxidase by copper. Pyridoxine phosphate oxidase is required for PLP formation from pyridoxine phosphate (Depeint *et al.*, 2006a). In this copper model, PLP was more cytoprotective than pyridoxal. Similarly, PLP was also more effective than pyridoxal in preventing iron-induced hepatocyte lipid peroxidation and cytotoxicity (Mehta and O'Brien, 2007). Greater protection by PLP rather than pyridoxal could be attributed to PLP:Copper (or iron) binding prior to crossing the hepatocyte plasma membrane.

Table 2.5 Copper cytotoxicity and ROS formation

Toxin and B Vitamin Treatment	Percent Cytotoxicity (trypan blue uptake)			ROS Formation (FI units)
	60 min	120 min	180 min	90 min
Control	19 ± 1	23 ± 2	25 ± 3	96 ± 8
+Copper 35µM	52 ± 3[a]	54 ± 3[a]	68 ± 3[a]	149 ± 7[a]
+Thiamin Pyrophosphate 1mM	34 ± 2[b]	38 ± 3[b]	42 ± 3[b,c]	129 ± 12[b]
+Thiamin 1mM*	33 ± 2[b]	38 ± 2[b]	39 ± 2[b.c]	122 ± 4[b]
+Pyridoxal Phosphate 3mM	21 ± 1[b]	25 ± 1[b]	29 ± 1[b,d]	111 ± 7[b]
+Pyridoxal 3mM	39 ± 4[b]	48 ± 4[b]	65 ± 6	132 ± 7[b]
+Pyridoxine 3mM	44 ± 3[b]	51 ± 4	65 ± 5	137 ± 9[b]
+Pyridoxamine 3mM	25 ± 2[b]	25 ± 2[b]	29 ± 2[b,d]	109 ± 7[b]

Please refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. Briefly, isolated rat hepatocytes (10^6 cells/ml) were incubated at 37°C in rotating round bottom flasks with 95% O_2 and 5% CO_2 in Krebs-Henseleit buffer (pH = 7.4). Varying treatments were incubated and cytotoxicity was determined using trypan blue uptake assay. ROS was determined by measuring DCFD oxidation which was expressed as fluorescence intensity (FI) units. λ_{ex}=490, λ_{em}=520. [a]significant as compared to control ($P < 0.05$), [b]significant as compared to CuSO4 35µM ($P < 0.05$). [c]significant as compared to pyridoxal and pyridoxine ($P < 0.05$). [d]significant as compared to thiamin, TPP, pyridoxal and pyridoxine ($P < 0.05$).*pre-incubation for 30 min.

2.5 Concluding remarks

The B vitamin cytoprotective ranking differs considerably and depends upon the intracellular target and whether carbonyl stress, oxidative stress or mitochondrial toxicity initiates the cytotoxic mechanism. Thiamin was the most effective B vitamin at preventing cell death induced by acrolein/glyoxal intermediates or mitochondrial toxins, but was not effective in the hydroperoxide oxidative stress model. Few cellular metabolic pathways are dependent on a

single B coenzyme. Thus, vitamin combination treatment or supplementation should be more beneficial for increasing cellular resistance to oxidative stress or preventing chronic diseases associated with oxidative stress such as diabetes and non-alcoholic fatty liver disease (NAFLD). This screening technique could prove useful for determining which agent combination provides maximal protection against each toxin and may also assist in target enzyme identification.

Chapter 3. Cytoprotective mechanisms of carbonyl scavenging drugs in isolated rat hepatocytes

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3.1 Abstract

Diabetes is a disease among several others that has been linked with the accumulation of carbonylated proteins in tissues. Carbonylation is an irreversible, non-enzymatic modification of proteins by carbonyls. In Diabetes, dicarbonyls are thought to be generated by the autoxidation of reducing sugars which react with proteins and eventually lead to the formation of advanced glycation end-products (AGEs). Carbonyl scavenging drugs containing thiol or amine functional groups have been suggested to act therapeutically by preventing protein carbonylation by trapping the dicarbonyls glyoxal and methylglyoxal to form non-toxic adducts. This study seeks to determine the mechanism by which carbonyl scavenging drugs prevent glyoxal toxicity in a cell-free system and in isolated rat hepatocytes. In a cell free system, the glyoxal trapping ability of the drugs was measured by following glyoxal disappearance using Girard's Reagent T. For the thiol-containing drugs, the order of effectiveness for glyoxal trapping was penicillamine > cysteine > N-acetyl-cysteine, whereas for the amine-containing drugs, the order of effectiveness for glyoxal trapping was aminoguanidine >> pyridoxamine > metformin. Furthermore, aminoguanidine, penicillamine, cysteine and hydralazine at concentrations equimolar to glyoxal prevented protein carbonylation in hepatocytes. Other scavenging drugs such as pyridoxamine or metformin did not prevent glyoxal-induced cytotoxicity by trapping glyoxal, but instead prevented cytotoxicity by acting as a mitochondrial vitamin or reactive oxygen species scavenger, respectively.

3.2 Introduction

Diabetes mellitus affects 4% of the global population. Patients with diabetes are at increased risk for developing long-term complications, such as nephropathy, retinopathy, atherosclerosis, neuropathy and cataracts. Hyperglycemia plays a critical role in the pathogenesis of these complications and diabetic patients with poor maintenance of blood glucose levels and poor dietary and exercise habits are predominantly at risk (Ahmed, 2005).

Under hyperglycemic conditions, reducing sugars can be autoxidized to form reactive dicarbonyls such as glyoxal and methylglyoxal (Manini *et al.*, 2006; Novotný, 2007; Usui *et al.*, 2007). Methylglyoxal may also be generated by the breakdown of triose phosphates under non-oxidative conditions or during anaerobic metabolism of amino acids (Voziyan *et al.*, 2002). Dicarbonyls can bind covalently to amino and thiol groups of proteins and undergo a series of non-enzymatic reactions to form advanced glycation end-products (AGEs) or advanced lipoxidation end-products (ALEs) which have been implicated in the pathogenesis of diabetes *in vivo* (Ahmed, 2005; Thornalley *et al.*, 1999). Reaction of proteins with reactive dicarbonyls can result in the inactivation of essential cellular proteins which can potentially lead to cytotoxicity and further chronic complications (Abordo *et al.*, 1999; Thornalley *et al.*, 1999). Accordingly, therapeutic drugs with carbonyl trapping mechanisms that contain amine or thiol functional groups have been proposed to prevent glyoxal-induced oxidative stress.

Aminoguanidine is a nucleophilic hydrazine that prevents AGE formation at an early stage by reacting with AGE precursors like glyoxal by means of its carbonyl scavenging properties (Chen *et al.*, 2004; Peyroux and Sternberg, 2006; Thornalley, 2003). It reacts both *in vitro* and *in vivo* with glyoxal to form 3-amino-1,2,4-triazine derivatives (Thornalley *et al.*, 2000; Thornalley, 2003). In animal models for diabetes, aminoguanidine has been shown to effectively inhibit pathological complications, such as nephropathy and atherosclerosis (Peyroux and Sternberg, 2006).

Metformin is similar in structure to aminoguanidine. It is a biguanide compound that is currently being used to control type 2 diabetes in the United States and Europe (Kiho *et al.*, 2005; Ruggiero-Lopez *et al.*, 1999; Zhou *et al.*, 2001). *In vitro*, metformin can trap glyoxal to form guanidine-glyoxal adducts (triazepinone derivatives) (Kiho *et al.*, 2005; Peyroux and Sternberg, 2006; Ruggiero-Lopez *et al.*, 1999). High dose metformin given daily for 10 weeks to streptozotocin (STZ)-induced diabetic rats has been shown to decrease AGE deposition in sciatic nerve and renal cortex (Peyroux and Sternberg, 2006). Furthermore, administration of high doses of metformin for 2 months to type 2 diabetic patients caused a decrease in methylglyoxal plasma levels (Peyroux and Sternberg, 2006).

Hydralazine is an antihypertensive drug that traps acrolein bound to protein, and thus prevents acrolein-induced hepatotoxicity (Burcham and Pyke, 2006; Kaminskas *et al.*, 2004; Mehta and O'Brien, 2007). This hydrazine derivative has been shown to prevent protein cross-linking in animal models (Burcham and Pyke, 2006; Kaminskas *et al.*, 2004), to inhibit renal damage in animal models with type 2 diabetes and also to prevent glyoxal- and methylglyoxal-induced low density lipoprotein (LDL) modification (Negre-Salvayre *et al.*, 2008; Nangaku *et al.*, 2003).

Pyridoxamine is one of the three natural forms of vitamin B_6. It has been shown to trap methylglyoxal to form a methylglyoxal-pyridoxamine dimer (Nagaraj *et al.*, 2002), and to trap glyoxal or glycolaldehyde to form a hemiaminal adduct (Voziyan *et al.*, 2002). Pyridoxamine administration prevents diabetic nephropathy, neuropathy and vaculopathy in STZ-induced diabetic rats and also inhibits AGE formation (Babaei-Jadidi *et al.*, 2003; Stitt *et al.*, 2002; Voziyan *et al.*, 2002; Voziyan and Hudson, 2005). Its therapeutic effect has been demonstrated in multiple animal models of diabetes and in phase 2 clinical trials (Chetyrkin *et al.*, 2008).

Penicillamine is an effective copper chelator used to treat Wilson's disease and rheumatoid arthritis. It scavenges dicarbonyls by forming a 2-acylthiazolidine derivative and has

been shown to prevent collagen cross-linking in experimental diabetic rat models (Chang *et al.*, 1980; Wondrak *et al.*, 2002). It may also protect human skin keratinocytes and fibroblasts from glyoxal- and methylglyoxal-induced carbonyl toxicity (Wondrak *et al.*, 2002). Furthermore, penicillamine exhibits antioxidant properties seemingly by forming stable copper-penicillamine complexes that render copper unable to participate in the Fenton reaction (Yoshida *et al.*, 1993).

N-acetyl-cysteine (NAC) is used in the clinical management of acetaminophen overdose. NAC (acetylated cysteine) and cysteine are precursors to glutathione that scavenge 4-hydroxy-2-nonenal (4-HNE) and also act as cellular antioxidants (De Flora *et al.*, 2001; Thong-Ngam *et al.*, 2007). NAC pre-treatment prevents brain malondialdehyde (MDA) increase and GSH decrease in an animal model for Alzheimer's disease (Fu *et al.*, 2006). In addition, cysteine has been reported to scavenge acetaldehyde and prevent ethanol hepatotoxicity *in vivo* (Hirayama *et al.*, 1983).

In the following, we have assessed in isolated rat hepatocytes the molecular cytotoxic mechanisms of glyoxal and investigated the molecular cytoprotective mechanisms of the therapeutic agents to determine whether they have more than one site of action.

3.3 Materials and methods

3.3.1 Chemicals

Aminoguanidine, penicillamine, cysteine, N-acetyl-cysteine, hydralazine, pyridoxamine, metformin, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL), trolox, butylated hydroxyanisole (BHA), mannitol, quercetin, glyoxal, sodium phosphate monobasic, anhydrous sodium borate, Girard's reagent T, thiobarbituric acid, trichloroacetic acid, 2',7'-dichlorofluorescin diacetate (DCFD), dinitrophenylhydrazine (DNPH) and rhodamine 123 were purchased from Sigma-Aldrich Corp. (Oakville, ON, CAN). Type II Collagenase was purchased from Worthington (Lakewood, NJ).

3.3.2 Animal treatment and hepatocyte preparation

Male-Sprague-Dawley rats weighing 275-300 g (Charles River Laboratories, MA, USA) were housed in ventilated plastic cages over PWI 8-16 hardwood bedding. There were 12 air changes per hour, 12 h light photoperiod (lights on at 08:00 h) and an environmental temperature of 21-23°C with a 50-60% relative humidity. The animals were fed with a normal standard chow diet and water ad libitum. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldéus and coworkers (Moldeus *et al.*, 1978). Isolated hepatocytes (10^6 cells/mL) (10mL) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50 mL round-bottomed flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37°C for 30 min (Moldeus *et al.*, 1978).

3.3.3 Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by the trypan blue (0.1% w/v) exclusion test (Moridani *et al.*, 2003). Hepatocyte viability was determined every 30 min during the 3 h incubation, and the cells were at least 80-90% viable before use. Glyoxal was prepared immediately prior to use and was added to the hepatocytes either before or directly after the addition of the therapeutic drugs. The pre-incubation time of glyoxal or of the therapeutic agents was specified in each table. Stock solutions of chemicals were made in double distilled water, dimethylsulfoxide (DMSO), or methanol. A lethal dose of glyoxal was used that caused approximately 50% cytotoxic death at 2 h. The minimal concentration of therapeutic drugs that prevented cytotoxicity was determined and recorded.

3.3.4 Reactive oxygen species (ROS) formation

Hepatocyte ROS generation induced by glyoxal was determined by adding dichlorofluorescin diacetate (DCFD) to the hepatocyte incubate. DCFD penetrates hepatocytes

and is hydrolyzed to form non-fluorescent dichlorofluorescin. Dichlorofluorescin then reacts with ROS to form the highly fluorescent dichlorofluorescein and effluxes the cell. Furthermore, dichlorofluorescin is also oxidized by the strong oxidant peroxynitrite (Possel *et al.*, 1997). ROS formation was assayed by withdrawing 1 mL samples at different time points after incubation with glyoxal and the therapeutic agents. These samples were then centrifuged for 1 min at 50 x *g*. The cells were resuspended in H_2O and 1.6 μM DCFD was added (Possel *et al.*, 1997). The cells were allowed to incubate at 37°C for 10 min. The fluorescence intensity of ROS product was measured using a Shimadzu RF5000U fluorescence spectrophotometer at 490 nm excitation and 520 nm emission wavelengths.

3.3.5 Hepatocyte lipid peroxidation

Glyoxal reacted with thiobarbituric acid (TBA) to form a chromophore that absorbed at 532 nm. Therefore, lipid peroxidation was assayed by measuring the formation of formaldehyde, a lipid hydroperoxide decomposition product (Bagchi *et al.*, 1998). Formaldehyde derivatization was determined colorimetrically using NASH's reagent. This reagent contained a mixture of 4 M ammonium acetate, 1 M acetic acid, 1 M acetyl acetone and Millipore water (Nash, 1953; Winters and Cederbaum, 1990). 1 mL samples of hepatocyte suspension were withdrawn at different time points. 56 μL of trichloroacetic acid (TCA) (30% w/v) was added to the samples to stop the reaction and lyse the cells. The mixture was centrifuged at high speed for 1 min to precipitate the cells. 500 μL of the supernatant was added to an equal volume of NASH's reagent. The solution was vortexed and incubated for 60 min at 37°C with shaking. The formaldehyde levels of the mixture were determined at 412 nm using a Shimadzu UV-vis spectrophotometer.

3.3.6 Protein carbonylation assay

The total protein-bound carbonyl content was measured by derivatizing the protein carbonyl adducts with dinitrophenylhydrazine (DNPH). Briefly, an aliquot of the suspension of cells (0.5 mL, 0.5 x 10^6 cells) at different time points was added to an equivalent volume (0.5 mL) of 0.1% DNPH (w/v) in 2.0 N HCl and allowed to incubate for 1 h at room temperature. This reaction was terminated and the total cellular protein precipitated by the addition of an equivalent of 1.0 mL volume of 20% TCA (w/v). Cellular protein was pelleted by centrifugation at 50 x *g*, and the supernatant was discarded. Excess unincorporated DNPH was extracted three times using an excess volume (0.5 mL) of ethanol:ethyl acetate (1:1) solution. Following extraction, the recovered cellular protein was dried under a stream of nitrogen and dissolved in 1 mL of Tris-buffered 8.0 M guanidine–HCl, pH 7.2. The resulting solubilized hydrazones were measured at 366–370 nm. The concentration of 2,4-DNPH derivatized protein carbonyls was determined using an extinction coefficient of 22,000 M^{-1} cm^{-1} (Hartley *et al.*, 1997).

3.3.7 Mitochondrial membrane potential assay

The uptake and retention of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. This assay is based on the fact that rhodamine 123 accumulates selectively in the mitochondria by facilitated diffusion. However, when the mitochondrial potential is decreased, the amount of rhodamine 123 that enters the mitochondria is decreased as there is no facilitated diffusion. Thus the amount of rhodamine 123 in the supernatant is increased and the amount in the pellet is decreased. Samples (500 µL) were taken from the cell suspension incubated at 37 °C at different time points, and centrifuged at 50 x *g* for 1 min. The cell pellet was then resuspended in 2 mL of fresh incubation medium containing 1.5 µM rhodamine 123 and incubated at 37 °C in a thermostatic bath for 10 min with gentle shaking. Hepatocytes were separated by centrifugation and the amount of rhodamine 123 appearing in the incubation medium was measured fluorimetrically using Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The

capacity of mitochondria to take up the rhodamine 123 was calculated as the difference in fluorescence intensity between control and treated cells (Andersson *et al.*, 1987).

3.3.8 Determination of reactive dicarbonyls

The concentration of glyoxal was determined using Girard's reagent T. Briefly, an aliquot of glyoxal was mixed with 120 mM sodium borate, pH 9.3. An aliquot of this mixture was then added to 100 mM Girard's reagent T. Once the reaction reached equilibrium, the amount of reacted dicarbonyls was determined spectrophotometrically at 326 nm (Mitchel and Birnboim, 1977; Voziyan *et al.*, 2002).

3.3.9 Statistical analysis

Statistical analysis was performed by one-way ANOVA (analysis of variance) test, and significance was assessed by employing Tukey's post-hoc test. Results were presented as the mean±standard error (S.E.) from there separate experiments, and a probability of less than 0.05 was considered significant.

3.4 Results

As shown in Table 3.1, incubation of isolated hepatocytes with 5mM glyoxal induced an approximate 50% loss in hepatocyte viability in 2 h (LD50) as measured by the Trypan blue exclusion assay. Glyoxal-induced cytotoxicity proceeded ROS formation, lipid peroxidation and mitochondrial toxicity. The cytoprotective trend of the therapeutic agents against glyoxal-induced cytotoxicity was hydralazine > aminoguanidine > penicillamine > pyridoxamine > metformin > cysteine > N-acetyl-cysteine. These agents prevented glyoxal-induced cytotoxicity and ROS production in a concentration dependent manner.

Table 3.1 Glyoxal-induced cytotoxicity, ROS production and protein carbonylation in isolated rat hepatocytes

Compounds added	Cytotoxicity (% trypan blue uptake)			ROS Formation (FI units)	Protein carbonylation (nmoles/10^6 cells)	Protein carbonylation (antidote effect)*
Incubation time (min)	*60*	*120*	*180*	*45*	*90*	*90*
Control (hepatocytes only)	21 ± 1	22 ± 2	25 ± 2	98 ± 5	6 ± 0.3	6 ± 0.3
+ Glyoxal 5mM	42 ± 3[a]	58 ± 3[a]	95 ± 5[a]	229 ± 11[a]	211 ± 7[a]	211 ± 7[a]
+ Aminoguanidine 1mM	22 ± 3[b]	28 ± 3[b]	46 ± 5[b]	163 ± 8[b]	215 ± 6	212 ± 6[b]
+ Aminoguanidine 5mM	22 ± 2[b]	27 ± 3[b]	27 ± 4[b,c]	132 ± 7[b]	7 ± 0.2[b,c]	10 ± 0.6[b,c]
+ Penicillamine 1mM	25 ± 4[b]	37 ± 5[b]	75 ± 5[b]	200 ± 10[b]	192 ± 9[b]	194 ± 8[b]
+ Penicillamine 5mM	24 ± 3[b]	35 ± 4[b]	39 ± 5[b,d]	195 ± 10[b]	7 ± 0.3[b,d]	25 ± 1[b,d]
+ Pyridoxamine 1mM	43 ± 4	61 ± 4	100	204 ± 10[b]	250 ± 8	244 ± 12
+ Pyridoxamine 3mM	25 ± 4[b]	49 ± 3[b]	50 ± 4[b]	130 ± 7[b]	193 ± 7[b]	149 ± 6[b]
+ Pyridoxamine 10mM	29 ± 3[b]	39 ± 4[b]	46 ± 3[b]	112 ± 6[b,e]	259 ± 9	144 ± 7[b]
+ Metformin 1mM	33 ± 3[b]	56 ± 6	98 ± 6	185 ± 9[b]	268 ± 8	256 ± 6
+ Metformin 3mM	32 ± 2[b]	49 ± 4[b]	80 ± 6[b]	167 ± 7[b]	221 ± 6	169 ± 8[b]
+ Metformin 5mM	28 ± 3[b]	28 ± 2[b]	68 ± 6[b]	155 ± 8[b]	201 ± 4	150 ± 6[b]
+ Cysteine 1mM	31 ± 2[b]	35 ± 4[b]	87 ± 7	219 ± 12[b]	204 ± 8	204 ± 8
+ Cysteine 5mM	32 ± 4[b]	54 ± 6	71 ± 5[b]	213 ± 9[b]	40 ± 2[b,f]	16 ± 0.8[b,f]
+ N-acetyl-cysteine 1mM	39 ± 4	60 ± 7	100	211 ± 9[b]	230 ± 6	216 ± 10
+ N-acetyl-cysteine 5mM	43 ± 5	60 ± 4	72 ± 5[b]	206 ± 8[b]	235 ± 5	193 ± 6[b]
+ N-acetyl-cysteine 10mM	31 ± 3[b]	33 ± 2[b]	69 ± 6[b]	199 ± 9[b]	91 ± 6[b,g]	145 ± 6[b]
+ Hydralazine 0.1mM	24 ± 3[b]	25 ± 2[b]	77 ± 6[b]	199 ± 7[b]	243 ± 5	233 ± 4
+ Hydralazine 0.5mM	24 ± 3[b]	41 ± 5[b]	56 ± 6[b]	142 ± 7[b]	230 ± 8	196 ± 6[b]
+ Hydralazine 1mM	24 ± 2[b]	25 ± 3[b]	47 ± 4[b]	102 ± 5[b]	212 ± 5	190 ± 5[b]
+ Hydralazine 5mM	27 ± 2[b]	31 ± 4[b]	48 ± 6[b]	110 ± 6[b]	20 ± 4[b,h]	45 ± 6[b,h]
+ TEMPOL 0.2mM	37 ± 4	40 ± 3[b]	77 ± 5[b]	211 ± 6[b]	221 ± 7	215 ± 6
+ Trolox 0.2mM	43 ± 4	48 ± 4[b]	57 ± 5[b]	196 ± 6[b]	248 ± 9	256 ± 9
+ BHA 0.5mM	34 ± 4[b]	47 ± 3[b]	78 ± 4[b]	141 ± 4[b]	220 ± 6	234 ± 7
+ Mannitol 50mM	24 ± 2[b]	25 ± 2[b]	38 ± 3[b]	194 ± 5[b]	214 ± 7	229 ± 8

Hepatocytes were either treated instantaneously with glyoxal 5mM and the therapeutic agents, or the cells were pre-treated wtih glyoxal for 1 h prior to the addition of the other agents (*).Glyoxal-induced cytotoxicity increased over time. The agents were most effective at preventing glyoxal-induced cytotoxicity and ROS generation at concentrations equimolar to or greater than glyoxal. Protein carbonylation was best inhibited by aminoguanidine, penicillamine > cysteine, hydralazine > N-acetyl-cysteine (NAC). Please refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate

experiments are given. TEMPOL, 4-hydroxy-2,2,6, 6-tetramethylpiperidene-1-oxyl, ROS scavenger; BHA, butylated hydroxyanisole, lipid antioxidant; mannitol, hydroxyl radical scavenger; trolox, antioxidant. [a]Significant as compared to control ($P < 0.05$). [b]Significant as compared to glyoxal 5mM ($P < 0.05$), [c]Significant as compared to penicillamine (5mM), pyridoxamine (5mM), metformin (5mM), cysteine (5mM) NAC (10mM) and hydralazine (5mM) ($P < 0.05$) [d]Significant as compared to pyridoxamine, metformin, cysteine, NAC and hydralazine ($P < 0.05$) [e]Significant as compared to aminoguanidine, penicillamine, metformin, cysteine and NAC ($P < 0.05$) [f]Significant as compared to NAC ($P < 0.05$) [g]Significant as compared to cysteine ($P < 0.05$), [h]Significant as compared to cysteine and NAC ($P < 0.05$).

Hydralazine inhibited glyoxal-induced hepatocyte cytotoxicity and ROS formation (Table 3.1) in a concentration dependent manner. At a low concentration (0.1 mM), it exhibited strong protection against lipid peroxidation (Figure 3.2). However, hydralazine only demonstrated a protective effect against glyoxal-induced protein carbonylation when it was used at a concentration equimolar to glyoxal (5 mM) (Table 3.1). As shown in Figure 3.1, hydralazine was ineffective at restoring the collapsed mitochondrial membrane potential.

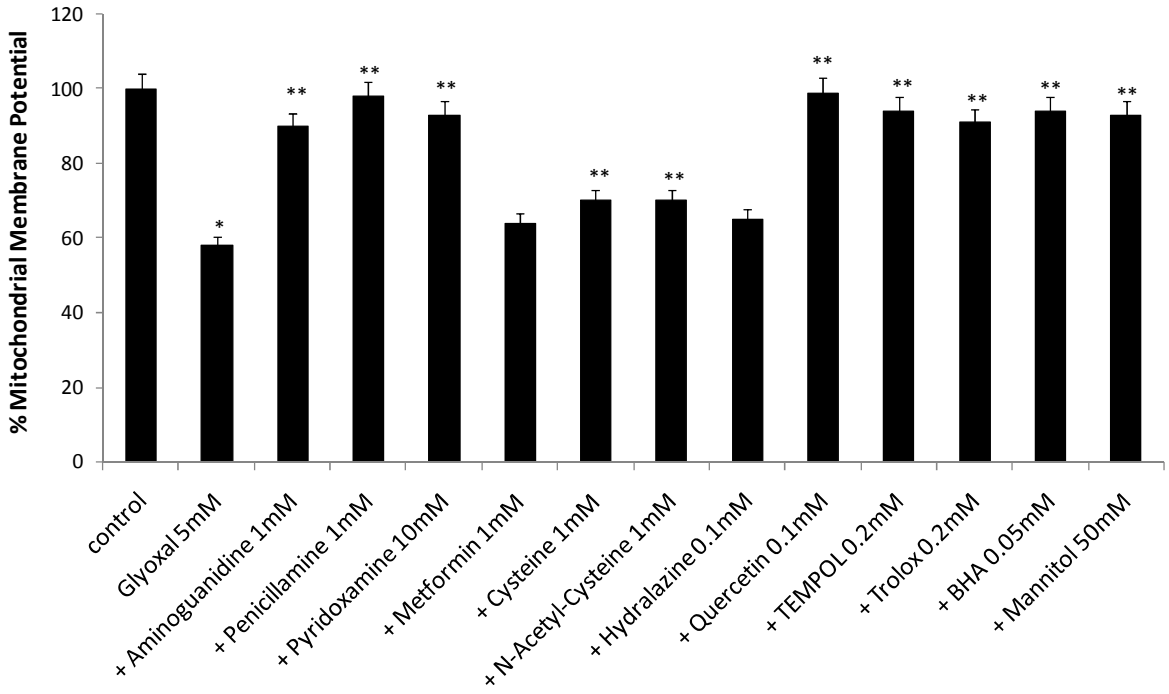

Figure 3.1 Glyoxal-induced mitochondrial toxicity in isolated rat hepatocytes. The mitochondrial membrane potential was best restored by aminoguanidine (1mM), penicillamine (1mM) and pyridoxamine (10mM). BHA, butylated hydroxyanisole; lipid antioxidant; TEMPOL, 4-hydroxy-2,2,6, 6-tetramethylpiperidene-1-oxyl, ROS scavenger; mannitol, hydroxyl radical scavenger; trolox, antioxidant; quercetin, ROS scavenger. Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. $^{\alpha}$Pyridoxamine 10mM pre-incubated for 60 min. Results above are shown for 45 min and are comparable at 90 min. *Significant as compared to control *(P* < 0.05). **Significant as compared to glyoxal 5mM ($P < 0.05$). Note: Lowest concentrations of therapeutic agents are shown that prevented mitochondrial toxicity.

Penicillamine and aminoguanidine (5 mM) significantly prevented protein carbonylation in isolated rat hepatocytes when added up to 1 h after glyoxal administration (Table 3.1), and quickly removed glyoxal as measured by Girard's method in a cell free system (Figure 3.3). Penicillamine and aminoguanidine also decreased glyoxal-induced ROS production (Table 3.1), mitochondrial toxicity (Figure 3.1) and lipid peroxidation (Figure 3.2) before cytotoxicity ensued.

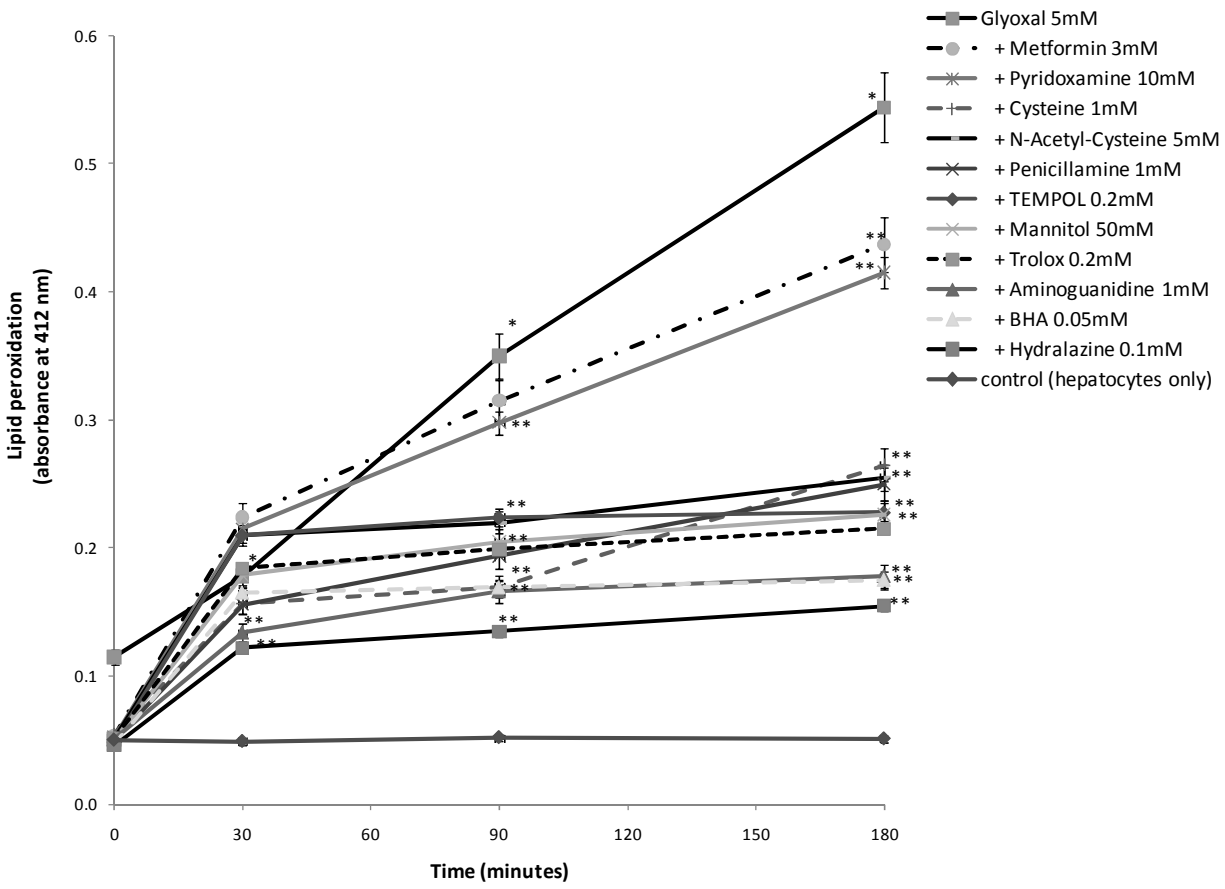

Figure 3.2 Measurement of lipid peroxidation in isolated rat hepatocytes. Glyoxal-induced lipid peroxidation increased over time. Of the carbonyl scavenging drugs, aminoguandine, pencillamine, cysteine and NAC were the best at inhibiting lipid peroxidation. Pyridoxamine and metformin delayed lipid peroxidation at 3 h, but were overall weak in their ability to protect against lipid peroxidation. BHA, butylated hydroxyanisole, lipid antioxidant; TEMPOL, 4-hydroxy-2,2,6, 6-tetramethylpiperidene-1-oxyl, ROS scavenger; mannitol, hydroxyl radical scavenger; trolox, antioxidant. Refer to the Experimental Procedures for a description of the experiments performed. Mean ± SE for three separate experiments are given. *Significant as compared to control *(P* < 0.05). **Significant as compared to glyoxal 5mM (*P* < 0.05). Note: Lowest concentrations of therapeutic agents are shown that prevented lipid peroxidation.

Metformin and pyridoxamine at concentrations up to 10mM failed to prevent glyoxal-induced protein carbonylation (Table 3.1) and in addition had a delayed effect on removing glyoxal in the cell free system (Figure 3.3). Metformin decreased glyoxal-induced ROS formation (Table 3.1), but had little effect on lipid peroxidation (Figure 3.2) and no effect on the

mitochondrial membrane potential (Figure 3.1). Pyridoxamine prevented ROS formation and restored the mitochondrial membrane potential, but was weak at inhibiting lipid peroxidation induced by glyoxal. Pyridoxamine was pre-incubated with hepatocytes to allow time for its phosphorylation and metabolic conversion to pyridoxal phosphate (transaminase coenzyme) which can enter the mitochondria and restore transaminase function (Depeint *et al.*, 2006a; Pourahmad and O'Brien, 2000).

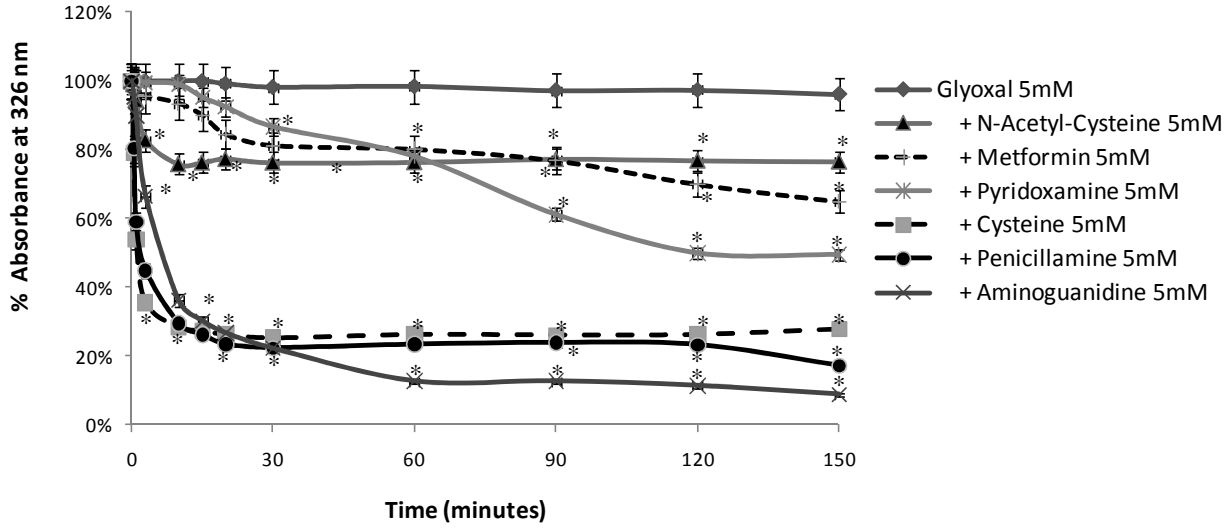

Figure 3.3 Measurement of glyoxal disappearance using Girard's Reagent T with equimolar concentrations of therapeutic agents. The order of glyoxal disappearance was: aminoguandine > penicillamine, cysteine > pyridoxamine > metformin, NAC. Refer to the Experimental Procedures for a description of the experiments performed. Mean ± SE for three separate experiments are given. *Significant as compared to glyoxal 5mM *(P* < 0.05).

As shown in Table 3.1, cysteine (5 mM) at a concentration equimolar to that of glyoxal significantly inhibited protein carbonylation when added up to 1h after glyoxal and also rapidly removed glyoxal (Figure 3.3). Cysteine prevented lipid peroxidation induced by gloyxal but exhibited only slight protection against mitochondrial toxicity and ROS formation. N-acetyl-cysteine at twice the concentration of glyoxal decreased protein carbonylation. However, it had

little effect on protein carbonylation and on glyoxal disappearance when it was added at a concentration equimolar to that of glyoxal. Like cysteine, N-acetyl-cysteine prevented lipid peroxidation but only slightly prevented mitochondrial toxicity and ROS generation.

3.5 Discussion

ROS formation, lipid peroxidation and mitochondrial toxicity occurred before cytotoxicity ensued. Cytotoxicity was also prevented by TEMPOL, a ROS scavenger, BHA, a lipid antioxidant, mannitol, a hydroxyl radical scavenger and trolox, an antioxidant, suggesting that cytotoxicity was attributed to ROS and lipid peroxidation. Furthermore, the mitochondrial membrane potential was restored by quercetin and TEMPOL, both ROS scavengers, suggesting mitochondrial toxicity was caused by mitochondrial ROS production. Lastly, the ROS scavengers and antioxidants did not have any effect on protein carbonylation levels, confirming that protein carbonylation was due to glyoxal-protein binding rather than protein (amino acid) oxidation to carbonyls by ROS (Requena *et al.*, 2003; Stadtman and Berlett, 1998).

Hydralazine protected hepatocytes at concentrations much lower than glyoxal suggesting that hydralazine was acting as an antioxidant. Additionally, we previously showed that hydralazine (0.5 mM) was the best inhibitor of cumene hydroperoxide-induced lipid peroxidation in microsomes and isolated rat hepatocytes which further strengthens its antioxidant effect (Mehta and O'Brien, 2007). A study completed by Munzel et al suggested hydralazine was an effective inhibitor of ROS formation because it prevented plasma membrane NADPH oxidase activation and ROS production (Munzel *et al.*, 1996). As previously mentioned, hydralazine is an efficient scavenger of acrolein (Burcham and Pyke, 2006). The inhibition of glyoxal-induced protein carbonylation by hydralazine when incubated with glyoxal at an equal concentration provides evidence of a glyoxal scavenging effect in addition to its suggested antioxidant activity in this model.

Penicillamine and aminoguanidine prevented glyoxal-induced protein carbonylation when added at concentrations equimolar to glyoxal, and also removed glyoxal very quickly using the Girard's method to measure glyoxal trapping. Therefore, the cytoprotectiveness of penicillamine and aminoguanidine against glyoxal-induced protein carbonylation probably resulted from scavenging glyoxal to form thiazolidine and 1,2,4 triazine derivatives, respectively (Wondrak *et al.*, 2002). These agents also decreased ROS production, lipid peroxidation and mitochondrial toxicity before cytotoxicity ensued at concentrations much lower than glyoxal suggesting a ROS scavenging and/or antioxidant effect. As further support, we previously showed that both agents at concentrations much lower than glyoxal significantly inhibited cumene hydroperoxide-induced lipid peroxidation in microsomes and isolated rat hepatocytes (Mehta and O'Brien, 2007). It has been shown that the therapeutic effect of aminoguanidine is due to its antioxidant and chelating abilities (Price *et al.*, 2001). Aminoguanidine like hydralazine has been shown to inhibit LDL modification induced by glyoxal, methylglyoxal and MDA, (Brown *et al.*, 2006) and this protection likely resulted from its antioxidant activity in addition to its carbonyl scavenging properties (Jedidi *et al.*, 2003). In addition, Wondrak et al. showed that the protective effects of penicillamine against glucose-induced glycation in the presence of oxygen was due to its antioxidant and metal chelatating activity (Fu *et al.*, 1994; Wondrak *et al.*, 2002).

The therapeutic mechanism of metformin and pyridoxamine is unlikely to be that of carbonyl scavenging activity as they did not have any effect on glyoxal protein carbonylation levels. Metformin likely acted as a ROS scavenger as it decreased glyoxal-induced ROS formation. Kanigur-Sultuybek et al. suggested that the therapeutic effect of metformin resulted from a direct effect on ROS or an indirect action on the superoxide anions generated during hyperglycemia (Kanigur-Sultuybek *et al.*, 2007). Two other groups also showed that metformin directly scavenged ROS or modified the intracellular generation of superoxide anion (Bonnefont-Rousselot *et al.*, 2003; Ouslimani *et al.*, 2005). Pyridoxamine likely acted as vitamin B6 that

restored mitochondrial transaminases that had been inhibited by mitochondrial ROS (Depeint *et al.*, 2006a; Mehta *et al.*, 2008). Kannan et al. reported that in the U937 promonocytic human cell line, pyridoxamine inhibited superoxide radical generation inside and outside the mitochondria, reduced lipid peroxidation and prevented damage to mitochondrial membrane integrity (Kannan and Jain, 2004).

Protection by cysteine was likely attributed to the formation of an adduct with glyoxal as it prevented glyoxal-induced protein carbonylation at a concentration equal to that of glyoxal. Similarly, N-acetyl-cysteine decreased protein carbonylation caused by glyoxal, thus also presenting the possibility of adduct formation with glyoxal. Schauenstein et al. reported the *in vitro* formation of a cysteine-aldehyde adduct (Schauenstein *et al.*, 1977) and Vasdev's group showed that N-acetyl-cysteine bound to aldehydes, thus preventing reaction of aldehydes with physiological proteins (Vasdev *et al.*, 1998).

3.6 Conclusion

Glyoxal is a key target for therapeutic intervention in pathological conditions involving carbonyl toxicity like diabetes. Our results showed that the cytotoxic mechanism of glyoxal was mediated by oxidative stress (Shangari and O'Brien, 2004) and mitochondrial membrane collapse as endogenous ROS, lipid peroxidation and protein carbonylation were markedly increased and the mitochondrial membrane potential was significantly decreased. Additionally, ROS scavengers and antioxidants that prevented ROS formation, lipid peroxidation, and mitochondrial toxicity also prevented the ensuing cytotoxicity. The concentration of glyoxal used in this study is much higher than what is found under physiological conditions (~12.5 µg/mL) (Shangari and O'Brien, 2004). However, a lethal concentration of glyoxal was required to cause cytotoxicity in 2 h in order to examine the molecular mechanisms of cytoprotection by the therapeutic agents. Our results further showed that the therapeutic agents tested have multiple mechanisms of protective action (Scheme 1).

Carbonyl trapping may therefore not be an exclusive mechanism for cytoprotection for each of the proposed agents as some showed strong protection against ROS formation, mitochondrial toxicity and lipid peroxidation. It may be possible that some of the carbonyl by-products formed during the lipid peroxidation assay reacted with the therapeutic agents present during the incubation. However, the antioxidant capacity of the therapeutic drugs was further confirmed by investigating their cytoprotective effectiveness against cumene hydroperoxide-induced lipid peroxidation in both microsomes and isolated rat hepatocytes (Mehta and O'Brien, 2007). Understanding the cytoprotective molecular mechanisms of the therapeutic agents could also help identify the molecular targets of glyoxal and thereby the sequence of events contributing to glyoxal-induced cell death.

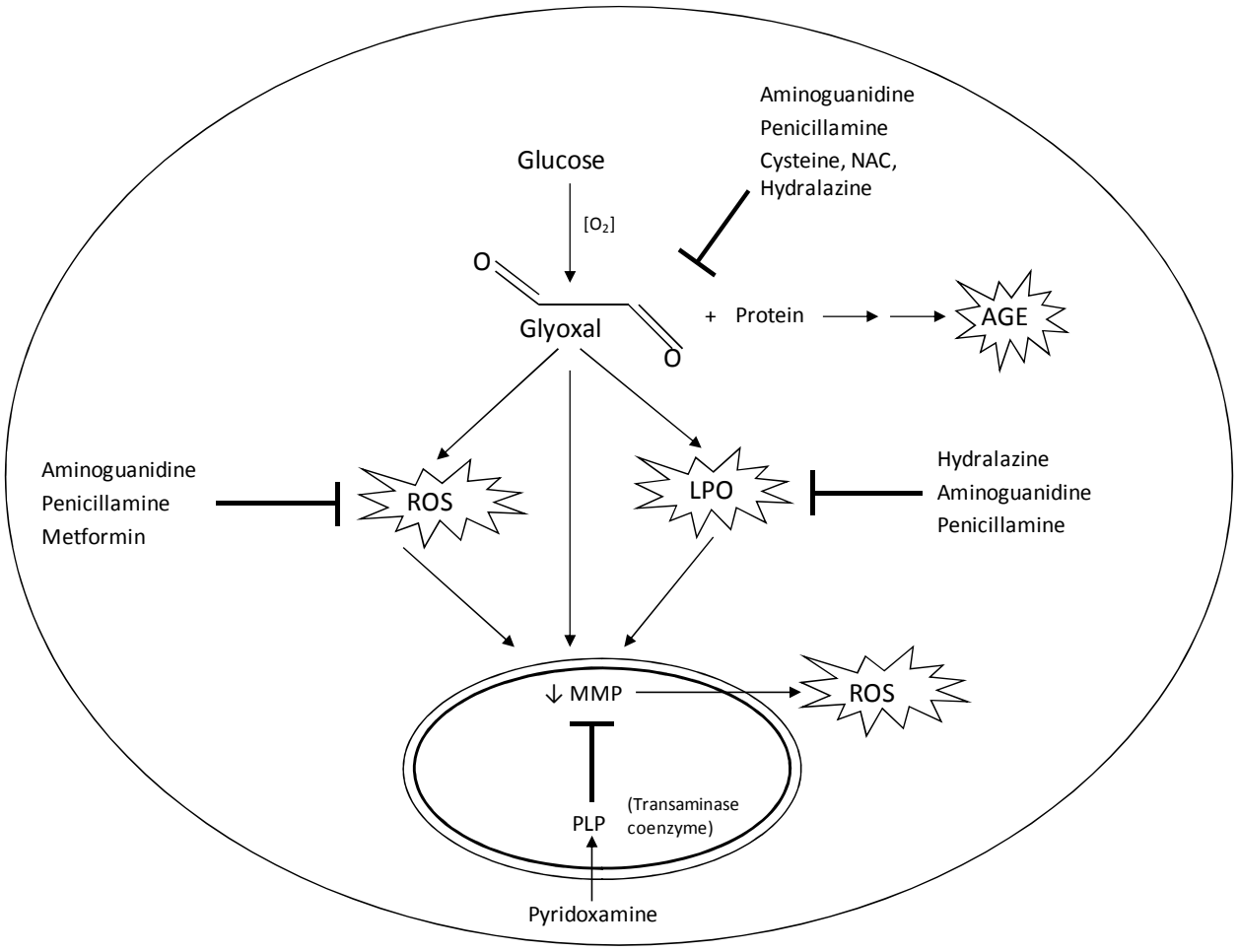

Figure 3.4 Proposed cytoprotective mechanisms of therapeutic agents against glyoxal toxicity. Aminoguanidine, penicillamine, cysteine and NAC can trap glyoxal and inhibit glyoxal-induced protein carbonylation. Hydralazine, aminoguanidine and penicillamine can also act as antioxidants and prevent glyoxal-induced lipid peroxidation which contributes to cytotoxicity. Glyoxal-induced ROS formation can be prevented by aminoguanidine, penicillamine and metformin and pyridoxamine can inhibit mitochondrial ROS formation. AGE, advanced glycation end products; ROS, reactive oxygen species; LPO, lipid peroxidation; MMP, mitochondrial membrane potential; NAC, N-acetyl-cysteine; PLP, pyridoxal phosphate

Chapter 4. Rescuing hepatocytes from iron-catalyzed oxidative stress using vitamin B1 and B6

Rhea Mehta, Liana Dedina and Peter J. O'Brien

4.1 Abstract

In the following rescue experiments, iron-mediated hepatocyte oxidative stress cytotoxicity was found to be prevented if vitamin B1 or B6 was added 1 h after treatment with iron. The role of iron in catalyzing Fenton-mediated oxidative damage has been implicated in iron overload genetic diseases, carcinogenesis (colon cancer), Alzheimer's disease and complications associated with the metabolic syndrome through the generation of reactive oxygen species (ROS). The objectives of this study were to interpret the cytotoxic mechanisms and intracellular targets of oxidative stress using "accelerated cytotoxicity mechanism screening" techniques (ACMS) and to evaluate the rescue strategies of vitamins B1 and B6. Significant cytoprotection by antioxidants or ROS scavengers indicated that iron-mediated cytotoxicity could be attributed to reactive oxygen species. Of the B6 vitamers, pyridoxal was best at rescuing hepatocytes from iron-catalyzed lipid peroxidation (LPO), protein oxidation and DNA damage, while pyridoxamine manifested greatest protection against ROS-mediated damage. Thiamin (B1) decreased LPO, mitochondrial and protein damage and DNA oxidation. Together, these results indicate that added B1 and B6 vitamins protect against the multiple targets of iron-catalyzed oxidative damage in hepatocytes. This study provides insight into the search for multi-targeted natural therapies to slow or retard the progression of diseases associated with Fenton-mediated oxidative damage.

4.2 Introduction

A large number of xenobiotics and metal ions have been implicated in cellular damage by oxidative stress through their intracellular production of reactive oxygen species (ROS), including hydroxyl radicals (HO·), superoxide radicals ($O_2{\cdot}^-$) and hydrogen peroxide (H_2O_2) (Aruoma *et al.*, 1989; Halliwell and Gutteridge, 1992; Otogawa *et al.*, 2008; Rashba-Step *et al.*, 1993). Iron (Fe) is an essential component of many enzymes involved in a number of vital biological processes. Although normally bound to proteins, Fe may be released and become free to catalyze the formation of ROS, particularly highly reactive hydroxyl radicals (Emerit *et al.*, 2001; Rauen *et al.*, 2004). The generation of hydroxyl radicals is achieved through the reaction of ferrous iron (Fe^{2+}) with H_2O_2 (Fenton reaction) (Kadiiska *et al.*, 1995; Minotti and Aust, 1989; Stohs and Bagchi, 1995). Once formed, these ROS can attack and oxidize lipids, proteins and DNA, leading to cell death and tissue injury (Kadiiska *et al.*, 1995; Oikawa and Kawanishi, 1998). On the basis that multiple intracellular targets are subject to attack by ROS, compounds that possess multifactorial antioxidant activity may offer pharmacological value against Fe-catalyzed oxidative damage.

Data obtained from literature is largely supportive of Fe's capacity to initiate oxidative damage and interfere with important cellular events. Fe-catalyzed oxidative damage to biological molecules has been linked to a number of chronic health disorders such as Alzheimer's disease, colorectal cancer and the spectrum of diseases within the metabolic syndrome, such as diabetes, hypertension and non-alcoholic steatohepatitis (NASH) (Depeint *et al.*, 2008; Fujita *et al.*, 2009; Knobel *et al.*, 2007; Lecube *et al.*, 2009; Oikawa and Kawanishi, 1998; Otogawa *et al.*, 2008; Sumida *et al.*, 2009; Tsuchiya *et al.*, 2010). Oxidative damage has also been linked to chronic Fe-overload in genetic disorders and to exposure to excess Fe through environmental and dietary

factors (Depeint *et al.*, 2008), occupational hazards and cookware (Camaschella and Strati, 2010; Kew and Asare, 2007; Pepper *et al.*, 2010).

In recent years, B1 and B6 vitamins have been investigated for their therapeutic mechanisms in experimental and clinical models of oxidative stress (Babaei-Jadidi *et al.*, 2003; Opara, 2002; Stitt *et al.*, 2002; Voziyan and Hudson, 2005). Our group previously demonstrated an increase in dicarbonyl protein adduct formation in vitamin B1 (thiamin) partially deficient rats (Shangari *et al.*, 2005), which was associated with aberrant crypt foci formation (Bruce *et al.*, 2003). Our group also elucidated the multiple mechanisms of protection of vitamins B1 and B6 against dicarbonyl-induced hepatotoxicity. In that model, the vitamins demonstrated a multitude of protective strategies that included antioxidant, ROS quenching, metal ion chelating and carbonyl scavenging activities (Mehta *et al.*, 2008; Mehta *et al.*, 2009).

Thiamin is a coenzyme for transketolase and mitochondrial alpha-ketoglutarate and pyruvate dehydrogenases that are part of the multienzyme complexes which form part of the Citric Acid cycle (Depeint *et al.*, 2006b). Thiamin has been investigated for treatment of epilepsy (Ranganathan and Ramaratnam, 2005) and other neurodegenerative disorders (Balk *et al.*, 2006; Thomson and Marshall, 2006), even though the underlying mechanisms involved were often unclear. Its use for preventing diabetic complications has also been well documented, (Ahmed and Thornalley, 2007; Ascher *et al.*, 2001; Bakker *et al.*, 2000; Thornalley *et al.*, 2001) and the role of thiamin in reversing protein carbonyl formation has been observed in acetaldehyde exposure (Aberle *et al.*, 2004).

The B6 vitamers are coenzymes for cytosolic and mitochondrial transaminases (Depeint *et al.*, 2006a). As a therapeutic agent, they have been described in relation to diabetes (Jain, 2007), epilepsy (Gaby, 2007) and cardiovascular disease (Wierzbicki, 2007). The antioxidant and

radical scavenging properties of the B6 vitamers have long been considered in literature, for example in reducing oxidative stress markers associated with homocysteinemia (Mahfouz and Kummerow, 2004) or in preventing ROS formation and lipid peroxidation in a cellular model (Kannan and Jain, 2004).

In the present work, Fe-loaded freshly isolated hepatocytes were used as an *in vitro* model to study hepatotoxicity mechanisms. To stimulate oxidative stress, we exaggerated the redox-active, chelatable Fe pool using the highly membrane permeable ferric iron:8-hydroxyquinoline (Fe^{3+}:8-HQ) complex to transport Fe^{3+} through the plasma membrane of hepatocytes (Lehnen-Beyel *et al.*, 2002; Oubidar *et al.*, 1996). Once inside, Fe^{3+} upon reduction to Fe^{2+}, caused significant oxygen-dependent cytotoxicity (Mehta and O'Brien, 2007).

The objective of this study was to use "accelerated cytotoxicity mechanism screening" techniques (ACMS) to determine the intracellular targets of Fe-catalyzed oxidative damage in order to investigate the protective properties of vitamins B1 and B6. Previously, ACMS techniques were used to show that high dose/short time *in vitro* rat hepatocyte cytotoxicity for 12 halobenzenes correlated with low dose/long term *in vivo* rat hepatotoxicity (Chan *et al.*, 2007). In our oxidative stress model, a lethal dose of 12 μM Fe^{3+}:8-HQ (1:2) caused approximately 50% hepatocyte cytotoxicity in 2 h. The addition of 1mM vitamin B1 and B6 1 h after hepatocyte incubation with Fe restored viability and prevented ROS formation. This shows that vitamins B1 and B6 can rescue hepatocytes from Fe-induced cytotoxicity and hepatocyte oxidative damage. To substantiate the cytotoxic mechanisms of oxidative stress in our system, we also determined the therapeutic value of the lipid antioxidant BHA, the free radical scavenger quercetin and the dicarbonyl traps penicillamine and aminoguanidine.

4.3 Materials and Methods

4.3.1 Materials

Toxin: Ferric chloride, 8-hydroxyquinoline, *B1/B6 vitamins*: Thiamin, pyridoxal, pyridoxamine; *Lipid antioxidant:* Butylated hydroxyanisole (BHA); *Free radical scavenger:* Quercetin; *Dicarbonyl trapping agents:* Aminoguanidine, penicillamine; *Fe chelators:* Deferiprone, pyridoxal isonicotinoyl hydrazone (PIH); *Assay ingredients:* Ferrous sulphate, hydrogen peroxide, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2',7'-dichlorofluorescin diacetate (DCFD), dinitrophenylhydrazine (DNPH), rhodamine 123, 2-deoxyribose, o-phenanthroline were purchased from Sigma–Aldrich Corp. (Oakville, ON, CAN). Type II Collagenase was purchased from Worthington (Lakewood, NJ).

4.3.2 Animal Treatment and Hepatocyte Preparation

Male Sprague–Dawley rats weighing 275–300 g (Charles River Laboratories, MA, USA) were housed in ventilated plastic cages over PWI 8–16 hardwood bedding. There were 12 air changes per hour, 12 h light photoperiod (lights on at 08:00 h) and an environmental temperature of 21–23°C with a 50–60% relative humidity. The animals were fed with a normal standard chow diet and water ad libitum. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldéus and coworkers (Moldeus *et al.*, 1978). Isolated hepatocytes (10^6 cells/mL, 10 mL) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50 mL round-bottomed flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37°C for 30 min (Moldeus *et al.*, 1978).

4.3.3 Hepatocyte Viability

Cell viability was assessed microscopically by plasma membrane disruption as determined by the trypan blue (0.1% w/v) exclusion test (Moridani *et al.*, 2003). Fe^{3+}:8-HQ was prepared and pre-mixed immediately prior to use and was added to the hepatocytes 1 h before the addition of the therapeutic agents in order to ensure that the hepatocytes were in a state of oxidative stress prior to the addition of the agents. Stock solutions of chemicals were made in double distilled water, DMSO, or methanol. Hepatocyte viability was determined every 30 min after the addition of the agents, and the cells were at least 80–90% viable at the start (time zero) of the experiment. A lethal dose of 12 μM Fe^{3+}:24 μM 8-HQ was used that caused approximately 50% cytotoxic death at 2 h. The optimal concentrations of therapeutic agents that prevented cytotoxicity were recorded and the concentrations used were not cytotoxic.

4.3.4 ROS Formation

ROS generation by hepatocytes was determined by adding dichlorofluorescin diacetate (DCFD) to the hepatocyte incubate. DCFD penetrates hepatocytes and is hydrolyzed intracellularly to form non-fluorescent dichlorofluorescin. Dichlorofluorescin then reacts with ROS to form the highly fluorescent dichlorofluorescein and effluxes the cell. ROS formation was assayed by withdrawing one-milliliter samples at different time points after incubation with Fe^{3+}:8-HQ and the therapeutic agents. These samples were then centrifuged for 1 min at 50 g. The cells were resuspended in H_2O and 1.6 μM DCFD was added (Possel *et al.*, 1997). The cells were allowed to incubate at 37°C for 10 min. The fluorescence intensity of ROS product was measured using a Shimadzu RF5000U fluorescence spectrophotometer at 490 nm excitation and 520 nm emission wavelengths.

4.3.5 Lipid Peroxidation

Lipid peroxidation (LPO) in hepatocytes was assayed by measuring the amount of thiobarbituric acid reactive substances (TBARS) formed during the lipid hydroperoxide decomposition. At different time points, each test tube containing 1 mL aliquots of hepatocyte suspension (10^6 cells/mL) was treated with trichloroacetic acid (TCA) (250 μL, 70%, w/v) to stop the reaction and lyse the cells. 1 mL of thiobarbituric acid (TBA) (0.8%, w/v) was then added to determine the formation of LPO products. The suspensions were boiled for 20 min, centrifuged at 1000 g for 5 min and the supernatant was read at 532 nm using a Pharmacia Biotech Ultrospec 1000 (Smith *et al.*, 1982). An extinction coefficient of 1.56 $mM^{-1}cm^{-1}$ was used to determine the concentration of TBARS produced.

4.3.6 Mitochondrial Membrane Potential Assay

The uptake and retention of the cationic fluorescent dye rhodamine 123 was used for the estimation of mitochondrial membrane potential (MMP) in hepatocytes. This assay is based on the fact that rhodamine 123 accumulates selectively in the mitochondria by facilitated diffusion. However, when the mitochondrial potential is disrupted, the amount of rhodamine 123 that enters the mitochondria is decreased as there is no facilitated diffusion. The amount of rhodamine 123 in the supernatant is therefore increased and the amount in the pellet is decreased. Samples (500 μL) were taken from the cell suspension incubated at 37°C, and centrifuged at 70 g for 1 min. The cell pellet was then resuspended in 2 mL of fresh incubation medium containing 1.5 μM rhodamine 123 and incubated at 37°C in a thermostatic bath for 10 min with gentle shaking. Hepatocytes were separated by centrifugation and the amount of rhodamine 123

appearing in the incubation medium (supernatant) was measured using a Shimadzu RF5000U fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths and expressed as fluorescence intensity units (Andersson *et al.*, 1987).

4.3.7 Alkaline Comet Assay

The capacity of Fe-induced oxidative stress to induce DNA single- and double-strand breaks in hepatocytes was assessed by the alkaline comet assay, also known as the single cell gel electrophoresis (SCGE) assay. The alkaline comet assay is a simple method to visualize and quantify DNA-damage in eukaryotic cells. It is based on the principle that denatured DNA fragments can be measured migrating out of the cell nucleus during electrophoresis. Cells that have accumulated DNA damage appear as fluorescent "comets" with a distinct head consisting of intact DNA, and a tail containing relaxed, unwound DNA fragments (Horvathova *et al.*, 2004). The alkaline comet assay was performed as per the protocol of the manufacturer. Briefly, samples taken from the cell suspensions (50 μL) were mixed with low melting-point (LMP) agarose at 37°C (1:10 v/v) and an aliquot was immediately spread out onto the comet slide. Slides were cooled, immersed in lysis solution and finally placed in the alkaline electrophoresis solution and subjected to electrophoresis (300 mA, 21 V). Slides were then immersed in distilled water, ethanol and then dried at room temperature. Diluted SYBR® green I solution was applied to the slides prior to viewing and could be read at excitation 494 nm and emission 521 nm (Lemay and Wood, 1999; Malyapa *et al.*, 1998). Slides were photographed under a Zeiss LSM510 deconvolusion fluorescence microscope (Carl Zeiss Canada Ltd., Toronto, ON, Canada) using AxioVision© software and a FITC filter. Once the images were captured, the metric of olive tail moment (OTM) was scored using CometScore© software (TriTek Corp., VA,

USA), which employed the following equation for OTM (μM): Olive Tail Moment = (Amount of Tail DNA)(Distance between centers of gravity of head and tail) (Olive *et al.*, 1990). A total of 100 comets per treatment were scored using a 20x objective.

4.3.8 Protein Carbonylation Assay

The total protein-bound carbonyl content in hepatocytes was measured by derivatizing the protein carbonyl adducts with DNPH. Briefly, an aliquot of the suspension of cells (0.5×10^6 cells, 500 μL) at different time points was added to an equivalent volume (500 μL) of 0.1% DNPH (w/v) in 2 N HCl and allowed to incubate for 1 h at room temperature. This reaction was terminated and the total cellular protein precipitated by the addition of an equivalent of 1 mL volume of 20% TCA (w/v). Cellular protein was pelleted by centrifugation at 1000 g, and the supernatant was discarded. Excess unincorporated DNPH was extracted three times using an excess volume (500 μL) of ethanol:ethyl acetate (1:1) solution. Following extraction, the recovered cellular protein was dried under a stream of nitrogen and dissolved in 1 mL of Tris-buffered 8 M guanidine–HCl, pH 7.2. The resulting solubilized hydrazones were measured at 370 nm using a Pharmacia Biotech Ultrospec 1000. The concentration of 2,4-DNPH derivatized protein carbonyls was determined using an extinction coefficient of 22,000 $M^{-1}cm^{-1}$ (Hartley *et al.*, 1997).

4.3.9 o-Phenanthroline Assay

The o-phenanthroline (OP) assay was used to examine the iron chelating properties of the therapeutic agents, measured as the disappearance of the OP-chelatable Fe^{2+} complex (Minotti and Aust, 1987). Mixtures containing 150 μM Fe^{2+} and the compounds of interest were

incubated for 5 min at 37°C to enable complex formation. Next, 1 mL of OP solution (15 mM) was added to 500 µL of the reaction mixture and allowed to incubate for 5 min at 37°C. The formation of complexes between free Fe^{2+} and the added compounds was assessed spectrophotometrically at 510 nm and corresponded to a decrease in absorbance and colour compared to control (OP- Fe^{2+}) (Puntel *et al.*, 2005).

4.3.10 Degradation of 2-Deoxyribose

The formation of hydroxyl radicals was measured using the 2-deoxyribose oxidative degradation method (Cheeseman *et al.*, 1988). Briefly, reaction mixtures contained 3 mM 2-deoxyribose, 50 µM KH_2PO_4 buffer (pH 7.4), 50 µM $FeSO_4$, 500 µM H_2O_2 and the therapeutic compounds of interest in a final volume of 800 µL and were incubated for 30 min at 37°C. The reaction was terminated by adding the equivalent volume of 2.8% TCA, proceeded by 400 µL of 0.6% TBA solution and the tubes were incubated for 20 min in boiling water. Deoxyribose degradation to MDA was assessed spectrophotometrically at 532 nm (Gutteridge, 1981; Halliwell and Gutteridge, 1981; Puntel *et al.*, 2005).

4.3.11 Statistical Analysis

Statistical analysis was performed by a one-way ANOVA (analysis of variance) test, and significance was assessed by employing Tukey's posthoc test. Results were presented as the mean±standard error (S.E.) from three separate experiments, and a probability of less than 0.05 was considered significant.

4.4 Results

Figure 4.1 displays the increase in Fe-induced cytotoxicity over a period of 4 h (results not shown for the first hour of Fe^{3+}:8-HQ pre-incubation). Time "0" was therefore recorded from the time the protective agents were added. Of the B vitamins, pyridoxamine (1mM) protected poorly against Fe-induced cytotoxicity until 2 h, at which time point its protection stabilized. Pyridoxal (1mM) and thiamin (1mM) were both effective and inhibited cytotoxicity by approximately 45% and 35%, respectively over the recorded time period. The dicarbonyl trapping agents, aminoguanidine (300µM) and penicillamine (300µM), were effective therapeutic agents until 1 h, at which point their protection declined to 20%. BHA (50µM) and quercetin (50µM), however, kept cytotoxicity to a minimum and protected against Fe-induced cell damage by approximately 50% throughout the time curve.

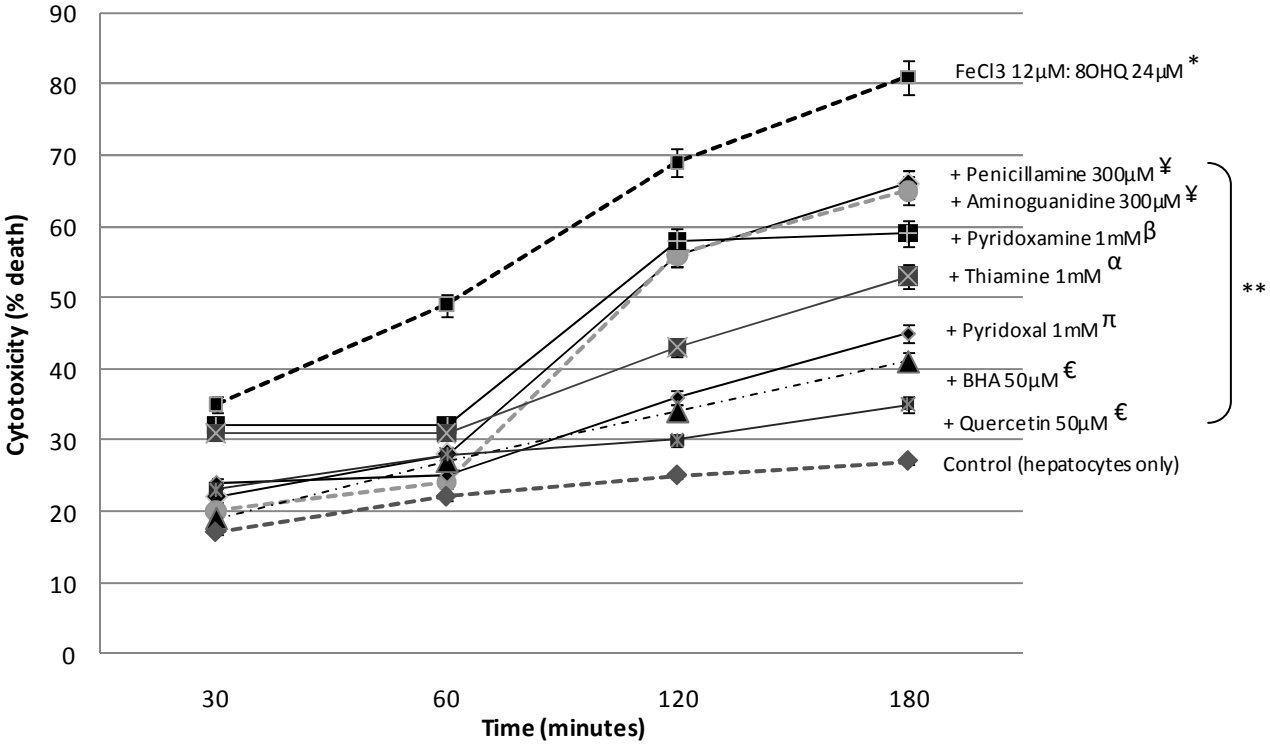

Figure 4.1 Effect of therapeutic agents against iron-induced cytotoxicity in isolated rat hepatocytes

Cells were pre-treated with Fe^{3+}:8-HQ for 1 h (results not shown), followed by a 3 h incubation with the therapeutic agents. Fe-induced cytotoxicity increased over time. At 2 h, the B vitamin order of protection against cytotoxicity was pyridoxal > thiamin > pyridoxamine. Protection by aminoguanidine and penicillamine grew weak over time, while BHA and quercetin prevented cytotoxicity throughout the time period. Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. *Significant as compared to control ($P < 0.05$), **Significant as compared to Fe^{3+} 12 µM:8-HQ 24 µM ($P < 0.05$), $^{\pi}$Significant as compared to thiamin 1mM and pyridoxamine 1mM ($P < 0.05$), $^{\alpha}$Significant as compared to pyridoxal 1mM and pyridoxamine 1mM ($P < 0.05$), $^{\beta}$Significant as compared to pyridoxal 1mM and thiamin 1mM ($P < 0.05$), $^{¥}$Significant as compared to BHA 50µM and quercetin 50µM ($P < 0.05$), $^{€}$Significant as compared to aminoguanidine 300µM and penicillamine 300µM ($P < 0.05$).

As shown in Table 4.1, Fe-induced cytotoxicity was accompanied by oxidative stress as measured by increased ROS formation and LPO. The B vitamins were assessed for their relative

effectiveness at preventing ROS formation and LPO. The order of effectiveness found for ROS scavenging activity was pyridoxamine > pyridoxal > thiamin, while the order of effectiveness for inhibiting LPO was pyridoxal, thiamin > pyridoxamine. Fe also caused a collapse of the MMP, and all agents were comparable in their level of protection against mitochondrial damage. BHA and quercetin were twice as effective compared to the dicarbonyl scavengers at preventing ROS formation, whereas they were only slightly more effective than aminoguanidine and penicillamine at decreasing LPO.

Table 4.1 Effect of therapeutic agents against iron-mediated oxidative stress in isolated rat hepatocytes

Compounds added	**ROS Formation** (FI units)		**Lipid Peroxidation** (μM)		**MMP** (FI units)	
Incubation time (45 min)						
Control (hepatocytes only)	84 ± 3		0.36 ± 0.05		481 ± 7	
+ $FeCl_3$ 12μM: 8OHQ 24μM	422 ± 11	[a]	2.60 ± 0.65	[a]	538 ± 7	[a]
+ Thiamin 1mM	276 ± 6	[b,c]	1.33 ± 0.04	[b,g]	488 ± 12	[b]
+ Pyridoxal 1mM	258 ± 5	[b,d]	1.27 ± 0.02	[b,g]	506 ± 9	[b]
+ Pyridoxamine 1mM	230 ± 6	[b,e]	1.69 ± 0.03	[b,e]	515 ± 12	[b]
+ BHA 50μM	210 ± 9	[b,f]	1.22 ± 0.04	[b,f]	514 ± 6	[b]
+ Quercetin 50μM	225 ± 6	[b,f]	1.15 ± 0.03	[b,f]	505 ± 6	[b]
+ Aminoguanidine 300μM	347 ± 12	[b,g]	1.49 ± 0.04	[b,g]	515 ± 7	[b]
+ Penicillamine 300μM	316 ± 8	[b,g]	1.42 ± 0.05	[b,g]	512 ± 5	[b]

Fe-induced cytotoxicity was initiated by ROS formation and LPO. Of the B vitamins, pyridoxamine was best at preventing ROS formation, and lipid peroxidation was best decreased by pyridoxal and thiamin. Quercetin and BHA were more effective than the dicarbonyl scavengers at inhibiting ROS formation and LPO. All agents were comparable in protection against mitochondrial toxicity. Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. [a]Significant as compared to control ($P < 0.05$), [b]Significant as compared to Fe^{3+} 12 μM:8-HQ 24 μM ($P < 0.05$), [c]Significant as compared to pyridoxal 1mM and pyridoxamine 1mM ($P < 0.05$),

[d]Significant as compared to thiamin 1mM and pyridoxamine 1mM ($P < 0.05$), [e]Significant as compared to thiamin 1mM and pyridoxal 1mM ($P < 0.05$), [f]Significant as compared to aminoguanidine 300µM and penicillamine 300µM ($P < 0.05$), [g]Significant as compared to BHA 50µM and quercetin 50µM ($P < 0.05$).

As shown in Figure 4.2 and 4.3, administration of Fe increased both protein carbonylation and DNA damage in hepatocytes. Figure 4.2 illustrates the protective effect of the B vitamins against protein carbonylation over the course of 90 min. At 30 min, all vitamins were comparable in their degree of protection against protein damage. However, protection by thiamin and pyridoxamine weakened over time, whereas pyridoxal maintained protection against protein damage. BHA and quercetin maintained protection against protein carbonylation over the course of 90 min, whereas protection by the dicarbonyl trapping agents grew weaker over time. As shown in Figure 4.3, all vitamins protected against Fe-catalyzed DNA damage. As DNA damage increased from 30 min to 90 min, pyridoxal and thiamin displayed the greatest inhibition of damage, followed by pyridoxamine. The free radical scavenger quercetin was best at preventing Fe-mediated DNA damage, and the remaining agents were equivalent in their level of protection.

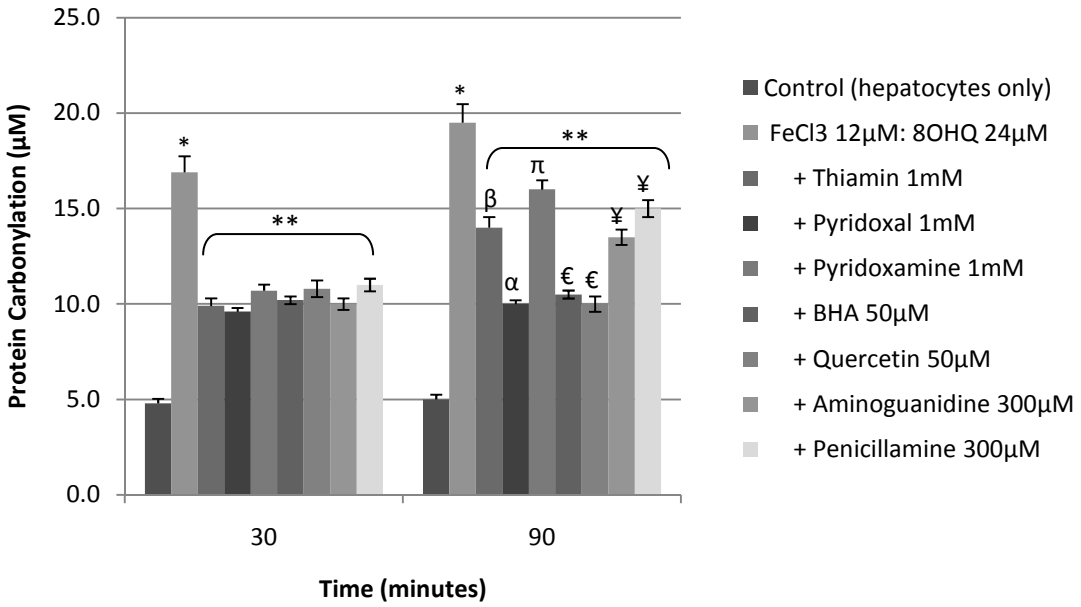

Figure 4.2 Effect of therapeutic agents against protein carbonylation in isolated rat hepatocytes

Protein carbonylation was inhibited by pyridoxal > thiamin > pyridoxamine. BHA and quercetin maintained their protection, while protection by aminoguanidine and penicillamine weakened over time. Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. *Significant as compared to control ($P < 0.05$), **Significant as compared to Fe^{3+} 12 μM:8-HQ 24 μM ($P < 0.05$), $^{\beta}$Significant as compared to pyridoxal 1mM and pyridoxamine 1mM ($P < 0.05$), $^{\alpha}$Significant as compared to thiamin 1mM and pyridoxamine 1mM ($P < 0.05$), $^{\pi}$Significant as compared to thiamin 1mM and pyridoxal 1mM ($P < 0.05$), $^{€}$Significant as compared to aminoguanidine 300μM and penicillamine 300μM ($P < 0.05$), $^{¥}$Significant as compared to BHA 50μM and quercetin 50μM ($P < 0.05$).

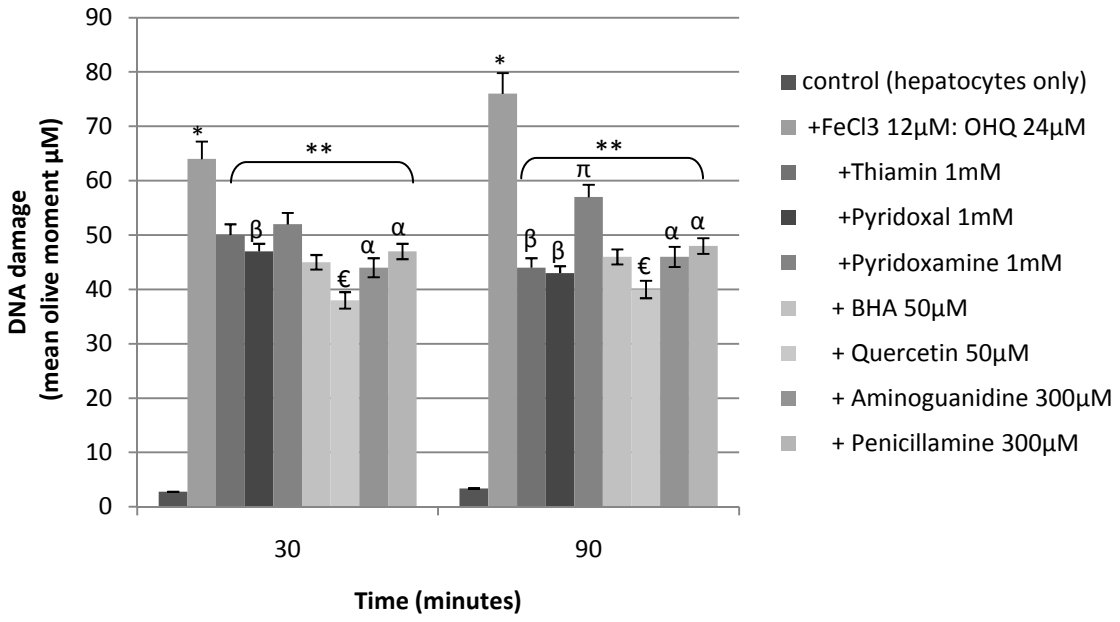

Figure 4.3 Protective effects of therapeutic agents against DNA damage in isolated rat hepatocytes

The therapeutic agents were evaluated for their ability to restore DNA single- and double strand breaks caused by Fe-mediated oxidative stress. Pyridoxal and thiamin displayed the greatest increase in protection over time, followed by pyridoxamine. Quercetin was best at decreasing DNA damage amongst the remaining agents. Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. *Significant as compared to control ($P < 0.05$), **Significant as compared to Fe^{3+} 12 µM:8-HQ 24 µM ($P < 0.05$), $^{\beta}$Significant as compared to pyridoxamine 1mM ($P < 0.05$), $^{\pi}$Significant as compared to thiamin 1mM and pyridoxal 1mM ($P < 0.05$), $^{€}$Significant as compared to aminoguanidine 300µM and penicillamine 300µM ($P < 0.05$), $^{\alpha}$Significant as compared to quercetin 50µM ($P < 0.05$).

As shown in Figure 4.4, pyridoxal inhibited the formation of hydroxyl radicals by 66%, and was comparable in activity to the lipid antioxidant BHA. Thiamin and pyridoxamine prevented formation by approximately 40% and 30%, respectively. Figure 4.5 illustrates the iron complexing potential of the B vitamins compared to the iron chelators deferiprone and pyridoxal isonicotinoyl hydrazone (PIH). This experiment confirmed that pyridoxal had the greatest Fe

complexing ability of the B vitamins, followed by thiamin. Pyridoxamine only demonstrated slight Fe chelating activity.

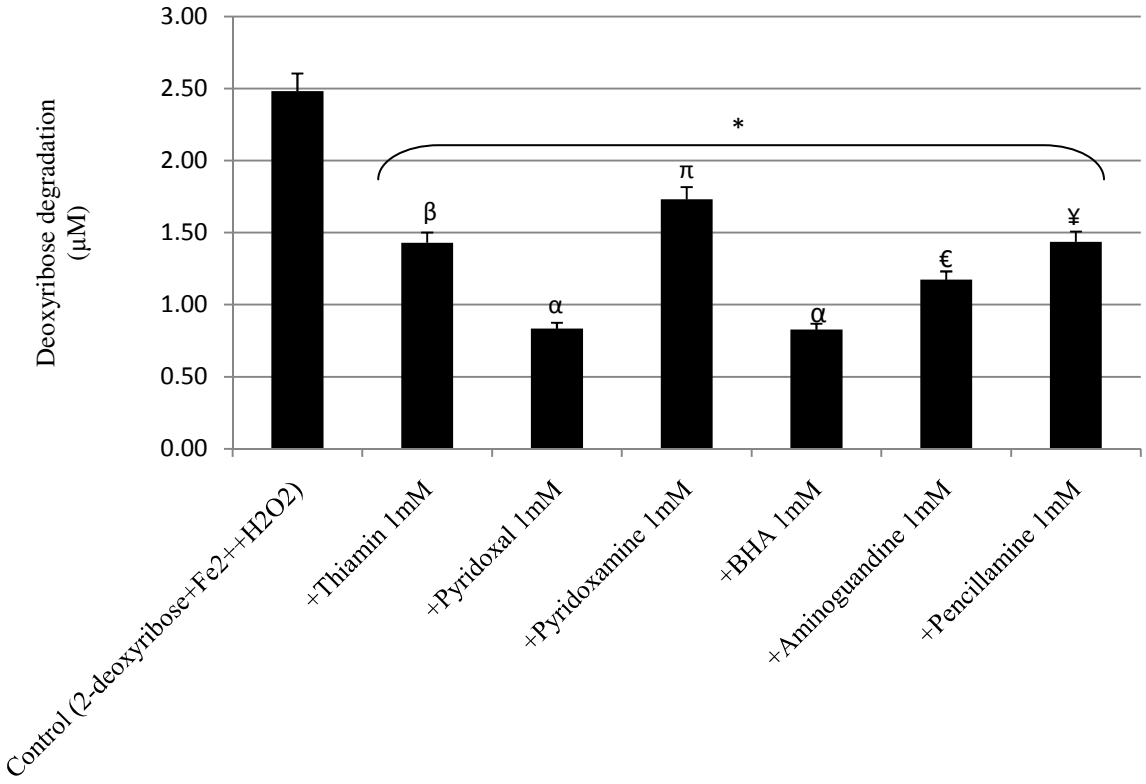

Figure 4.4 B vitamin hydroxyl radical scavenging activity

The formation of hydroxyl radicals was measured using the 2-deoxyribose oxidative degradation method. Pyridoxal exhibited the most hydroxyl radical scavenging activity that was comparable to the lipid antioxidant BHA (positive control). Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. *Significant as compared to 50 μM Fe^{2+} + 500 μM H_2O_2 + 3mM 2-deoxyribose ($P < 0.05$), $^{\beta}$Significant as compared to pyridoxal 1mM, pyridoxamine 1mM, BHA 50μM and aminoguanidine 300μM ($P < 0.05$), $^{\alpha}$Significant as compared to thiamin 1mM, pyridoxamine 1mM, aminoguanidine 300μM and penicillamine 300μM ($P < 0.05$), $^{\pi}$Significant as compared to thiamin 1mM, pyridoxal 1mM, BHA 50μM, aminoguanidine 300μM and penicillamine 300μM ($P < 0.05$), $^{€}$Significant as compared to thiamin 1mM, pyridoxal 1mM, pyridoxamine 1mM, BHA 50μM and penicillamine 300μM ($P < 0.05$), $^{¥}$Significant as compared to pyridoxal 1mM, pyridoxamine 1mM, BHA 50μM and aminoguanidine 300μM and penicillamine 300μM ($P < 0.05$).

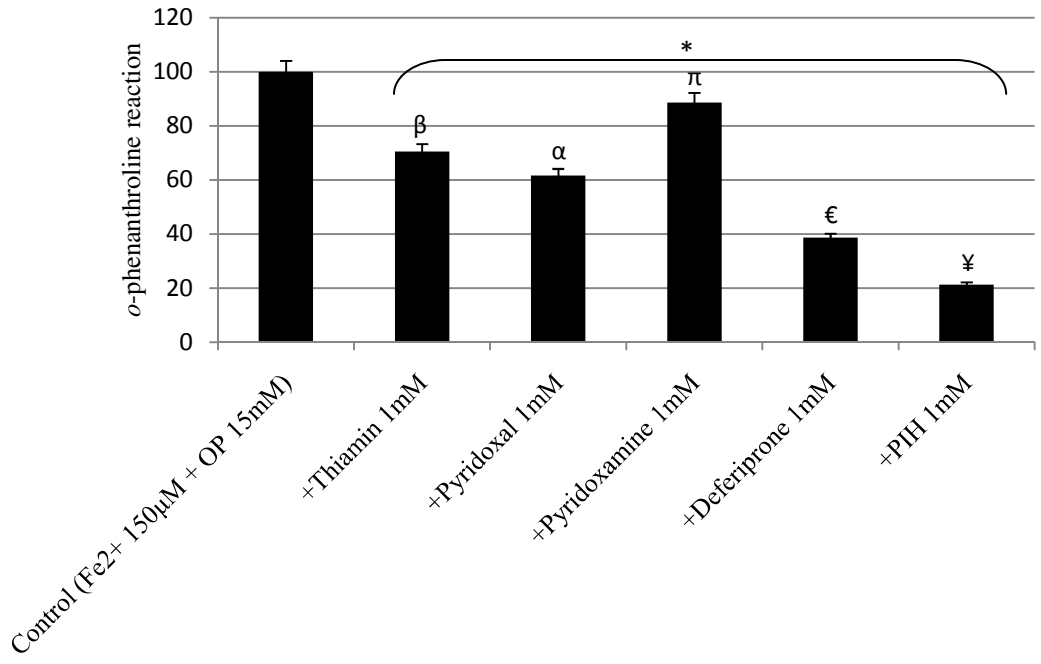

Figure 4.5 B vitamin:iron complexing activity

The order of B vitamin chelating activity found was pyridoxal > thiamin > pyridoxamine. The Fe chelators PIH > deferiprone were used as positive controls. Results were expressed as a percentage of the control. Refer to the Experimental Procedures for a complete description of the experiments performed. Mean±S.E. for three separate experiments are given. PIH; pyridoxal isonicotinoyl hydrazone. *Significant as compared to 150 µM Fe^{2+} + OP 15 mM ($P < 0.05$), $^{\beta}$Significant as compared to pyridoxal 1mM, pyridoxamine 1mM, deferiprone 1mM and PIH 1mM ($P < 0.05$), $^{\alpha}$Significant as compared to thiamin 1mM, pyridoxamine 1mM, deferiprone 1mM and PIH 1mM ($P < 0.05$), $^{\pi}$Significant as compared to thiamin 1mM, pyridoxal 1mM, deferiprone 1mM and PIH 1mM ($P < 0.05$), $^{€}$Significant as compared to thiamin, pyridoxal 1mM, pyridoxamine 1mM and PIH 1mM ($P < 0.05$), $^{¥}$Significant as compared to thiamin, pyridoxal 1mM, pyridoxamine 1mM and deferiprone 1mM ($P < 0.05$).

4.5 Discussion

The role of Fe in catalyzing ROS production is well known and evidence exists whereby oxidative radicals generated by Fe or other endogenous mechanisms may be associated either directly or indirectly with the pathogenesis of many chronic health conditions, such as those within the metabolic syndrome (Fujita *et al.*, 2009; Otogawa *et al.*, 2008; Sumida *et al.*, 2009).

Current research on vitamin B1 and B6 nutrient therapy is insufficient to confidently assess their mechanisms of action in such chronic conditions. Accordingly, in the present work we showed that Fe-mediated hepatocyte oxidative stress was prevented or delayed by vitamins B1 and B6 through several intracellular modes of action. Hepatocytes were pre-treated with Fe^{3+}:8-HQ for 1 h prior to the addition of the vitamins to verify whether hepatocyte cytotoxicity could be reduced or inhibited.

4.5.1 Cytotoxic Mechanisms of Fe-Mediated Oxidative Stress

Hepatocyte incubation with Fe^{3+}:8-HQ resulted in a substantial increase in cytotoxicity at 2 h and confirmed the transport of Fe into cells. However, before cytotoxicity ensued, early measurements of oxidative stress biomarkers indicated increased levels of ROS and LPO in hepatocytes. Protection by the lipid antioxidant BHA and the free radical scavenger quercetin provided further evidence that Fe toxicity was reactive-oxygen mediated. Fe also caused extensive hepatocellular protein carbonylation and DNA damage—both of which were prevented not only by the antioxidants, but also by the dicarbonyl scavengers aminoguanidine and penicillamine, suggesting the formation of LPO products, such as malondialdehyde and glyoxal, as a possible secondary outcome to ROS-mediated toxicity (Aldini *et al.*, 2007).

Protection against protein carbonylation by the lipid antioxidant BHA further supported this hypothesis. Protein carbonylation in hepatocytes was therefore presumably caused by (1) the oxidation of protein amino acid side chains or protein peptidic backbone by ROS and/or (2) the formation of carbonyl groups on protein or intramolecular cross-links by LPO products (Kim and Gladyshev, 2007; Mehta *et al.*, 2009; Petropoulos and Friguet, 2006). Extensive protection by

quercetin suggested ROS was the primary source of protein oxidation, but damage by lipid hydroperoxides could not be excluded as a potential mechanism based on the effectiveness of BHA.

The genotoxic potential of Fe was also investigated to determine whether Fe-mediated toxicity caused nuclear DNA damage in hepatocytes. Extensive attenuation of DNA damage by quercetin pointed to hydroxyl radicals as the primary cause of DNA damage in our system (Altman *et al.*, 1995; Aruoma *et al.*, 1989; Morel *et al.*, 1997; Wardman and Candeias, 1996). Earlier studies on Fe-induced DNA damage reported that Fenton-mediated hydroxyl radicals or other oxidative species were the cause of a range of DNA base modifications in rat kidney chromatin and DNA-protein cross-links in isolated DNA (Altman *et al.*, 1995; Toyokuni *et al.*, 1994). DNA damage by lipid hydroperoxides was possible but requires further investigation as both dicarbonyl trapping agents penicillamine and aminoguanidine also demonstrated hydroxyl radical scavenging activity as a key protective mechanism in our system (Figure 4.5) (Mehta *et al.*, 2009). The antioxidant activity of penicillamine was also supported by Russell's group who reported its significant effectiveness in scavenging hydroxyl radicals in a cell free system (Russell *et al.*, 1994) and by Fu's group who showed that its inhibitory effects against glucose-induced glycation were due to its antioxidant activity (Fu *et al.*, 1994). Gogasyavuz's group further demonstrated the protective effect of aminoguanidine against LPO in diabetic rat kidneys (Gogasyavuz *et al.*, 2002). Protection by both penicillamine and aminoguanidine therefore provided additional support for hydroxyl radical-mediated DNA oxidation.

4.5.2 Cytoprotective Mechanisms of B Vitamins

Vitamin B6

Pyridoxal was the most cytoprotective agent against Fe-catalyzed toxicity in hepatocytes, followed by thiamin. Protection by pyridoxal was attributed to its antioxidant activity, and to a lesser effect, its Fe chelating activity. The ability to complex Fe was demonstrated *in vivo* when Fe excretion in the rat increased upon intravenous administration of pyridoxal (Johnson *et al.*, 1982). The ability of pyridoxal to chelate Fe was verified using the OP assay, which measured a decrease in Fe^{2+}:OP levels by pyridoxal. However, its ability to reduce Fe^{2+}:OP binding activity was weak in comparison to the administered Fe chelators, suggesting that Fe chelation was not the primary mechanism of pyridoxal protection. The protective effect of pyridoxal against protein carbonylation and DNA damage was maintained over time, and in the case of DNA oxidation, pyridoxal exhibited an antidotal or rescue effect. Substantial protection by pyridoxal against deoxyribose degradation, which measured the formation of hydroxyl radicals, further substantiated its antioxidant activity. Finally, the ability of pyridoxal to decrease mitochondrial toxicity presumably resulted from its antioxidant activity or from its role as a mitochondrial transaminase cofactor (Depeint *et al.*, 2006a). Based on our findings and in order of priority, the antioxidant and Fe chelating activities of pyridoxal were the therapeutic mechanisms in our model for oxidative stress.

Cytoprotection by pyridoxamine was weakest amongst the B vitamins and only stabilized after 2 h. However, pyridoxamine was best at decreasing ROS formation. Its significant protection against protein carbonylation in hepatocytes resulted from its ROS scavenging or dicarbonyl scavenging activity (Mehta *et al.*, 2008; Mehta *et al.*, 2009). Pyridoxamine was less effective than pyridoxal at degrading deoxyribose and preventing DNA damage in hepatocytes, suggesting it did not specifically prevent hydroxyl radical formation. Rather, pyridoxamine may

have specifically inhibited other types of ROS, such as superoxide radicals, as Kannan et al. demonstrated the inhibition of superoxide radical generation by pyridoxamine in human monocytes (Kannan and Jain, 2004). Another recent study by Mahfouz's group reported the reduction of superoxide levels by pyridoxamine in cultured endothelial cells exposed to hydrogen peroxide-mediated oxidative stress (Mahfouz *et al.*, 2009). The dicarbonyl scavenging mechanism of pyridoxamine was supported by Metz et al. who claimed the B6 vitamin trapped lipid hydroperoxide intermediates *in vivo* (Metz *et al.*, 2003). Additional studies also reported that pyridoxamine trapped glyoxal or methylglyoxal and the adducts formed *in vitro* were identified (Nagaraj *et al.*, 2002; Voziyan *et al.*, 2002). Finally, pyridoxamine was least effective among the B vitamins at decreasing Fe:OP binding activity suggesting it was a poor Fe chelator (Mehta and O'Brien, 2007).

Vitamin B1

Cytoprotection by thiamin, like pyridoxamine, resulted from its ROS quenching or dicarbonyl trapping ability. Unlike pyridoxamine however, thiamin was more effective at preventing LPO in hepatocytes and inhibiting the oxidative degradation of deoxyribose. This suggests that thiamin may play a role in hydroxyl radical scavenging in addition to trapping of other types of ROS. The antioxidant properties of thiamin were previously demonstrated by our group as it decreased cumene-hydroperoxide-induced microsomal LPO (Mehta and O'Brien, 2007). Lukienko's group also showed the inhibition of microsomal LPO by thiamin in rat hepatocytes. His group reported that thiamin prevented free radical oxidation by interacting with free radicals and hydroperoxides to form non-toxic thiochrome and thiamin disulfide (Lukienko *et al.*, 2000; Mehta *et al.*, 2008; Sheline *et al.*, 2002). In further support of its proposed protective

mechanisms, we showed that thiamin delayed protein oxidation, and like pyridoxal, displayed an antidotal effect against DNA oxidation. Thornalley's group also demonstrated that thiamin inhibited plasma protein glycation in diabetic rats when it was used at a high dose (Karachalias *et al.*, 2005). Finally, thiamin also decreased mitochondrial toxicity in hepatocytes which was likely connected to its role as a mitochondrial coenzyme for pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase, both of which may have been deactivated by ROS (Depeint *et al.*, 2006b).

Our study was designed to probe the molecular cytotoxic targets of Fe as a model for oxidative stress in order to better understand the therapeutic value of vitamins B1 and B6. Our results demonstrated a great sensitivity of lipids, protein and DNA to oxidative stress induced by Fe in primary rat hepatocytes. To our knowledge, we are the first to report the antidotal and/or inhibitory activity of vitamins B1 and B6 against Fe-mediated protein oxidation and DNA damage in hepatocytes. The ability of the therapeutic agents to maintain protection and/or rescue hepatocytes from damage over time suggests that the B vitamin protective mechanisms in hepatocytes are effective and may function in combination with the oxidative stress repair processes in hepatocytes. DNA repair capacity is essential in order to prevent genotoxicity, and the repair of most types of DNA oxidation is mediated by base-excision enzymes (Abalea *et al.*, 1998; Jaruga and Dizdaroglu, 1996; Krokan *et al.*, 1997). During oxidative stress, protein modifications that are irreversible must be eliminated by degradation. However, certain types of protein damage, such as oxidation to sulfur containing amino acids like cysteine and methionine, can be reduced by several intracellular enzymes and hence may be deemed reversible (Petropoulos and Friguet, 2006). It is clear from our study that pharmacological intervention is crucial to the rescue and repair processes of hepatocytes that are subjected to oxidative damage.

Such exogenous repair mechanisms are necessary in addition to the existing intracellular processes in order to reduce damage to the greatest capacity.

The role of nutrient therapy in preventing or retarding disease progression could be important in Western societies where diseases within the metabolic syndrome are becoming more prevalent and are fueled by poor diet and inadequate nutrient supplementation. The ability to reverse or inhibit toxicity using naturally-occurring agents could prove useful as therapy to minimize the complications raised by oxidative stress, for two critical reasons: (a) their ability to target the multiple endpoints of oxidative stress, (b) their predicted clinical safety profile and reduced likelihood of adverse events. Although the concentrations of the therapeutic agents used in our system were significantly higher than their physiological levels, such concentrations were required in order to detect an accelerated protective response against an exaggerated dose of Fe within the 4 h hepatocyte viability period. The high concentrations used were further supported by the ACMS technique described previously. Additional studies are required to assess this technique in our model and further explore the interconnections of oxidative stress, free radicals and B1 and B6 vitamin therapy. However, we hope that our research in isolated rat hepatocytes has provided preliminary insight into this fundamental relationship.

Chapter 5. General Conclusions and Future Perspectives

5.1 Hypotheses Revisited

5.1.1 Hypothesis 1: *The endogenous toxins, glyoxal and Fenton-mediated ROS, can cause oxidative damage to hepatocytes by a) oxidizing lipids, generating further RCS; b) forming protein carbonyls; c) oxidizing DNA. The increased damage by ROS and RCS can overcome intracellular antioxidant defense and subsequently lead to cytotoxicity.*

In the General Introduction we described two endogenous toxins that are associated with the Western diet and metabolic syndrome: 1) RCS (glyoxal) and 2) iron-mediated ROS. Glyoxal, as mentioned, is a reactive dicarbonyl that can be formed endogenously through the autoxidation or degradation of glycolytic intermediates, such as fructose or glucose and other downstream intermediates. Dietary iron overload, as described, increases the risk of several chronic diseases through its intracellular Fenton-mediated pro-oxidant effect; that is, its ability to catalyze the formation of deleterious ROS. In this study, we investigated the cytotoxic mechanisms of glyoxal and iron in isolated rat hepatocyes. In order to determine the cytotoxic targets of the specific toxins, we incubated the toxins with compounds with well-established protective mechanisms, such as antioxidants, ROS scavengers, metal chelators and carbonyl scavenging agents.

An LD50 concentration of glyoxal incubated with hepatocytes caused increased ROS formation, lipid peroxidation and mitochondrial toxicity (Chapter 3). Cytotoxicity was prevented by ROS scavengers, antioxidants (including the lipid antioxidant BHA) providing evidence that cytotoxicity (including mitochondrial damage) was caused by ROS formation and lipid peroxidation. Glyoxal was proposed to target and damage proteins by two means—directly or indirectly through the generation of ROS. However, weak protection by ROS scavengers and

antioxidants versus strong protection by carbonyl scavenging agents pointed to direct glyoxal-protein binding as the most likely mechanism of glyoxal-induced protein carbonylation. Unpublished evidence of glyoxal-induced DNA damage suggested however that DNA was oxidized by ROS as observed by the significant inhibition of damage by the hydroxyl radical scavenger mannitol and the antioxidant trolox (see Appendix I).

Like glyoxal, we investigated the cytotoxic mechanisms of iron-mediated oxidative damage and determined that toxicity was ROS-mediated in our hepatocyte model (Chapter 4). Protection by the lipid antioxidant BHA and the free radical scavenger quercetin confirmed this effect. Iron also caused extensive protein carbonylation and DNA damage—both of which were prevented not only by antioxidants, but also by dicarbonyl scavenging agents. Iron-mediated damage to proteins resulted from either direct oxidation by ROS, or through indirect damage by products of lipid peroxidation, such as glyoxal and malondialdehyde. DNA damage, however, was more likely to have occurred as a result of ROS-mediated oxidation as observed by the strong protective effect by quercetin, a hydroxyl radical scavenger.

The schematic in Figure 5.1 depicts a summary of the results obtained based on Hypothesis 1.

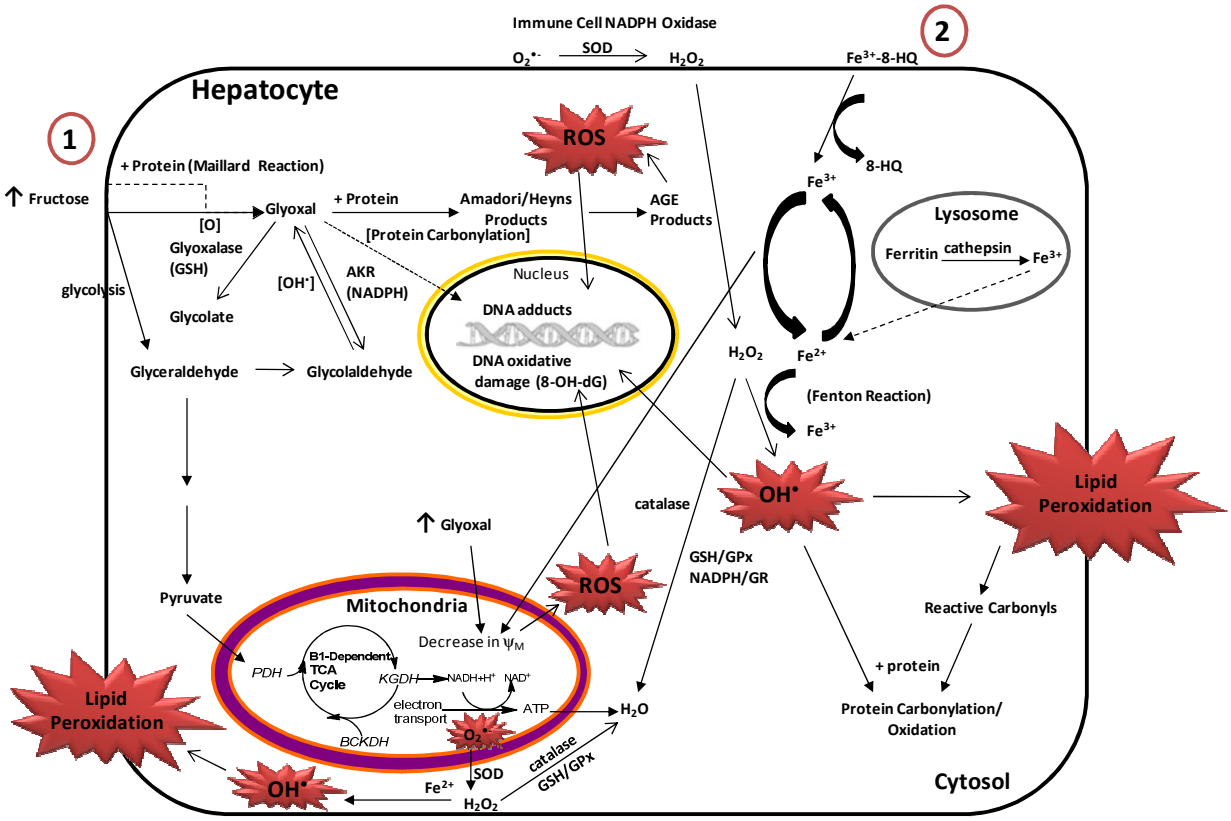

Figure 5.1 Schematic of endogenous toxin (RCS, ROS) cytotoxic mechanisms in the hepatocyte oxidative stress model. This illustration shows that the endogenous toxins researched in this thesis (RCS (glyoxal) (1) and Fenton-mediated ROS (2)) can cause oxidative damage to lipids, proteins and DNA. Lysosomal damage and mitochondrial toxicity induced by the endogenous toxins can also lead to deleterious ROS production. The pathways of RCS and ROS detoxification are also outlined in the schematic. In (1), dietary fructose can form endogenous glyoxal through autoxidation, the Maillard reaction or through Fenton-mediated oxidation of fructose or its glycolytic intermediates. Once formed, glyoxal can react with the amino groups of proteins and form AGE. This process also disrupts the mitochondrial membrane potential which causes ROS formation and lipid peroxidation. Glyoxal or its resulting formation of ROS can also cause DNA oxidation. In (2), ferrous iron (reduced) can catalyze the formation of hydroxyl radicals from H_2O_2 (Fenton reaction), which can cause lipid peroxidation, protein oxidation or DNA damage. Products of lipid peroxidation may also oxidize protein amino groups. Iron also disrupts the mitochondrial membrane potential, thereby initiating an increase in the formation of mitochondrial ROS.

5.1.2 Hypothesis 2: *B1 or B6 vitamins are multifunctional agents which can delay, prevent or rescue hepatocytes from oxidative damage induced by RCS (as exemplified by glyoxal) or Fenton-mediated ROS.*

The therapeutic properties of vitamins B1 or B6 are multi-fold. To the general public, they may not be known as a single entity but rather as part of a complex of multivitamins that should be consumed altogether to maintain good health. Vitamins have diverse biochemical functions—those within the B vitamin complex are of utmost importance because they can function as cofactors for enzymes that are vital to cellular function. One such example is the role of vitamn B6 in the 1-carbon metabolic pathway, which involves the transfer of 1-carbon groups for DNA synthesis and DNA methylation (Larsson *et al.*, 2010). The therapeutic properties of vitamins B1 and B6, however, go beyond their coenzyme status. As demonstrated throughout the chapters of this thesis, they may also function as antioxidants, ROS scavengers, metal chelators, AGE breakers or dicarbonyl traps. The therapeutic functions of vitamins B1 or B6 are of special interest in the area of diabetes and colon carcinogenesis. Oxidative stress in these conditions, as well as in other diseases associated with the metabolic syndrome, is characterized by multiple toxic endpoints—many of which are thought to be suppressed by vitamins B1 or B6. Furthermore, such diseases are correlated with deficiencies in these vitamins. Knowing what has been suggested for the function of vitamins B1 and B6, we aimed to investigate their key protective mechanisms in endogenous toxin-induced oxidative stress models. We hope that such an approach will move us one step closer to bridging the gap between the shortcomings of the Western diet and the causes of various chronic diseases.

5.1.2.1 Chapter 2

In Chapter 2, we set out to develop an initial understanding of the effects of vitamins B1 and B6 and their relative cytoprotective ranking against cell death, lipid peroxidation or ROS generated by carbonyls, hydroperoxides and mitochondrial toxins.

Of the vitamins investigated, the B1 vitamer thiamin was suggested to exhibit mitochondrial coenzyme activity as its dominant protective effect against carbonyl toxicity, whereas the B6 vitamers varied in their mechanisms of protection against glyoxal and acrolein. Protection by pyridoxal and/or PLP was suggested to occur through transition metal (iron) chelating activity or through the interaction of the B6 vitamer with protein as a safety mechanism to hinder glyoxal-protein binding. Pyridoxamine displayed ROS scavenging and antioxidant activity against glyoxal and acrolein, respectively, but was overall weak in preventing cytotoxicity compared to the other agents.

Hydroperoxide toxicity was best inhibited by the B6 vitamers, while B1 was ineffective in its protective capacity against hydroperoxide-mediated oxidative stress toxicity. Of the B6 vitamers, pyridoxine was cytoprotective when pre-incubated with hepatocytes prior to the addition of the toxin, likely resulting from its intracellular conversion to PLP. Pyridoxal, however, displayed the greatest antioxidant activity in both hepatocytes and microsomes in comparison to the other B6 vitamins, and was also cytoprotective. Overall, the B6 vitamins demonstrated antioxidant activity as their primary mechanism of protection against hydroperoxide-induced toxicity.

Mitochondrial toxicity induced by cyanide was inhibited by pre-incubated thiamin, pyridoxamine and pyridoxine. Thiamin was protective presumably through its coenzyme role in the mitochondria, whereas protection by pyridoxamine and pyridoxine was likely attributed to

their ROS scavenging or mitochondrial coenzyme activity. Copper toxicity and ROS formation was inhibited by PLP, pyridoxamine and vitamin B1. Protection by PLP and thiamin or TPP was likely a result of mitochondrial coenzyme activity, whereas pyridoxamine probably acted as a mitochondrial ROS scavenger.

The initial screening of the B vitamins provided the following conclusions of protective capacity against carbonyl, hydroperoxide and mitochondrial toxin-induced cytotoxicity, ROS formation and lipid peroxidation in hepatocytes: Thiamin/TPP acted primarily as a mitochondrial enzyme cofactor; pyridoxamine and pyridoxine as a ROS scavenger, pyridoxal/PLP as a metal chelator or antioxidant.

5.1.2.2 Chapter 3

The cytoprotective screening of the B vitamins against carbonyl stress triggered further interest in the cytotoxic mechanisms of glyoxal, with a specific focus on the role of glyoxal in protein carbonylation and preventative mechanisms. The focus of chapter 3 was to investigate the dicarbonyl trapping mechanisms of a group of therapeutic agents with amine or thiol functional groups.

Pyridoxamine was among the group of agents tested, in addition to other carbonyl scavenging drugs with anti-diabetic, anti-hypertensive, copper chelating and antioxidant properties. The mechanisms involving the inhibition of thiamin against glyoxal-induced protein carbonylation *in vivo* had been previously elucidated by another member of our lab (Shangari *et al.*, 2005), but additional research was performed involving the glyoxal trapping ability of thiamin and can be found in Appendix I.

The agents were either incubated in conjunction with glyoxal or were added to the hepatocyte incubate one hour after glyoxal to allow for the observation of an antidotal effect. The agents were also used at concentrations above, equal to, or below glyoxal to determine whether the observed therapeutic effects varied when different concentrations were used.

Interestingly, glyoxal-induced protein carbonylation was only inhibited by agents with concentrations equimolar to glyoxal (5 mM), apart from NAC, which only demonstrated an effect at twice the concentration of glyoxal. But the remaining agents—aminoguanidine, penicillamine, cysteine and hydralazine all inhibited and reversed glyoxal-induced protein carbonylation when incubated with hepatocytes at a concentration equal to the dicarbonyl.

A similar effect was observed when the agents were tested for their glyoxal trapping activity in a cell-free system. Aminoguanidine, penicillamine and cysteine displayed the most immediate and pronounced glyoxal trapping effect, whereas glyoxal trapping by pyridoxamine was delayed and weak. Metformin and NAC demonstrated the weakest glyoxal trapping ability.

When incubated at lower concentrations, aminoguanidine and penicillamine demonstrated ROS scavenging or antioxidant activity. Similarly, hydralazine, cysteine and NAC prevented glyoxal-induced lipid peroxidation at lower concentrations, and thus acted as antioxidants. Pyridoxamine and metformin were poor glyoxal scavengers, but were effective in decreasing ROS formation. Moreover, pyridoxamine was among the best at restoring the mitochondrial membrane potential, crediting its primary therapeutic role in preventing glyoxal toxicity to its mitochondrial coenzyme activity.

Although carbonyl trapping was an observed therapeutic mechanism for many of the agents tested, it was only apparent when the agents were used at concentrations equal to glyoxal. In *in vitro* studies, it is possible to investigate cytoprotective and cytotoxic effects using varying

concentrations, however the *in vivo* environment is unpredictable and highly variable. It is therefore crucial to identify the various mechanisms involved for agents with therapeutic value and to understand the scope of protection.

5.1.2.3 Chapter 4

As mentioned in Chapter 1, the Western diet is abundant in its supply of fructose, and studies also indicate the profusion of heme iron from the diet. This is of particular concern in the pathogenesis of chronic liver disease and colorectal cancer (Bastide *et al.*, 2011; Corpet *et al.*, 2010; Freedman *et al.*, 2010; Pierre *et al.*, 2003). Recent evidence published by our group suggested that fructose toxicity was attributed to the formation of endogenous glyoxal, in the presence of an iron catalyst. Fenton-mediated oxidation of fructose therefore led to the generation of glyoxal (Feng *et al.*, 2009; Lee *et al.*, 2009). Our final objective was to elucidate the cytoprotective mechanisms of vitamins B1 and B6 against Fenton-mediated ROS formation in keeping with the goal of identifying natural, multifunctional therapies for intervention studies against Western diet-derived chronic diseases.

Pyridoxal was the most cytoprotective agent against iron-catalyzed toxicity in hepatocytes. Protection by pyridoxal was primarily a result of its antioxidant activity, as observed by the inhibition of iron-mediated lipid peroxidation and deoxyribose degradation by pyridoxal. Pyridoxal also exhibited iron chelating activity, but to a lesser degree compared to its pronounced antioxidant effect. The protective effect observed by pyridoxal against protein carbonylation and DNA damage was maintained over time, and the most attractive result was in the case of DNA oxidation whereby pyridoxal further reduced DNA damage over time. The cytoprotective outcome of pyridoxal against protein and DNA oxidation was more likely a result

of its antioxidant capacity as opposed to its dicarbonyl trapping ability, since pyridoxal was a poor glyoxal trapping agent (Appendix I).

Parallel to its role against glyoxal-induced toxicity, pyridoxamine was also most effective as a ROS scavenger in the Fenton-mediated oxidative stress model. Pyridoxamine also demonstrated significant protection against iron-mediated protein carbonylation, providing evidence of a dicarbonyl trapping effect in addition to its ROS scavenging activity. As shown in Chapter 3, pyridoxamine was initially weak in trapping glyoxal, but by 2 h it had scavenged 40% of the dicarbonyl. Therefore, pyridoxamine may be effective as a late stage trapping agent, which is consistent with its proposed role in the literature as an AGE inhibitor (Stitt *et al.*, 2002).

Thiamin acted like both pyridoxal and pyridoxamine in that it was an effective antioxidant, ROS scavenger and possible dicarbonyl trapping agent. However, based on the poor glyoxal trapping ability of thiamin (Appendix I), it is unlikely that dicarbonyl trapping was a primary mechanism of protection. Thiamin was effective at preventing lipid peroxidation and deoxyribose oxidation, therefore pointing to its antioxidant or ROS scavenging effect as its primary protective function against Fenton-mediated oxidative stress. Thiamin prevented iron-mediated protein carbonylation but grew weaker over time. However thiamin, like pyridoxal, acted as an antidote against DNA oxidation and was effective at reducing DNA damage over the course of time. Protection by thiamin against mitochondrial toxicity was either attributed to its coenzyme activity or its ROS scavenging function.

Based on our observations of the cytoprotective mechanisms of vitamins B1 and B6 against RCS and Fenton-mediated ROS formation, we conclude the following primary mechanisms of protection:

1) Thiamin is an antioxidant or ROS scavenger

2) Pyridoxal is an antioxidant or transition metal chelator

3) Pyridoxamine is a ROS scavenger and late stage dicarbonyl scavenger

The schematic in Figure 5.2 illustrates a summary of the results obtained based on Hypothesis 2.

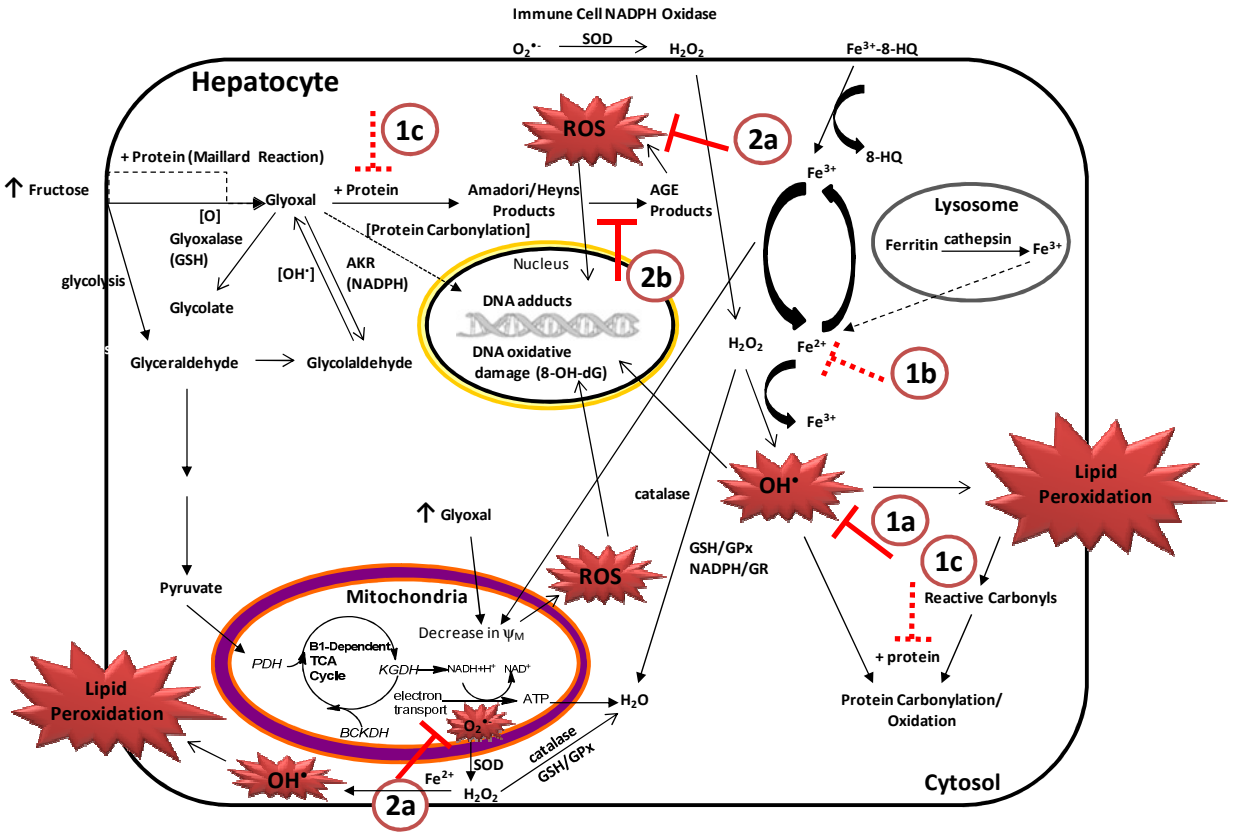

Figure 5.2 Schematic of B1/B6 vitamin protective mechanisms against endogenous toxin (RCS, ROS)-induced oxidative damage in isolated rat hepatocytes. The cytoprotective function of pyridoxal can be primarily attributed to the inhibition of hydroxyl-radical mediated damage (1a), and to a lesser extent, its iron chelating activity (1b). An additional protective mechanism of pyridoxal results from the formation of Schiff base adducts between pyridoxal and protein amino groups, thereby limiting the amount of amino groups available for reaction with dicarbonyls (1c). Pyridoxamine scavenges ROS (including mitochondrial ROS) (2a) and also scavenges glyoxal or downstream Amadori/AGE products (2b). Thiamin scavenges ROS (including mitochondrial ROS) (2a) and inhibits hydroxyl-radical mediated damage (1a). Note: This schematic is identical to Figure 5.1, but with the addition of the B1/B6 protective mechanisms.

5.2 Future directions

In this thesis, we developed an understanding of the specific protective mechanisms of vitamins B1 or B6 against endogenous toxins which we believe play a significant role in the pathology of many chronic conditions. Although the results obtained in this thesis address the stated hypotheses, we have yet to establish a direct relationship between vitamin B1 or B6 targeted therapy and diet-induced chronic metabolic diseases. Future avenues of research are described below which address the current knowledge gaps.

5.2.1 *In vivo* animal model

The results of this thesis suggest that vitamins B1 or B6 could act as useful nutritional interventions with the potential to alleviate or prevent the multiple endpoints of oxidative stress in the pathogenesis of acute hepatic conditions. Further investigation is necessary to determine which vitamin or combination of vitamin supplements and which doses are most useful for preventing oxidative damage and normalizing RCS and ROS levels. However, the first step in assessing the feasibility of vitamins B1 or B6 as micronutrient therapy in our proposed study is to develop an animal model that tests our objectives and hypotheses.

We therefore conducted a pilot experiment that mimicked our *in vitro* conditions (Hypothesis 1) and designed an animal model based on increased fructose (as excess energy from the diet) and increased dietary iron (to stimulate oxidative stress). Our model also included a group with reduced calcium. As dietary calcium is known to reduce the bioavailability of iron (Shawki and Mackenzie, 2010), we presumed a diet low in calcium would increase the availability of redox active iron and hence increase oxidative stress. Our hypothesis was based on the idea that a high-fructose and high-iron diet can lead to oxidative stress (short-term study)

and subsequent chronic hepatic disease and/or colon carcinogenesis (long-term study) through the formation of reactive protein carbonyls, formed by the interaction of early glycolysis products (derived primarily from fructose) with reactive oxygen (resulting from Fenton iron).

In summary, the results from the pilot study showed that the concentration of serum and liver malondialdehyde (MDA) was increased in the combined fructose, iron and reduced calcium group (Group C). Protein carbonyl formation in the liver and colonic epithelium was also markedly increased (> 10 times) in the same group. The experimental description, design and the results obtained from the pilot experiment can be found in Appendix II of the thesis.

5.2.1.1 Additional *in vivo* studies

Our next goal is to use the above animal model to evaluate dietary intervention with vitamin B6 to determine whether it can decrease oxidative damage (assessed by measuring levels of protein carbonyls and ROS) and chronic metabolic disease risk. The Western diet not only induces the formation of endogenous toxins, but it is also said to have a direct impact on the micronutrient status of the body. We will therefore investigate the effect of vitamin B6 supplementation as well as deficiency on the development of chronic metabolic disease. Our decision to assess the function of B6 arose from recent epidemiological studies that support the importance of vitamin B6 as a risk factor for CRC. Specifically, these recent studies successfully determined an inverse association between plasma levels of vitamin B6 (PLP and pyridoxal) and CRC (Eussen *et al.*, 2010; Larsson *et al.*, 2010; Le Marchand *et al.*, 2009).

Other avenues of research include the use of copper-deficient diets in place of increased iron. This is based upon the notion that diets with fructose and reduced copper caused oxidative stress as a result of increased hepatic iron (Fields and Lewis, 1997) and by recent studies

showing that low copper induces hepatic fatty acid synthesis and NAFLD (Aigner *et al.*, 2010). The substitution of fructose with other sweeteners, such as glucose or sucrose (glucose+fructose), represents another possible future study to identify whether results are conflicting or unexpectedly similar. Finally, dietary interventions with other therapeutic agents, such as omega-3 fatty acids (reduced formation of inflammatory lipids) and vitamin C and E (antioxidants) can also be investigated and compared with studies on vitamin B6 in the proposed *in vivo* model.

Expanding our knowledge from *in vitro* hepatocyte studies to an *in vivo* animal model will allow for a more complete assessment and understanding of the role that the Western diet plays in contributing to chronic metabolic disease. Further understanding will in turn provide us with a greater platform for determining natural preventative measures and therapies against obesity, the metabolic syndrome and chronic disease.

5.2.2 Limitations of research

The ACMS technique was effective at predicting the molecular cytotoxic and cytoprotective mechanisms of the endogenous toxins and therapeutic agents researched in this hypothesis, but the method is limited to the study of acute effects. The short-term viability of isolated primary rat hepatocytes restricts the opportunity for long-term *in vitro* analysis. Although a cultured hepatocyte model may extend the viability of hepatocytes to over 24 h, the down-regulation of CYP450 activity after only 4 h consequently affects phase 1 metabolism and therefore weakens the model. Nevertheless, the isolated hepatocyte model allows for the efficient modulation of conditions and treatments given and provides the ability to rapidly measure biochemical oxidative stress markers and endpoints.

The high sensitivity of isolated hepatocytes and the variability between isolation procedures delineates further drawbacks. The isolation procedure utilized in our lab involves numerous intricate steps which may as a result cause mechanical disruptions to hepatocytes and subsequently affect the viability of hepatocytes. An additional concern lies in the higher than physiological concentrations of toxins and therapeutic agents used in the isolated hepatocyte model. These concentrations often do not correlate with *in vivo* concentrations or acute levels of exposure and are a recurring subject of enquiry among reviewers of our work, despite the explained ACMS method.

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Appendices

Appendix I: Unpublished results supporting Chapter 3 findings

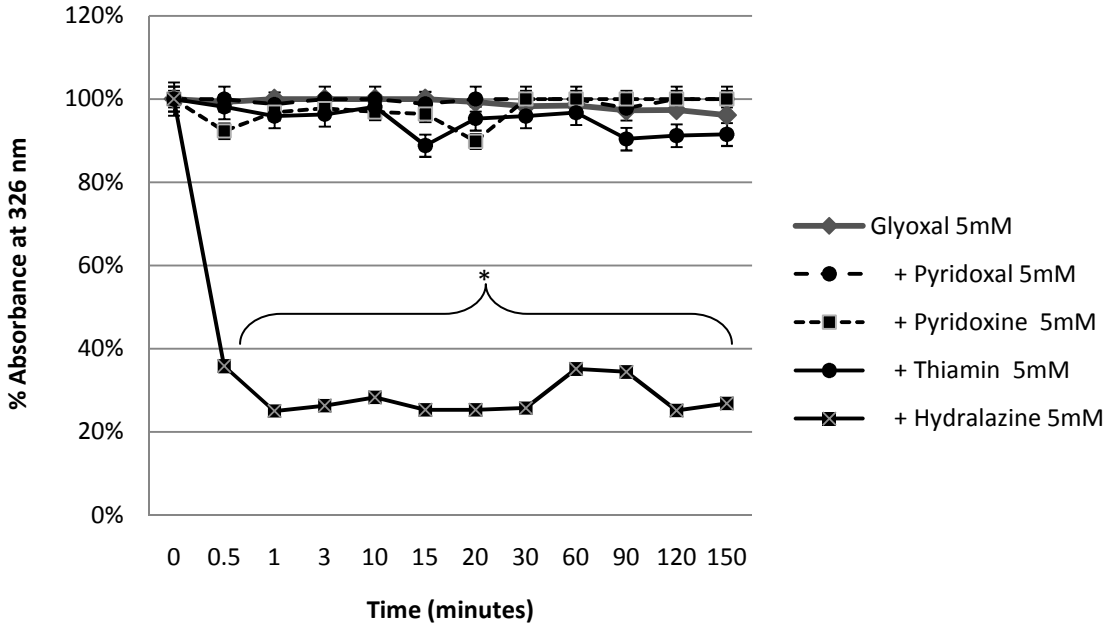

Figure AI.1 Measurement of glyoxal disappearance using Girard's Reagent T with equimolar concentrations of pyridoxal, pyridoxine, thiamin and hydralazine (5mM). This figure depicts results which were not relevant to the original manuscript but are relevant to the thesis. The absorbance corresponds to the percentage of glyoxal present in the medium. In the figure, the only agent which demonstrated effective glyoxal trapping ability was hydralazine. Pyridoxal, pyridoxine and thiamin were all ineffective at trapping equimolar glyoxal in the cell-free system, suggesting they were poor scavengers of glyoxal. Mean ± SE for three separate experiments are given. *Significant as compared to glyoxal 5mM ($P < 0.05$). Please refer to the methods section in Chapter 3 for a complete description of the experiment.

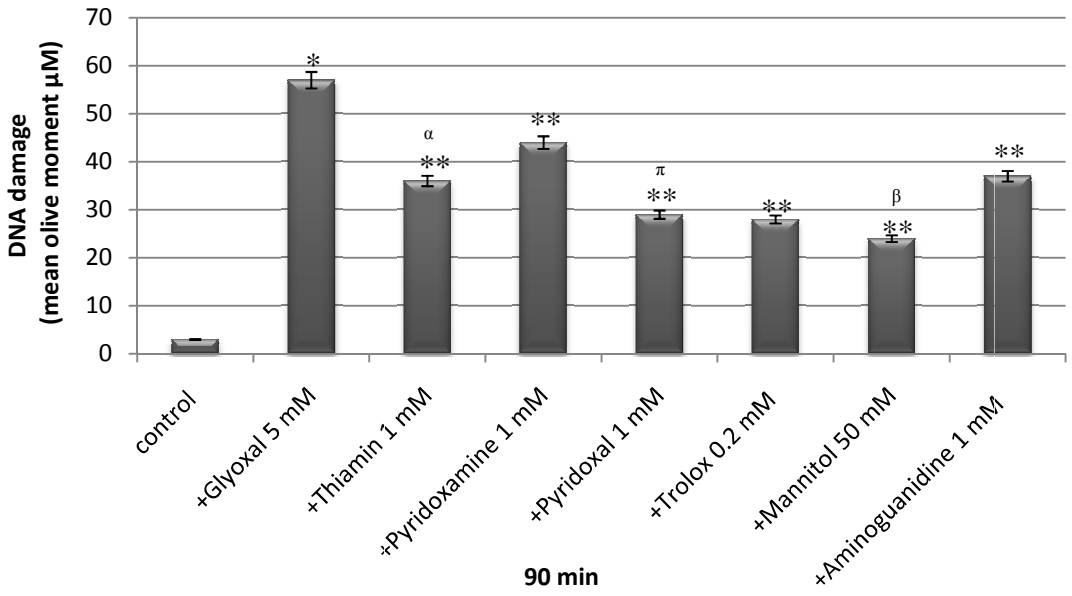

Figure AI.2 Protective effect of therapeutic agents against glyoxal-induced DNA damage in isolated rat hepatocytes. The therapeutic agents were evaluated for their ability to inhibit DNA damage, as measured by the Comet assay. Of the B vitamins, DNA damage was best inhibited by pyridoxal > thiamin > pyridoxamine. Inhibition of DNA damage by the hydroxyl radical scavenger mannitol and the antioxidant trolox, and to a lesser extent by the dicarbonyl trapping drug aminoguanidine, provide evidence that DNA was more likely oxidized by ROS than glyoxal. *Significant as compared to control ($P < 0.05$), **Significant as compared to Glyoxal 5 mM ($P < 0.05$), $^{\alpha}$Significant protection as compared to pyridoxamine 1mM ($P < 0.05$), $^{\pi}$Significant protection as compared to thiamin 1mM and pyridoxamine 1mM ($P < 0.05$), $^{\beta}$Significant protection as compared to trolox 200 µM and aminoguanidine 1mM ($P < 0.05$). Mean ± SE for three separate experiments are given. Please refer to the methods section in Chapter 4 for a complete description of the Comet assay.

Appendix II: Pilot *in vivo* study in F344 rats

AII.1 Study Description

The recent World Cancer Research/American Institute for Cancer Research review identified the risk of CRC as convincingly associated with increased obesity (particularly visceral adiposity), physical inactivity, the consumption of red/processed meats and alcohol consumption, and inversely associated with increased intake of calcium, milk/dairy products, garlic and foods containing dietary fiber.

We have interpreted these factors as ones that are associated with 1) excess energy, 2) inflammation, a result of decreased calcium and decreased colonic barrier function, and 3) oxidative stress with increased iron. We have designed an animal diet that simulates these conditions based on 1) increased fructose (rather than starch), 2) decreased calcium, and 3) increased dietary iron.

Previous studies have demonstrated the effects of each of these variables on chronic disease and/or cancer risk in animals but they have never been assessed together (Fields and Lewis, 1997; Ilsley *et al.*, 2004; Newmark *et al.*, 2009). Our expectation from *in vitro* studies and a review of the literature is that the concomitant exposure to factors 1-3 will expose the liver and colon to increased RCS and ROS and give rise to liver disease and/or colon carcinogenesis (McKeown-Eyssen *et al.*, 2010).

We propose to test the effect of the combined diet with:

1) A pilot study to assess the methods and measure oxidative stress and protein carbonyls in plasma, liver and colon in a period of 3 months **(study completed and described below)**;

2) A medium term test of the combined effect of the risk factors versus the control diet on colonic aberrant crypt foci (ACF), and biochemical markers of oxidative stress in animals euthanized at 4 months **(study in progress)**;

3) A long term carcinogenesis, NASH and/or diabetes test of the combined effect of the risk factors versus the control diet on colonic tumors and/or AGE formation in rats euthanized at 18 months (or earlier if in distress) **(study in progress)**.

We also propose to test the effect of related diets:

4) Short term studies to assess whether vitamin B6 inhibits the formation of protein carbonyls **(study in progress)**:

a. Fructose, aFe, aB6
b. Fructose, eFe, aB6
c. Fructose, eFe, mB6
d. Fructose, eFe, eB6

m = marginal; a = adequate (normal levels); e = excess

5) Short term studies to determine whether fructose-based and oxidative stress diets increase protein carbonyl formation synergistically **(study in progress)**:

a. Glucose, aCa, aFe
b. Glucose, mCa, eFe
c. Fructose, aCa, eFe
d. Fructose, mCa, eFe

m = marginal; a = adequate (normal levels); e = excess

Markers for analysis include measures of inflammation (C-reactive protein), oxidative stress (TBARS, ROS, ferrous iron), energy excess (insulin, triglycerides), circulating carbonyls (4-HNE, glyoxal, MDA) and plasma and tissue protein carbonyls with animals euthanized at approximately 2 months (or earlier if in distress). Blood and tissue samples will be collected with euthanasia/necropsy.

Our expectation is that the combined diet will result in colonic inflammation and ACF and NAFLD (or NASH), with an inverse relationship to vitamin B6 or calcium, and that the studies will identify the dietary components and risk biomarkers most important for the conduct of future clinical studies.

AII.2 Hypotheses:

1. A high-fructose and high-iron diet can lead to oxidative stress and subsequent chronic disease or colon cancer through the formation of reactive carbonyls, formed by the interaction of early glycolysis products (derived primarily from fructose) with reactive oxygen (resulting from Fenton iron). A reduction in calcium in the proposed diet will result in more permeable colonic epithelium, bacterial fragment translocation, colonic inflammation and oxidative stress **(pilot study completed, long term study in progress)**.

2. Dietary intervention in a high-fructose and high-iron diet with vitamin B6 can decrease oxidative damage and chronic metabolic disease risk. Similarly, a high-fructose, high-iron and reduced B6 diet can further increase oxidative damage and chronic disease risk **(future study)**.

AII.3 Materials and Methods (Pilot Study)

AIII.3.1 Materials

TBARS Assay Kit# 10009055 and Protein Carbonyl Assay Kit #10005020 were purchased from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Animal diets were prepared by Research Diets Inc. (New Brunswick, NJ).

AIII.3.2 Animal Treatment

6 Fischer 344 male rats weighing 155-160 g were assigned to the experiment and housed in ventilated plastic cages. Care and treatment of the rats were in compliance with the guidelines of the Canadian Council on Animal Care, and the protocol was approved by the University of Toronto Animal Care Committee. The following environmental conditions applied throughout the course of the study: 12 air changes per hour, 12 h light/day cycle, and an ambient temperature of 21-23°C with a 50-60% relative humidity. After one week acclimatization (on standard rat chow and water *ad libitum*) the rats were randomized into 3 groups of 2 animals each, housed in 2 animals per cage, and provided with the following diets:

Group A) Control diet, AIN-76A with carbohydrate as corn starch (57% kcal);

Group B) Fructose (57% kcal), high-iron diet, with iron as ferric citrate (0.2 % kcal); and

Group C) Fructose, high-iron, low calcium diet (reduced from 1.3% to 0.89 % kcal).

The animals were checked weekly for any evidence of distress or bloody feces and the rats were weighed every second week. The rats were anesthetized after 12 weeks by CO_2 inhalation. Blood was collected by cardiac puncture and livers were removed at sacrifice and frozen for later

analysis of oxidative stress (TBARS, clinical chemistry tests) and protein carbonyl formation. Colonic epithelial cells from half of the colons were isolated upon sacrifice and then frozen for further analysis of oxidative stress and protein carbonyls. Upon isolation, liver slices and the other half of the colons were sent for histological examination for evidence of ACF and acute liver disease. Serum samples were collected by centrifugation of blood and were aliquoted at -80°C for later analysis. A description of liver and colonic epithelium homogenate preparation is described below under the appropriate assay sections.

AIII.3.3 Colonic epithelial cell preparation

Colonic epithelial cells were collected as described by Bjerknes et al. (Bjerknes and Cheng, 1981). Briefly, the colon from anus to cecum was harvested, rinsed with 3 mL of Hank's (-Ca, -Mg) then split lengthwise and spread, mucosa surface up, on filter paper. The paper was then placed in a Petri dish and the colon covered gently with Hank's (-Ca, -Mg) containing EDTA (10 mM), with gentle swirling. After 10 minutes, the mucosal cells were harvested with a single sweep of a blunt spatula as a pile of mucosal cells as crypts that could be easily picked up and transferred to a collection tube. The preparation was completed at room temperature. Colonic cells were separated by centrifugation. The supernatant was discarded, and the pellet was used for analysis.

AIII.3.4 TBARS Assay

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) was used for determining lipid peroxidation in serum, liver and colonic epithelium (Kit #10009055, Cayman Chemical Co.). The MDA-TBA adducts formed by the reaction were measured fluorometrically

at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Liver and colonic epithelium were weighed and homogenized in radioimmunoprecipitation buffer (RIPA) containing protease inhibitors (e.g. EDTA). Samples were centrifuged at 1,600 g for 10 minutes at 4°C. The supernatant was used for the determination of lipid peroxidation. Standards of MDA, ranging from 0-5 µM were also prepared prior to beginning the experiment.

Briefly, tubes containing 100 µL aliquots of the samples or standards were mixed with an equal volume of sodium dodecyl solution (SDS) and 4 mL of colour reagent. Tubes were boiled vigorously for 1 h and then immediately incubated in an ice bath to stop the reaction. The tubes were centrifuged at 1,600 g for 10 minutes at 4°C, and fluorescence was measured using a Shimadzu RF5000U fluorescence spectrophotometer. The values of MDA for each sample were calculated from the standard curve (Yagi, 1998).

AIII.3.5 Protein Carbonyl Assay

Protein carbonyl formation in serum, liver homogenate and colonic epithelium cells were measured by method of DNPH reaction (Kit #10005020, Cayman Chemical Co.). The amount of protein-hydrazone produced was quantified spectophotometrically at an absorbance of 370 nm. Protein concentrations of the samples were standardized to 10 mg/mL before performing the assay. Liver and colonic cells were weighed, rinsed with PBS and homogenized in cold phosphate buffer, pH 6.7 containing EDTA. The homogenates were centrifuged at 10,000 g for 15 minutes at 4°C. Contaminating nucleic acids were removed from the supernatant by incubating samples with streptomycin sulphate 1% for 15 minutes and centrifuging the samples at 6,000 g for 10 minutes at 4°C. The remaining supernatant was used for determining protein carbonyl content.

Briefly, samples were incubated for 1 h with DNPH or 2.5 M HCl (as a sample control). Samples were then lysed with 20% TCA and 10% TCA, with centrifugation after each step. Samples were resuspended in a 1:1 ethanol/ethyl acetate mixture and centrifuged, with the process repeated three times. After the final wash, pellets were resuspended in guanidine hydrochloride and samples and the corresponding controls were measured at 370 nm using a Shimadzu UV-vis spectrophotometer. The protein carbonyl concentration was calculated using the extinction coefficient of 11,000 $M^{-1}cm^{-1}$ (Levine *et al.*, 1994; Reznick *et al.*, 1992).

AII.4 Results

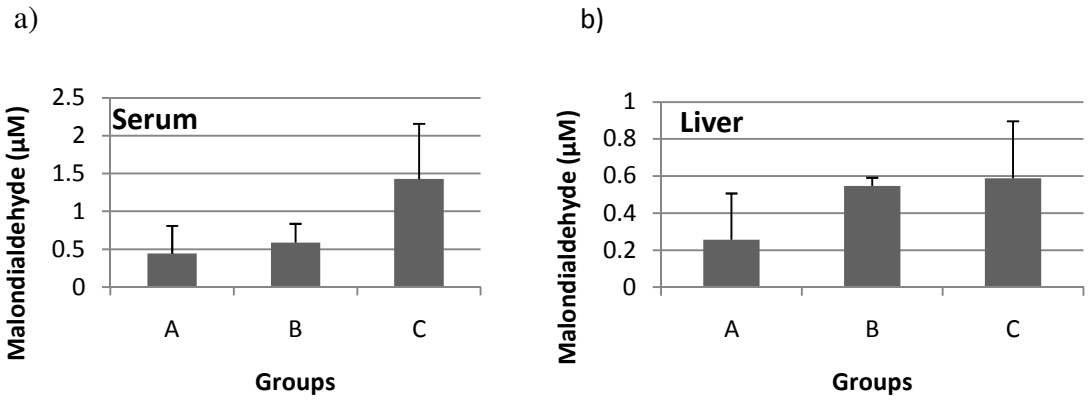

Figure AII.1 Measurement of malondialdehyde in a) serum and b) liver samples. Malondialdehyde (MDA) was measured as a marker of oxidative stress. MDA concentration was highest in group C > group B > group A in serum and was elevated in both groups B and C in the liver. Colonic epithelial cells did not show any evidence of malondialdehyde formation. Group A: control (starch-based diet); Group B: high-fructose, high-iron (as ferric citrate, 10x); Group C: high-fructose, high-iron, low calcium. n=2.

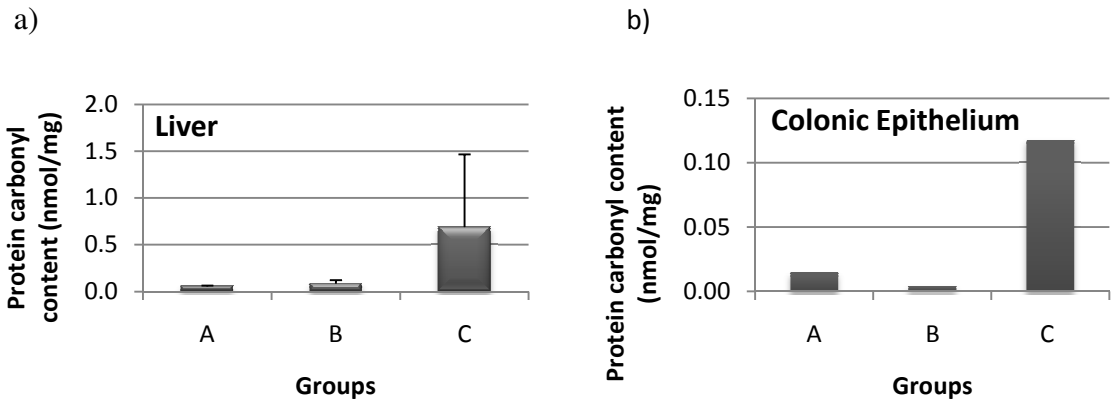

Figure AII.2 Protein carbonyl formation in a) liver and b) colonic epithelium samples. In the liver and colon, protein carbonyl formation was markedly increased in group C, as compared to Groups A and B. Protein carbonyl content was marginal in serum samples. Group A: control (starch-based diet); Group B: high-fructose, high-iron (as ferric citrate, 10x); Group C: high-fructose, high-iron, low calcium. a) n=2; b) n=1.

AII.5 Preliminary Considerations

Although the results obtained are not statistically significant, it was necessary to conduct a preliminary experiment to assess the effectiveness and reliability of our experimental protocols, as well as to ensure that a positive trend was observed to provide a basis for moving forward with longer term studies. The pilot study comprised of 6 animals in total (2 per group) and the results were variable within individual groups. Therefore it is crucial to assess a minimum of 4-6 animals per group in order to account for the variability.