REGULATION OF OXIDATIVE STRESS AND ITS MODULATION BY NATURAL HEALTH PRODUCTS

By

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ABSTRACT

Oxidative stress is characterized by the cellular accumulation of reactive oxygen species (ROS). Increased production of ROS, such as the superoxide anion ($O_2^{\cdot -}$), or a deficiency in their clearance by antioxidant defences, mediates the cellular pathology. Non-alcoholic fatty liver disease (NAFLD) is a broad spectrum liver disorder commonly manifesting in a milieu of the metabolic syndrome. Oxidative stress is an important pathogenic mediator in NAFLD, and in its associated morbidities like atherosclerosis. The objective of my research was to investigate the regulation of oxidative stress and the antioxidant actions of natural health products (NHPs) in the context of NAFLD and its associated disorders.

The $O_2^{\cdot -}$ generating reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase contributes to atherogenesis by facilitating macrophage induced vascular injury. In manuscript I, the plant alkaloid berberine effectively abolished NADPH oxidase mediated $O_2^{\cdot -}$ production in lipopolysaccharide (LPS) stimulated macrophages. Real-time PCR analysis and siRNA transfection studies revealed that berberine mediated its effects through down-regulation of the oxidase's catalytic subunit $gp91^{phox}$. Berberine also restored the activity of the $O_2^{\cdot -}$ clearing enzyme superoxide dismutase (SOD).

High fat diet (HFD) fed rodents are a popular model for investigating NAFLD pathogenesis. In manuscript II, folic acid supplementation significantly reduced HFD-induced hepatic oxidative stress and liver injury in mice. Folic acid decreased NF-κB/DNA binding, down-regulated the gene expression of NADPH oxidase, and inhibited its activation. The antioxidant activities of SOD and catalase were also restored and the reduced to oxidized glutathione ratio (GSH:GSSG)

was re-established with folic acid supplementation. Folic acid's hepatoprotective antioxidant effects were associated with a marked improvement in liver histology.

Homocysteine (Hcy) levels are perturbed in NAFLD, but the etiology is unclear. In manuscript III, HFD fed mice exhibited decreased Hcy levels. Real-time PCR and Western Immunoblotting analysis revealed that Hcy catabolising enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) were increased in the liver of these animals. The transsulfuration activities of these enzymes were elevated and coincided with enhanced hepatic hydrogen sulfide (H_2S) biosynthesis. Glutathione was maintained despite increased hepatic oxidative stress.

Taken together, NHPs such as berberine and folate, and Hcy catabolising enzymes CBS and CSE might have therapeutic potential for managing oxidative stress in NAFLD and its associated co-morbidities.

FORWARD

This thesis is presented in a manuscript style format. Each manuscript has been published. The list of co-authors and journal names for each manuscript are as follows:

Manuscript I:

Lindsei K. Sarna, Nan Wu, Sun-Young Hwang, Yaw L. Siow and Karmin O. 2010. Berberine Inhibits NADPH Oxidase Mediated Superoxide Anion Production in Macrophages. *Canadian Journal of Physiology and Pharmacology*. 88: 369-378.

Manuscript II:

Lindsei K. Sarna, Nan Wu, Pengqi Wang, Sun-Young Hwang, Yaw L. Siow and Karmin O. 2012. Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase. *Canadian Journal of Physiology and Pharmacology*. 90: 155-165.

Manuscript III:

Sun-Young Hwang*****, Lindsei K. Sarna*****, Yaw L. Siow, Karmin O. High fat diet stimulates hepatic cystathionine β-synthase and cystathionine γ-lyase expression. 2013. *Canadian Journal of Physiology and Pharmacology*. 91: 913-919. ***** co-first author

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In loving memory of my Didos

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and **Michael Sarna**

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LIST OF ABBREVIATIONS

(AP-1)	activator protein-1
(ALT)	alanine aminotransferase
(AST)	aspartate aminotransferase
(AFLD)	alcoholic fatty liver disease
(CVD)	cardiovascular disease
(CBS)	cystathionine-*b*-synthase
(CSE)	cystathionine-γ-lyase
(DHF)	dihydrofolate
(DMSO)	dimethyl sulfoxide
(MTT)	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(DTNB)	5,5’-dithio-bis(2-nitrobenzoic acid)
(e^-)	electrons
(EMSA)	electrophoretic mobility shift assay
(eNOS)	endothelial nitric oxide synthase
(FAD)	flavin adenine dinucleotide
(FR)	folate receptor
(GDI)	guanyl nucleotide dissociation inhibitors
(H&E)	hematoxylin and eosin
(HFD)	high fat diet
(Hcy)	homocysteine
(H_2O_2)	hydrogen peroxide
(H_2S)	hydrogen sulfide
(8-OH-dG)	8-hydroxy-2’-deoxyguanosine
(HNE)	4-hydroxy-2-nonenal
($OH^{\cdot}$)	hydroxyl radical
(HOCl)	hypochlorous acid

($LOO^{\cdot}$)	lipid peroxyl radical
(LPS)	lipopolysaccharide
(MDA)	malondialdehyde
(MCD)	methionine choline deficient
(5-MTHF)	5-methyl-tetrahydrofolate
(5,10-methylene-THF)	5,10-methylene tetrahydrofolate
(NHPs)	natural health products
($NO^{\cdot}$)	nitric oxide
(NOS)	nitric oxide synthases
(NBT)	nitroblue tetrazolium
(NAFLD)	non-alcoholic fatty liver disease
(NASH)	non-alcoholic steatohepatitis
(NRF2)	nuclear factor erythroid 2–related factor 2
(NF-kB)	nuclear factor kappa B
(ANOVA)	one-way analysis of variance
(GSSG)	oxidized glutathione
($ROO^{\cdot}$)	peroxyl radicals
($ONOO^{-}$)	peroxynitrite
(PMA)	phorbol 12-myristate 12-acetate
(PCFT)	proton coupled folate transporter
(PLP)	pyridoxal 5′-phosphate
(ROS)	reactive oxygen species
(qRT-PCR)	real-time polymerase chain reaction
(RFC)	reduced folate carrier
(GSH)	reduced glutathione
(NADPH oxidase)	reduced nicotinamide adenine dinucleotide phosphate oxidase
(redox)	reduction/oxidation
(SAM)	s-adenosylmethionine

($O_2^{\cdot -}$)	superoxide anion
(SOD)	superoxide dismutase
(THF)	tetrahydrofolate
(TBARS)	thiobarbituric acid reactive substances
(T2DM)	type 2 diabetes mellitus

I. INTRODUCTION

1.1. Background

Reactive oxygen species (ROS) is a generalized term referring to a broad range of naturally occurring oxygen derived molecules. These small and chemically reactive molecules share the ability to donate or accommodate electrons (e^-). ROS may be sub-classified as radical or non-radical species depending on whether the molecule in question contains unpaired or paired e^-, a feature that ultimately determines its chemical instability and reactive properties. Direct evidence supporting the biological relevancy of ROS was first reported in 1954 by Commoner *et al.* who demonstrated that free radicals are a common component of life, and that their levels are associated with the metabolic activity of respective tissues (Commoner et al., 1954). In the years to follow, Denham Harman would propose the "free radical theory of aging", which would position free radical species as a major underlying cause of cellular damage, tissue degeneration and aging (Harman, 1956). The early impression of ROS as only accidental, cytotoxic by-products of cellular metabolism would however be challenged by subsequent studies that identified specialized enzymes that could metabolize and clear ROS from cells (McCord & Fridovich, 1969) as well as those which implicated ROS in the mediation of protective (Johnston et al., 1975) and normophysiological (Gruetter et al., 1979) processes. With perception shifted, a new paradigm would emerge in the field of free radical biology. Today, this paradigm recognizes that: 1) ROS are regulated endogenously by their rate of generation and clearance; and 2) ROS are inherently involved in both normophysiological and pathophysiological processes. Investigating the complex mechanisms involved in the regulation of ROS, and how it translates to physiological versus pathophysiological events, remains an active and ongoing area of biomedical research.

Non-alcoholic fatty liver disease (NAFLD) is defined by a broad spectrum of related hepatic disorders. It ranges from simple steatosis or "fatty liver", to non-alcoholic steatohepatitis (NASH) and cirrhosis in its most advanced stage. Although the first characterization of NAFLD was reported more than 60 years ago (Zelman, 1952), it was not until 1980 that Ludwig *et al* distinguished it as a disease (Farrell & Larter, 2006; Kleiner et al., 2014). Over the past decade, insight into the etiology and pathogenesis of NAFLD has begun to emerge (Farrell & Larter, 2006). Today, it is estimated that NAFLD manifests in 1 billion people worldwide (Loomba & Sanyal, 2013). NAFLD is strongly associated with metabolic syndromes, and is particularly prevalent among obese and type 2 diabetic populations. Although a number of underlying pathogenic mediators have been identified, the mechanisms by which they contribute to NAFLD pathogenesis remain poorly understood. With the growing number of hepatic and extra-hepatic complications associated with NAFLD, as well as the limited strategies approved to manage the disorder clinically, NAFLD has become a serious global health issue (Loomba & Sanyal, 2013).

Natural health products (NHPs) encompass a wide range of products that are sold primarily in a medicinal format. Although these products are regulated in Canada, they are available over the counter and do not require a prescription for use. NHPs are sold for the purpose of maintaining or promoting health, re-establishing function or with the intent of preventing, treating or diagnosing disease. NHPs range from macro and micronutrient supplements (vitamins, minerals, amino acids, essential fatty acids, probiotics, or any other substance obtained from plants, animals or other organisms), to traditional medicines and homeopathic preparations. In Canada, all NHPs are regulated by Health Canada's *Natural Health Product Regulations*, which ensures

all products sold in Canada are safe, effective and of highest quality. Almost 3/4 of Canadians have used a NHP (Health Canada, 2014)**.**

1.2. Research objectives

The overall objective of the research is to investigate the regulation of oxidative stress and the antioxidant actions of NHPs in the context of NAFLD and its associated co-morbidities.

II. LITERATURE REVIEW

2.1. Oxidative Stress

2.1.1. Reactive oxygen species (ROS)

ROS is a generalized term referring to both radical and non-radical oxygen containing molecules. Radical species such as superoxide anion ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$) and the hydroxyl radical ($OH^{\cdot}$) are collectively defined by unpaired e^-, while non-radical species such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) are similar in that they contain only paired e^- (Rada et al., 2008; Siow et al., 2011a). Each ROS is however characterized by its own chemical and biological properties (Table 1), which in turn determines the overall stability of the molecule as well as its potential chemical and biological interactions. Of the various ROS, $O_2^{\cdot -}$ is of particular interest since its formation facilitates the generation of a number of other ROS including, but not limited to H_2O_2, $OONO^-$ and $OH^{\cdot}$. Fluctuations in $O_2^{\cdot -}$ levels can therefore profoundly influence ROS levels in cells.

2.1.2. Sources of ROS

There are a number of sources that may contribute to ROS formation in cells. Certain extrinsic factors such as UV and ionizing radiation can liberate ROS (Winterbourn, 2008) leading to unintentional and uncontrolled generation of these species in cells. Oxidation of phenols, as well as auto-oxidation of certain compounds following exposure to transition metals, may be intrinsic sources that can also lead to ROS formation (Siow et al., 2011a; Winterbourn, 2008). While there are a variety of sources that can lead to involuntary cellular ROS generation, there are also a number of enzymes and enzymatic systems that contribute to the production of these reactive molecules in cells. Complexes belonging to the mitochondrial electron transport chain, drug

Table 1. Chemical and biological properties of ROS

ROS	Stability (half life)	Membrane permeable	Chemical reactions with other ROS	Biological reactions	Reference
$O_2^{\cdot-}$		No	$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$ $O_2^{\cdot-} + H_2O_2 \xrightarrow[Fe]{} OH^{\cdot} + O_2 + OH^-$ $O_2^{\cdot-} + NO^{\cdot} \rightarrow OONO^-$	thiols transition metals ascorbate	(Winterbourn & Hampton, 2008)
H_2O_2		Yes	$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe} OH^{\cdot} + O_2 + OH^-$	thiols transition metals methionine	(Rada et al., 2008)
$OH^{\cdot}$	10^{-9}s	(N/A)		very reactive and non-discriminatory DNA Carbohydrates phenylalanine lipids (peroxidation)	(Valko et al., 2007)
$NO^{\cdot}$	5s	Yes	$NO^{\cdot} + O_2^{\cdot-} \rightarrow OONO^-$	protein (nitrosylation)	(Valko et al., 2007)
$OONO^-$	10ms	Yes	$OONO^{\cdot} \rightarrow OH^{\cdot} + NO_2^{\cdot}$	thiols ascorbate tocopherol methionine DNA protein (tyrosine nitration) lipids (peroxidation)	(Pacher et al., 2007) (Valko et al., 2007) (Winterbourn & Hampton, 2008)

Abbreviations: $O_2^{\cdot-}$, superoxide anion, H_2O_2; hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical; $OONO^-$, peroxynitrite; ($NO_2^{\cdot}$) nitrogen dioxide radical.

metabolizing flavoenzymes such as cytochrome p450, as well as xanthan oxidase and nitric oxide synthases (NOS) are among many of the endogenous enzymatic systems that have been shown to generate ROS. The mitochondria is considered one of the major subcellular sources of ROS in cells (Droge, 2002). Molecular oxygen is converted to $O_2^{\cdot -}$ as a result of e^- leakage from respiratory complexes I and III, and by reverse e^- transport at complex II. It is estimated that approximately 2% of the oxygen consumed in the liver is converted to $O_2^{\cdot -}$ while in other organs, such as the kidney, brain, heart and skeletal muscle, only 0.1-0.2% is converted to $O_2^{\cdot -}$ (Tahara et al., 2009).

2.1.3. NADPH oxidase

Arguably, one of the most intriguing ROS generating systems discovered to date is the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase. Much like other ROS generators, this family of $O_2^{\cdot -}$ and H_2O_2 generating enzymes are heterogeneously distributed among virtually all cell and tissue types (Bedard & Krause, 2007; Lambeth, 2007). In contrast to other ROS producing enzymes however that have additional metabolic and functional roles, the only known biological function of NADPH oxidases is the generation of ROS (Bedard & Krause, 2007). This primary function for endogenous ROS production makes the NADPH oxidase truly unique among the intrinsic enzymatic ROS generators. To date, the best characterized and understood family member is the classical phagocytic NADPH oxidase (Fig. 1.1) (Lambeth, 2007). This $O_2^{\cdot -}$ generating oxidase was first identified in immune cells where it participates in, and functions as, an essential component of the microbicidal respiratory burst. The phagocytic NADPH oxidase is comprised of a membrane-bound dimer composed of

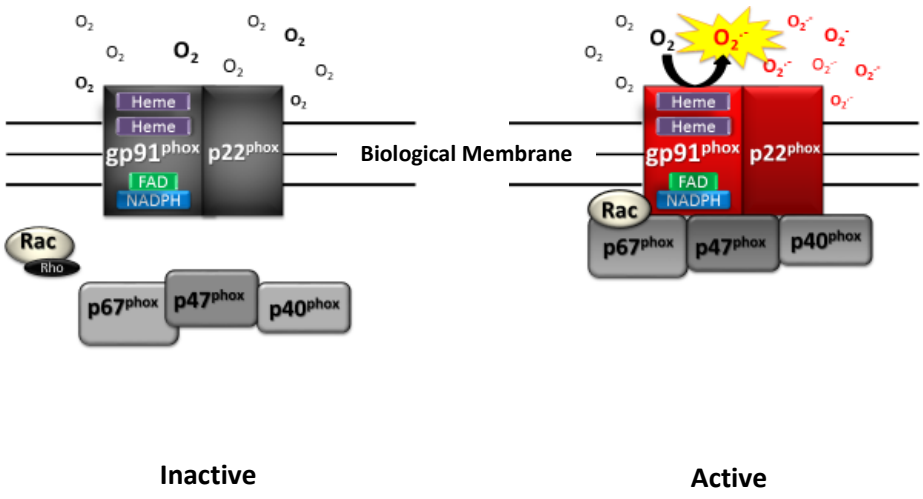

Fig. 1.1. Schematic representation of the phagocytic NADPH oxidase

A schematic representation of the classical phagocytic NADPH oxidase in both its inactive and activated states. (Image based on Bedard & Krause, 2007, Physiological Reviews)

gp91^{phox} and p22^{phox} subunits (collectively termed cytochrome b_{558}), as well as p47^{phox}, p67^{phox}, p40^{phox} and Rac 1/2 subunits, which remain localized in the cytosol when the enzyme is in an inactivated state. Activation of the oxidase complex requires translocation and interaction of cytosolic subunits with the membrane bound dimer. The gp91^{phox} is considered the main catalytic subunit of the phagocytic NADPH oxidase, as it contains the co-factors that facilitate transmembrane e^- transfer from NADPH donor, leading to the 1 e^- reduction of molecular oxygen. The remaining subunits of the NADPH oxidase perform essential stabilizing and regulatory functions that collectively support the full activation of the complex (Bedard & Krause, 2007).

Since the initial discovery of the phagocytic NADPH oxidase, six additional homologues of gp91^{phox} have been identified in the human genome (Brandes et al., 2014). These include Nox1-5 isoforms, which like the phagocytic (Nox2) isoform, generate $O_2^{\cdot -}$, as well as dual oxidases Duox 1 and 2, which generate H_2O_2 through a $O_2^{\cdot -}$ intermediate (Bedard & Krause, 2007). The various homologues differ by their structure, regulation, levels of expression, and cellular distribution. Regulation of NADPH oxidases are complex and isoform specific. Post-translational modifications and translocation of regulatory subunits from the cytosol to the membrane, are required for the activation of Nox1-3, while Nox 5 isoenzyme and Duox1 and 2 require binding of cytosolic calcium to the membrane bound subunits for enzyme activation (Brandes et al., 2014). In contrast, Nox4 appears to be constitutively expressed and is localized in the membrane with p22^{phox}. Transcription, translation and stabilization of the mature Nox4 protein appear to be the only mechanisms regulating Nox4 activity, with no additional control mechanisms or regulatory subunits having been identified (Brandes et al., 2014). Several transcription factors,

including nuclear factor kappa B (NF-kB) and activator protein (AP)-1 regulate NADPH oxidase gene expression (Bedard & Krause, 2007). Other factors including cytokines (Brandes et al., 2014), hormones (Kim et al., 2012), growth factors (Brandes et al., 2014), lipids (Colston et al., 2005), micronutrients (Banfi et al., 2001), mechanical stress (Brandes et al., 2014), and even certain ROS (Colston et al., 2005; Selemidis et al., 2007) work concertedly to control NADPH oxidase activity through both transcriptional and post-translational regulatory mechanisms.

2.1.4. Antioxidant defenses

The levels of ROS are not only regulated endogenously by their rate of production, but also by their rate of clearance. To avoid the accumulation of these highly reactive molecules, cells are equipped with a broad range of enzymatic and non-enzymatic antioxidant defense mechanisms (Table 2). Although functionally diverse, all antioxidants share a common property in that they neutralize, or at least limit the reactivity of ROS by increasing their chemical stability (Winterbourn, 2008). A number of enzymes function to efficiently metabolize and clear ROS both intra- and extra-cellularly (Nordberg & Arner, 2001). Superoxide dismutase (SOD), which catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2, was among the first enzymatic antioxidants discovered (McCord & Fridovich, 1969). Other enzymatic antioxidants have since been identified, and include catalase, which rapidly decomposes H_2O_2, as well as others such as glutathione peroxidase and glutathione reductase which perform similar functions (Nordberg & Arner, 2001; Winterbourn, 2008). There are also a variety of non-enzymatic small molecule antioxidants that can directly quench cellular ROS. Vitamins including ascorbic acid (vitamin C) and α-tocopherol (vitamin E) have long been known for their superior antioxidant function. Although

Table 2. Major endogenous antioxidant defenses

Antioxidant	Type	Substrate	Reaction	Reference
Superoxide dismutase (SOD)	Enzymatic antioxidant	$O_2^{\cdot-}$	$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$	(Winterbourn & Hampton, 2008) (Nordberg & Arner, 2001)
Catalase	Enzymatic antioxidant	H_2O_2	$2H_2O_2 \rightarrow O_2 + 2H_2O$	(Winterbourn & Hampton, 2008) (Nordberg & Arner, 2001)
Glutathione peroxidase	Enzymatic antioxidant	H_2O_2	$2GSH + H_2O_2 \rightarrow 2H_2O + GSSG$	(Nordberg & Arner, 2001)
Glutathione reductase	Enzymatic antioxidant	GSSG	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$	(Nordberg & Arner, 2001)
Glutathione	Direct antioxidant (hydrophilic)	$OH^{\cdot}$ H_2O_2 $LOO^{\cdot}$	H transfer	(Aquilano et al., 2014) (Galano & Alvarez-Idaboy, 2011)
Ascorbate	Direct antioxidant (hydrophilic)	$O_2^{\cdot-}$ $OH^{\cdot}$ $ONOO^-$	e^- transfer	(Winterbourn & Hampton, 2008) (Sharma & Buettner, 1993)
Tocopherol	Direct antioxidant (lipophilic)	$OH^{\cdot}$ $ONOO^-$ $ROO^{\cdot}$ $LOO^{\cdot}$	e^- transfer	(Winterbourn & Hampton, 2008) (Sharma & Buettner, 1993) (Packer et al., 1979)

Abbreviations: $O_2^{\cdot-}$, superoxide anion; H_2O_2; hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical; $LOO^{\cdot}$, lipid peroxyl radical; $OONO^-$, peroxynitrite; $ROO^{\cdot}$, peroxyl radicals; reduced glutathione GSH; oxidized glutathione, GSSG.

these vitamins increase the stability of ROS by donating their own e^-, their resultant radical species are significantly less reactive (Winterbourn, 2008) and can be efficiently neutralized through various dismutation reactions (Packer et al., 1979; Sharma & Buettner, 1993). In fact, the 1 e^- oxidation of ascorbate was shown in both chemical (Packer et al., 1979) and biological (Sharma & Buettner, 1993) based systems to facilitate the regeneration of α-tocopherol from the tocopheroxy radical. Not only does this interaction demonstrate the synergistic action of these vitamins, but is also suggest that ascorbate is an important terminal small molecule antioxidant (Sharma & Buettner, 1993). The tri-peptide glutathione is one of the most abundant cellular thiols and it is synthesized in cells from glutamic acid, cysteine and glycine. Although glutathione serves multiple functions in cells, one of its best known roles is reduction/oxidation (redox) buffering. Glutathione can directly quench a variety of radical species including $OH^{\cdot}$ which is achieved exclusively by hydrogen transfer reactions (Galano & Alvarez-Idaboy, 2011). Glutathione also participates in the clearance of tocopherol and ascorbate radicals by re-generating these species into their active antioxidant forms (Valko et al., 2007; Winterbourn, 2008). Glutathione is also a co-factor in glutathione peroxidase and glutathione transferase dependant reactions and therefore facilitates the enzymatic clearance of H_2O_2 (Nordberg & Arner, 2001) and lipid hydroperoxides (Hayes et al., 2005) respectively. The effectiveness by which endogenous antioxidants quench ROS depends on their relative concentrations and efficiency for performing e^- transfer reactions. The multifaceted antioxidant functions of glutathione, along with its high cellular concentrations, make it one of the most important antioxidant buffering systems in cells. Enzymatic antioxidants, ascorbate and a-tocopherol are by contrast present in low levels, however their efficiency for clearing ROS is high (Droge, 2002). Free amino acids such as tyrosine, cysteine and methionine, are inefficient scavengers by

comparison, however they can have antioxidant function due to their high levels (Droge, 2002; Winterbourn & Hampton, 2008). The subcellular localization and proximity of antioxidants to sources of ROS production, as well as their solubility (lipophilic vs hydrophilic) and capacity for performing e^- transfer reactions, also determines the ROS scavenging potential of antioxidants (Winterbourn, 2008).

2.1.5. Redox homeostasis & signaling

In addition to the potential interaction that may occur between various ROS, it is also evident that ROS have the potential to rapidly react with, and chemically modify a variety of biological targets (Table 1.1). These interactions can be controlled by cells and have been harnessed as part of a regulated process known as redox signaling (Kaludercic et al., 2014). There is strong evidence that spatio-temporal fluctuations arise in ROS levels under normal physiological conditions and are attributed to regulated imbalances in ROS generation and their clearance by antioxidant defenses (Kaludercic et al., 2014). In turn, these controlled oscillations in ROS elicit signals that appear to control many of the aspects of cell physiology including normophysiological and stress related responses (Winterbourn & Hampton, 2008). Although the exact mechanisms by which ROS mediate redox dependant signals is incompletely understood, there are at least three mechanisms that are generally accepted. The first mechanism is mediated through changes in the cell's redox potential, which is often reflected by the ratio of reduced to oxidized glutathione (GSH:GSSG). A decrease in this ratio would indicate a shift to a more oxidizing state, which in turn would increase the disulfide content of target thiol proteins through equilibration (Winterbourn & Hampton, 2008). Alternatively, ROS may directly interact with specific down-stream targets (cysteine residues), or may be scavenged by sensors which in turn mediate the oxidation of intended targets (Winterbourn & Hampton, 2008). These redox

modifications can lead to gain or loss of protein function, and thus have the potential to regulate the activity of certain receptors, transporters, transcription factors and enzymes along with the trafficking of these proteins (Kaludercic et al., 2014). ROS dependant signals have been implicated in inflammatory and mechanical stress related responses (Droge, 2002) as well as in the regulation of programmed cell cycle events such as proliferation, senescence, cell death, survival responses (Burhans & Heintz, 2009; Fisher, 2009) and even circadian rhythms (Stangherlin & Reddy, 2013). The precision of redox signaling is dependent on the particular ROS, its efficiency for the reaction ($1e^-$ or $2e^-$ oxidation), and its proximity to the target. H_2O_2, with its low activity and ability to traverse biological membranes, has the potential for widespread diffusion and it appears to target and regulate a number of downstream signaling pathways (Winterbourn, 2008; Winterbourn & Hampton, 2008). It is unclear whether $O_2^{\cdot -}$ is directly involved in cell signaling, or whether its effects are mediated through its spontaneous or enzymatic decomposition to H_2O_2 (Fisher, 2009). It is likely that other ROS such as $OH^{\cdot}$, do not participate in redox signaling because of their high instability and potential for non-discriminate reactions (Winterbourn & Hampton, 2008). The ability for cells to utilize these highly reactive molecules for the purpose of cell signalling requires that not only their production be regulated, but that their reactions are specific, fast and reversible (Winterbourn & Hampton, 2008). Antioxidant defences, which are also activated during cellular redox signalling are an important "brake" that rapidly terminates specific signals to maintain redox homeostasis (Droge, 2002; Valko et al., 2007).

2.1.6. Oxidative stress

Although regulated fluctuations in ROS levels may perform important signalling roles, uncontrolled fluctuations in these chemically unstable and reactive molecules may have profound and deleterious effects (Droge, 2002). Loss of redox homeostasis, whereby ROS levels remain chronically elevated above normophysiological thresholds, gives rise to oxidative stress and its associated pathologies (Kaludercic et al., 2014; Winterbourn, 2008) (Fig. 1.2). Oxidative stress is a complex biological phenomenon that manifests as a result of excessive ROS generation and/or a deficiency in ROS clearance by antioxidant defences. The adverse effects associated with oxidative stress are mediated through defective redox signalling and by unfavourable oxidative modification of biomolecules. Radicals such as $OH^{\cdot}$ may react with polyunsaturated fatty acids and initiate a self-propagating chain reaction known as lipid peroxidation, which can damage cellular membranes and produce cytotoxic lipid end products (Halliwell & Whiteman, 2004). The cysteine and methionine residues of proteins are also highly susceptible to oxidation and ROS induced disulfide formations can lead to structural and functional impairments in proteins (Droge, 2002; Valko et al., 2007). ROS mediated modifications to DNA might also affect gene expression, and therefore could have widespread deleterious effects when unmanaged (Winterbourn, 2008). The highly reactive nature of $OH^{\cdot}$ potentiates its reaction with the purine and pyrimidine bases as well as the deoxyribose backbone of DNA which can ultimately lead to DNA lesions and strand breaks (Halliwell & Whiteman, 2004).

2.1.7. Biomarkers of oxidative stress

There are a variety of probes that are available to detect ROS and measure redox in cells. The nitroblue tetrazolium (NBT) and lucigenin probes may be used to detect particular ROS such as

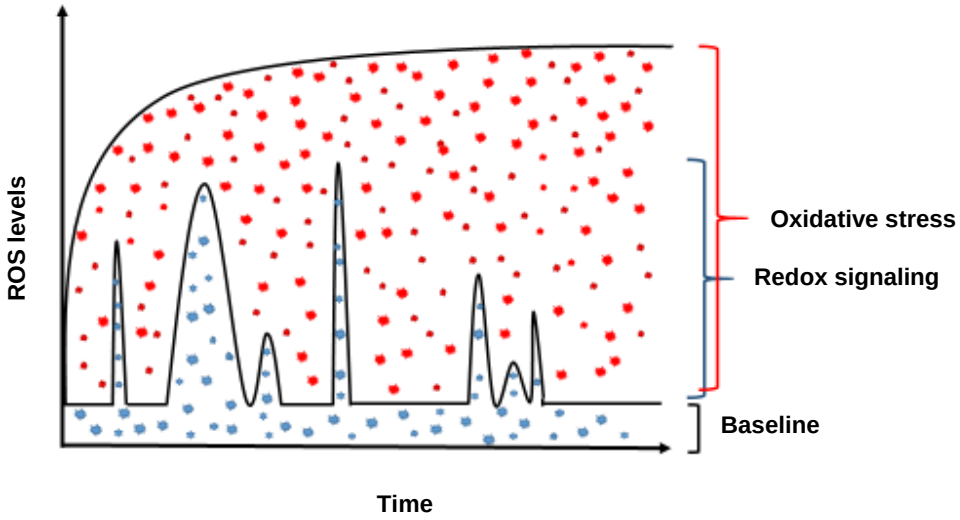

Fig. 1.2. Regulation of redox homeostasis: redox signaling versus oxidative stress

A schematic representation of redox homeostasis in cells. Reactive oxygen species (ROS) (*) levels are regulated in cells under normophysiological conditions. Acute fluctuations in ROS levels above baseline can be regulated in cells and exploited as part of cellular redox signaling. By comparison, chronic elevations in ROS above baseline levels gives rise to oxidative stress and its associated pathology. (Image based on Droge, 2002, Physiol Rev)

$O_2^{\cdot-}$ in cells (Choi et al., 2006; Li et al., 1998) while others such as dichlorodihydrofluroescein may be applied as a sensitive but general indicator of a pro-oxidative shift in cells (Winterbourn & Hampton, 2008). In light of their short half-lives, and the technical challenges associated with the direct measurement of ROS in biological samples, oxidation end-products have been widely used as *in-vivo* biomarkers of oxidative stress. Malondialdehyde (MDA), a stable end-product of lipid peroxidation is a common indices of oxidative stress as are other lipid end-products such as isoprostanes and 4-hydroxy-2-nonenal (HNE) (Siow et al., 2011a; Valko et al., 2007). Protein carbonyls (Valko et al., 2007) and 3-nitrotyrosine (Halliwell & Whiteman, 2004) are examples of protein oxidation end-products, while 8-hydroxy-2'-deoxyguanosine (8-OH-dG) is a common marker of oxidatively modified DNA (Siow et al., 2011a; Valko et al., 2007). The ratio of reduced to oxidized glutathione (GSH:GSSG) also provides an indication of the redox potential of cells and tissues (Lu, 1999). Elevated levels of these biomarkers have been detected in cell and animal based model systems and are strongly associated with cell and tissue injury. Similarly, these biomarkers of oxidative stress have also been measured clinically in a number of chronic diseases (Droge, 2002; Lambeth, 2007; Valko et al., 2007). For instance, we recently reported that systemic levels of MDA correlate with sub-clinical atherosclerosis in a healthy multi-ethnic cohort (Lear et al., 2012). Though these biological markers provide a strong indication that oxidative stress is a common underlying feature among various pathologies, to date, antioxidant based therapies have proven unsuccessful in clinical intervention studies. Therefore, it is clear that there remains much to learn about oxidative stress, its regulation and its contribution to disease pathogenesis (Winterbourn, 2008). Improving our understanding of the underlying mechanisms that are responsible for regulating oxidative stress under different disease conditions remains an ongoing objective in the field of free radical biology.

2.2. Non-alcoholic Fatty Liver Disease (NAFLD)

2.2.1. Epidemiology and risk factors

NAFLD is growing at alarming rates worldwide, and although the global incidence is not yet known, estimates suggest that 1 billion people may suffer from some form of the disorder (Loomba & Sanyal, 2013). Alarmingly however, this approximation might still underestimate the true prevalence of the disease since NAFLD continues to remain significantly underdiagnosed even in at risk populations (Kleiner et al., 2014). There are a number of risk factors for NAFLD, but increased adiposity and insulin resistance are regarded as the most influential factors (Cohen et al., 2011). Other modifiable risk factors include hypertriglyceridemia and hyperglycemia (Farrell & Larter, 2006). Conditions such as obesity, type 2 diabetes mellitus (T2DM), dyslipidemia, hyperglycemia and metabolic syndrome, in which these risk factors are established (Chalasani et al., 2012), have all been strongly correlated with the severity of NAFLD (Farrell & Larter, 2006). Non-modifiable risk factors include gender, age, and ethnicity (Cohen et al., 2011; Farrell & Larter, 2006). Although NAFLD affects one-third of the average adult population (Berlanga et al., 2014; Cohen et al., 2011), the incidence may be as high as 70-99% in obese and/or type 2 diabetic populations (Adams & Angulo, 2005; Kleiner & Brunt, 2012). Since NAFLD most often presents alongside a cluster of other metabolic disorders, it is often referred to as the hepatic manifestation of the metabolic syndrome (Than & Newsome, 2015). The global rise in obesity and type 2 diabetes is considered therefore, to be the major force responsible for raising NAFLD incidence worldwide (Loomba & Sanyal, 2013). NAFLD is now the most common chronic liver disorder in North America (Loomba & Sanyal, 2013), and is the major cause of elevated liver enzymes in the United States (Clark et al., 2003). The high rates of diagnosis in children (Manco et al., 2008; Sartorio et al., 2007) and adolescents (Fraser et al.,

2007; Strauss et al., 2000) strongly indicates that NAFLD will continue to grow as a major medical burden in the future.

2.2.2. NAFLD diagnosis

There are no biomarkers or positive serological tests available to effectively diagnose NAFLD (Kleiner & Brunt, 2012). Diagnosis requires evidence of liver fat accumulation manifesting in the absence of any secondary causes including malnutrition/starvation, certain medications and excessive alcohol intake (Chalasani et al., 2012; Kleiner & Brunt, 2012). It is imperative that the patient be screened for a history of alcohol abuse since NAFLD presents with similar histological features as alcoholic fatty liver disease (AFLD). Intakes of less than 10g alcohol/day for women (equivalent to one standard drink) and 20g alcohol per day for men are generally accepted as the cut off for NAFLD diagnosis (Farrell & Larter, 2006). Since NAFLD manifests asymptomatically in most patients, diagnosis generally occurs incidentally following routine blood exams which may detect elevations in liver aminotransferases or during unrelated hepatic ultrasonography (gallstones) (Berlanga et al., 2014; Farrell & Larter, 2006). Cirrhosis may however lead patients to seek medical attention since gastrointestinal bleeding, jaundice or muscle weakness are common symptoms (Farrell & Larter, 2006). Definitive categorization of NAFLD can only be achieved through histological evaluation, and so a liver biopsy remains the gold standard for accurate diagnosis (Chalasani et al., 2012; Cohen et al., 2011). Although liver tests such as alanine aminotransferase (ALT) are often elevated in NAFLD patients, they may also be normal despite significant histological evidence of the disorder (Kleiner et al., 2014; Mofrad et al., 2003). While non-invasive imaging techniques such as ultrasonography or magnetic resonance imaging can effectively detect simple steatosis, they fail to identify NASH

or fibrosis in patients (Chalasani et al., 2012). NAFLD is typically used as an umbrella term when histology is undefined.

2.2.3. The NAFLD spectrum

i) Steatosis (fatty liver)

Steatosis is an essential diagnostic criteria, and is defined as cytosolic lipid accumulation in more than 5% of hepatocytes. Steatosis, or "fatty liver", is histologically simple and presents as small and large lipid vacuole formations in the parenchymal tissue that are comprised predominantly of free fatty acids and triglycerides (Yeh & Brunt, 2014). Using isotopic tracer techniques, Donnelly et al, reported that 59% of hepatic triglycerides originate from peripheral tissues, 26% from hepatic *de novo* lipid synthesis and 15% from the diet (Donnelly et al., 2005). These significant findings may provide an underlying rationale for the particularly high incidence of steatosis among overweight and obese individuals. While steatosis presents in 16-24% of the general population (Farrell & Larter, 2006), upwards of 99% of patients undergoing bariatric surgery were found to have fatty liver (Kleiner & Brunt, 2012). Although free fatty acids and triglycerides have traditionally received much of the attention for their pathophysiological role in fatty liver, more recently the focus has broadened to also include esterified and non-esterified cholesterol and the significance of its hepatic accumulation in this disorder (Woo et al., 2005; Wu et al., 2013).

ii) NASH

In contrast to fatty liver, NASH presents with a complex histopathology that is characterized by steatosis, hepatocyte injury, inflammatory infiltrate, and/or fibrosis (Cohen et al., 2011; Kleiner

& Brunt, 2012). Hepatocyte ballooning, as evidenced histologically by cell swelling and a wispy cytoplasm, is indicative of parenchymal injury (Yeh & Brunt, 2014). While hepatocyte ballooning is essential for NASH diagnosis, the degree of steatosis and inflammation can vary considerably and fibrosis, although often present, is not a required feature (Kleiner & Brunt, 2012). Mixed immune cell populations comprised of neutrophils, macrophages and lymphocytes are notable in NASH, and tend to localize to areas of overt steatosis (Takahashi et al., 2012). It is currently unknown whether steatosis precedes NASH or whether these two disorders may manifest independently as distinct entities (Cohen et al., 2011). Though fatty liver is often considered to be a benign entity, in a follow up study 32% of cases reportedly evolved to NASH (Pais et al., 2013). It is unclear why fatty liver progresses to NASH in some, but not in others. Central obesity exhibits the strongest correlation with NASH (Farrell & Larter, 2006). NASH is thought to be present in 2-7% of the population (Farrell & Larter, 2006), however this value might significantly underestimate the true prevalence of the disorder. Elevation in circulating ALT, although often used to screen for NAFLD, may not reliably predict NASH (Kleiner et al., 2014), and in some cases levels were found to decrease in NASH (Pais et al., 2013). A recent study, involving patients who underwent bariatric surgery, suggest that upwards of 88% of NASH cases may not be biopsied and may go undiagnosed even within this at risk population (Kleiner et al., 2014).

iii) Cirrhosis

Cirrhosis also falls under the NAFLD spectrum and is characterized by the replacement of viable parenchymal tissue with collagenous fibrotic lesions (Cohen et al., 2011). NASH is the only entity under the NAFLD that may directly progress to cirrhosis (Yeh & Brunt, 2014). Since

evidence of other active histological parameters may be absent at this stage, NAFLD-related cirrhosis can only be diagnosed in those patients with biopsies previously indicating NASH (Brunt & Tiniakos, 2010). Over a 10 year period, NASH was found to progress to severe fibrosis in 20-30% of patients (Argo et al., 2009). Although hepatocyte injury best correlates with fibrogenesis (Farrell & Larter, 2006), the factors responsible for mediating the clinical evolution of NASH to cirrhosis also remain unknown (Cohen et al., 2011). Age and the degree of inflammation observed in initial biopsies may independently predict advanced fibrosis, but other metabolic related risk factors such as obesity and diabetes may not have significant predictive value (Argo et al., 2009). Due to the reduction in functional tissue, cirrhosis can lead to liver failure and is also associated with a pre-disposition to portal hypertension and hepatocellular carcinoma (Argo et al., 2009; Cohen et al., 2011).

2.2.4. Health risks associated with NAFLD

The health risks associated with NAFLD are becoming better appreciated and a number of hepatic and extra-hepatic complications have been correlated with the disorder. Over 7 years, the risk of developing liver related complications may be as high as 30-60% in NASH patients (Farrell & Larter, 2006). Liver failure is a serious and life threatening complication related with the advanced stages of NAFLD. It has been predicted that by 2025, NAFLD associated end-stage liver disease will be the primary cause of liver transplants (Kleiner et al., 2014). Patients with NAFLD associated cirrhosis are also at an increased risk for developing hepatocellular carcinoma. While mortality rates attributed to hepatocellular cancer reportedly account for less than 1% of deaths in control subjects, it may account for up to 24% of deaths in NAFLD patients (Onnerhag et al., 2014). In addition to liver related complications, there is strong evidence to

suggest that NAFLD may increase the risk of cardiovascular disease (CVD). The histological severity of NAFLD has been strongly correlated with carotid intima-media thickness and impaired endothelial flow mediated vasodilation, markers of subclinical atherosclerosis (Targher & Arcaro, 2007). Though NAFLD is associated with a number of factors that increase cardiovascular risk, including atherogenic dyslipidemia and obesity, the sum of the evidence suggests that NAFLD might increase CVD risk independent of these classical risk factors (Targher & Arcaro, 2007). It has also been proposed that atherosclerosis and NASH may be manifestations of the same disease mediated by the localization of activated macrophages (Bieghs et al., 2012). Overall, NAFLD is associated with a higher mortality rate (Onnerhag et al., 2014), with the major cause of death among NAFLD patients being CVD followed by cancer and liver related complications (Loomba & Sanyal, 2013). Management of CVD risk factors and early screening for CVD is highly recommended in those patients diagnosed with NAFLD (Chalasani et al., 2012; Targher et al., 2010). In addition to increasing CVD risk, current evidence from a wide range of epidemiological studies suggest that NAFLD may also independently increase the chances of developing T2DM, chronic kidney disease and colorectal cancers (Armstrong et al., 2014). Although more studies are needed to confirm these findings and determine the underlying cause(s) for such associations, NAFLD might be best perceived as a multisystem disease rather than an isolated hepatic pathology (Anstee et al., 2013).

2.2.5. NAFLD pathogenesis

It is evident that NAFLD is a complex and progressive hepatic disorder. While a number of risk factors have been strongly associated with NAFLD, the underlying mechanisms mediating NAFLD pathogenesis remain poorly understood. The co-manifestation of NAFLD with obesity,

T2DM and CVD suggests a shared underlying pathological mechanism(s) (Farrell & Larter, 2006). In light of the ethical concerns related with obtaining biopsy specimens in patients and the limitations associated with studying a complex and multimodal disease *in situ*, animal models have become an instrumental tool for investigating the underlying aspects of NAFLD pathology (Larter & Yeh, 2008). A number of NAFLD models are currently available, each presenting with its own characteristic advantages and disadvantages (Larter & Yeh, 2008; Takahashi et al., 2012). In order to provide valuable insight into the human disorder, the model selected for study should meet three criteria: the model should a) develop a hepatic histopathology that is consistent with NAFLD; b) the disorder should manifest within an appropriate metabolic context (Farrell & Larter, 2006; Larter & Yeh, 2008) and c) the method used to induce NAFLD should be relevant to the human disorder.

i) Genetic models for studying NAFLD pathogenesis

A number of genetic animal models have been used to investigate NAFLD. The most commonly reported are ob/ob mice and db/db mice, in which spontaneous mutations result in defective leptin signalling. As a result of these mutations, these mice are hyperphagic, obese, insulin resistant, and develop T2DM. Although the metabolic phenotype of these animals is consistent with the clinical pathology, these mice only develop steatosis. The ob/ob mice are resistant to fibrosis, which has brought to light the importance of leptin in fibrogenesis. NASH can be induced in these animals with the addition of a secondary stressor such as the bacterial endotoxin lipopolysaccharide (LPS). Fibrosis and cirrhosis can also be induced in db/db mice via a similar method. Other genetic models that have emerged more recently include the Zucker fatty (fa/fa) rat and foz/foz mice. Both models are hyperphagic and develop a phenotype that is consistent

with the metabolic syndrome. High fat diet feeding exacerbates the metabolic abnormalities and appears to induce NASH in these animals. More studies are needed however to fully characterize these models and their representation of the human disorder (Larter & Yeh, 2008; Takahashi et al., 2012). It is important to consider that although genetic defects are known to result in severe cases of steatosis, these conditions are rare (Cohen et al., 2011). Leptin also poorly correlates with NASH in clinical studies (Chalasani et al., 2003).

ii) Nutritional models for studying NAFLD pathogenesis

Methionine choline deficient (MCD) model

The histopathology of the methionine choline deficient (MCD) model is the most extreme of the nutritional models currently available (Takahashi et al., 2012). Diets deficient in MCD rapidly induce severe steatosis, NASH and fibrosis in the liver of mice after only 8-10 weeks. Although these hepatic features are desirable in a NAFLD model, MCD animals lose weight rather than gain weight, resulting in a metabolic phenotype that is inconsistent with the human disorder (Larter & Yeh, 2008). Moreover, the depletion of dietary nutrients opposes the generally accepted theory that over-nutrition is an important driving factor in the human pathology (Larter & Yeh, 2008; Takahashi et al., 2012). Diets are also not generally deficient in methionine and choline in NAFLD populations. For these reasons there is considerable controversy over the validity of using the MCD model to extrapolate and understand human NAFLD (Larter & Yeh, 2008; Takahashi et al., 2012).

Overfeeding

Over-nutrition is generally accepted to be one of the most influential factors responsible for raising NAFLD incidence over the past 30 years (Cohen et al., 2011). Because rodents tend to adapt to high caloric diets by reducing their dietary intake (Deng et al., 2005; Gaemers et al., 2011), techniques have been developed to induce chronic over-nutrition in rodents. Intra-gastric overfeeding of liquid high fat diets can increase caloric intake up to 185% in mice and produces many of the metabolic (obesity, insulin resistance) and histological features that are consistent with NASH after 9 weeks (Deng et al., 2005). However, the high mortality rate (10-15%) and the technical expertise required to perform these studies are significant drawbacks that have limited the use of this model (Deng et al., 2005; Larter & Yeh, 2008). Oral gavage with a fat emulsion has also been used to induce over-nutrition, but this method is labour intensive and is not considered to be practical for long term studies (Larter & Yeh, 2008).

High fat diet (HFD)

Although no animal model fully represents the clinical pathology (Farrell & Larter, 2006), the high fat diet (HFD) model is considered to be among the best NAFLD models currently available (Larter & Yeh, 2008). Typically, the standard HFD used in these studies contains 60-70% of its energy (kcal) in the form of dietary fat, and is fed to rodents *ad libitum*. Traditionally, these diets are also comprised of fats with a high saturated fat content (coconut oil, lard, tallow) which promotes optimal development of the model in comparison to other fats rich in mono- or poly-unsaturated fatty acids (Buettner et al., 2007; Gajda, 2008). Feeding such a HFD induces a phenotype in rodents that is consistent with the metabolic syndrome (obesity, hyperinsulinemia, hyperlipidemia) as well as induces histological features that resemble NAFLD pathology (Ito et al., 2007). Although steatosis is easily induced in this model, NASH may take longer to develop.

Long term studies have shown that it may take 35-50 weeks for NASH to be established in male C57BL/6 mice when fed a 60% kcal fat diet (Ito et al., 2007). In male Sprague-Dawley rats, feeding a 58% kcal fat diet induced steatosis and mild inflammation with intermittent hepatocyte balooning after 1 month, but established NASH with increasing grades of severity after 3-6 months (Svegliati-Baroni et al., 2006). Although the severity of liver injury induced by this model may be less than the MCD model (Takahashi et al., 2012), and *ad libitum* HFD feeding may require studies of longer duration than overfeeding studies, the HFD model might better represent the human pathology since it develops NASH gradually over time (Ito et al., 2007). Moreover, Vilar *et al* reported that the diet of obese NAFLD patients may not always be hypercaloric. Rather, total energy provided by dietary fat was found to be significantly higher in patients with NASH compared to those with simple steatosis in this study. Although the intake of all forms of dietary lipid increased in these patients, saturated fat consumption reached the recommended limit of 10% of the total energy intake, and in some NASH patients even exceeded these guidelines (Vilar et al., 2008). Other studies have also made similar associations between a higher fat intake and NAFLD (Musso et al., 2003). For these reasons, the HFD mouse model has been widely adopted for NAFLD research (Anstee et al., 2013). One of the shortcomings associated with the model is its potential for variability (Larter & Yeh, 2008). Dietary composition, the total and type of dietary lipids, and the study duration can all add variables that may influence the phenotype of the model (Takahashi et al., 2012).

iii) Mechanisms underlying NAFLD pathogenesis

The pathogenesis of NAFLD has not been fully elucidated. Rather, our understanding of the underlying mechanisms responsible for mediating NAFLD pathogenesis has been continuously

evolving (Cohen et al., 2011; Day & James, 1998b; Matherly & Puri, 2012). From the original "two-hit hypothesis" (Day & James, 1998b), to the subsequent "multi-hit theories" (Tilg & Moschen, 2010), to the multifaceted causes of hepatic steatosis (Matherly & Puri, 2012), it has become evident that a vicious cycle of interrelated and highly complex events concertedly mediate NAFLD pathogenesis. While the two-hit hypothesis recognizes perturbed lipid metabolism as the initiating factor responsible for sensitizing the liver to secondary insults like oxidative stress (Day & James, 1998b), the multi-hit theory acknowledges the parallel contributions of a variety of factors including lipotoxicity, oxidative stress, inflammation, insulin resistance, gut-derived endotoxins and genetics in the pathogenesis of NAFLD (Polyzos et al., 2012a; Tilg & Moschen, 2010).

Obviously, the visualization of hepatic lipid vacuole formations in fatty liver and NASH has implicated perturbed lipid metabolism as a central mediating factor in the disorder. While triglycerides may be perceived as protective, free fatty acids are hepatotoxic and render hepatocytes susceptible to injury (Day & James, 1998a; Polyzos et al., 2012a). Insulin resistance is another underlying pathogenic factor. A strong relationship between NAFLD and hepatic insulin resistance exists (Cohen et al., 2011) and visceral adiposity, steatosis and insulin resistance are also strongly associated (Farrell & Larter, 2006). Hepatic insulin resistance in particular disrupts insulin's inhibitory effect on glucose production but maintains the lipogenic effect of the hormone in the liver, the net effect which increases both glucose and lipid synthesis in the liver. The sum of the evidence suggests that although insulin resistance may cause steatosis, steatosis does not always cause insulin resistance (Cohen et al., 2011). Inflammation is another important pathogenic mediator in NAFLD. Inflammation is visualized histologically by

the presence of inflammatory infiltrates which are comprised of clusters of inflammatory cells including lymphocytes, monocytes, macrophages and Kupffer cells (Kleiner & Brunt, 2012). Inflammatory cytokines such as TNF-α and IL-6 may be important therapeutic targets in the disorder (Sarna et al., 2015). Although inflammatory response is an important part of the wound healing process, in NAFLD it leads to hepatocyte injury, contributes to hepatic and extrahepatic insulin resistance, and activates fibrogenesis (Cai et al., 2005; Marra et al., 2008).

Oxidative stress is a distinguishing feature in NAFLD. In clinical studies, upwards of 90% of patients with NAFLD exhibit elevated levels of hepatic lipid peroxides (Hardwick et al., 2010; Loguercio et al., 2004; Seki et al., 2002). Patients with NAFLD also present with compromised hepatic and systemic antioxidant defenses (Hardwick et al., 2010; Loguercio et al., 2004). In contrast to other molecular mediators such as lipid accumulation or fibrosis that tend to manifest in particular stages of the disorder, oxidative stress is notable throughout the entire NAFLD spectrum. In fact, markers of oxidative stress have been localized to hepatocytes in all spectrums of the disorder, and occasionally the sinusoidal endothelial cells of NASH patients (Seki et al., 2002). Pre-clinical studies have implicated oxidative stress as a causal factor in hepatocyte injury and as an important mediating factor in inflammatory and fibrogenic processes (Rolo et al., 2012). In fact, lipid peroxidation was one of the first molecular mediators reported to drive simple steatosis to NASH (Berson et al., 1998). Commenting on this work, Day and James identified oxidative stress as one of the crucial "second hits" responsible for initiating the evolution of fatty liver to NASH (Day & James, 1998b) and led to the classical "two hit hypothesis". Results from the PRIVENS trial, a large, multicenter phase 3 study reported improvements in the histological endpoints of NASH in patients receiving the antioxidant

vitamin E. The ability for vitamin E to improve NASH was found to be independent of changes in insulin sensitivity and weight loss in this study (Sanyal et al., 2010). Though there is convincing evidence that oxidative stress is involved in the pathogenesis of NAFLD, the underlying mechanisms responsible for regulating oxidative stress in NAFLD remain to be defined.

2.2.6. NAFLD management

There is no approved pharmaceutical based approach to treat NAFLD. Currently, the disorder is managed clinically by the implementation of lifestyle modifications that correct for risk factors (Polyzos et al., 2012a). Gradual weight loss is regarded as one of the safest and most effective methods for improving NAFLD (Chalasani et al., 2012; Farrell & Larter, 2006). While weight loss of approximately 7% of body weight over 48 weeks was shown to improve NASH in overweight and obese patients (Promrat et al., 2010), rapid weight loss greater than 2 lbs per week (>2.2kg), could have deleterious pro-fibrotic effects and may even lead to NASH and liver failure despite improving steatosis (Farrell & Larter, 2006; Luyckx et al., 1998). Therefore weight loss should be carefully monitored and controlled in NAFLD patients particularly in those who undergo gastric surgery which can lead to rapid weight reduction. Gradual weight loss may be achieved through increased physical activity and/or consumption of hypocaloric diets (Chalasani et al., 2012; Promrat et al., 2010). It is typically recommended that patients adopt a low fat high carbohydrate diet to manage NAFLD and its associated cardiometabolic disorders. A Mediterranean style diet might also have beneficial effects in these patients (Kontogianni et al., 2014; Ryan et al., 2013).

Because lifestyle modifications are often difficult to maintain over the long term (Polyzos et al., 2012a), studies investigating the therapeutic efficacy of pharmacological based interventions for NAFLD remains an active and ongoing area of research. In light of the gravity and growing number of disorders linked to NAFLD, there has been much research aimed at identifying pharmacological based therapies. Lipid lowering drugs like statins might be beneficial for managing hyperlipidemia and reducing CVD risk in NAFLD patients (Chalasani et al., 2012). On the other hand, insulin sensitizing agents have shown mixed results in clinical trials. Although metformin may increase insulin sensitivity, it does not appear to improve liver histology and is therefore not recommended for treating NAFLD (Chalasani et al., 2012). Thiazolidinediones have been shown to improve serum aminotransferases, insulin sensitivity and liver histology however, weight gain is an unwanted side effect (Farrell & Larter, 2006) and discontinuation of the drug has been found to worsen liver histology (Polyzos et al., 2012a). Vitamin E was shown to improve liver histology in non-diabetic NASH patients (Sanyal et al., 2010) and has been considered as an important first line drug therapy in these patients (Chalasani et al., 2012). However, it remains unclear whether vitamin E is similarly effective in other spectrums of the disorder as well as in those patients with diabetes. Long term intervention studies are also needed to confirm safety and efficacy for many of these aforementioned therapeutics. Since no single agent has been shown to effectively treat NAFLD, and because the underlying pathology of NAFLD is multifaceted, it is anticipated that multimodal and combined therapeutics will phase out monotherapy based approaches in future clinical trials (Polyzos et al., 2012a).

2.3. Natural health products (NHPs)

2.3.1. Berberine

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine; $C_{20}H_{18}NO_4^+$) is a bright yellow isoquinoline plant alkaloid (Fig. 2.1). Berberine is extensively distributed throughout plants belonging to the genus Coptis, Berberis and Hydrastis. These plants, which have a shrub like appearance are native to North and South America, Europe, Africa and Asia, and present with concentrated levels of berberine in their roots, rhizomes and bark. The stems, leaves, flowers and fruits also contain trace amounts of berberine (Siow et al., 2011a).

i) History of use

Berberine has a strong bitter taste so typically only the berries of berberine containing plants are traditionally consumed as a food item (Siow et al., 2011a). Berberine containing plants have been used for more than 2500 years in traditional Chinese, Ayeuvedic and North American medicines (Affuso et al., 2010). The roots of the goldenseal (*Hydrastis canadensis)* and berries of the Oregon grape *(Berberis aquifolium)* plants were used in Native American medicine while the rhizomes and roots of goldenthread *(Coptis chinensis)* and barberry (*Berberis vulgaris*) have respectively been used in Traditional Chinese Medicine and Ayurvedic medicines. The berberine rich fractions of these plants have been used medicinally to treat diarrhea, infection, and correct various gastrointestinal issues (Siow et al., 2011a). Berberine has also been used in traditional medicines to treat diabetes (Derosa et al., 2012).

(Source: http://www.chemspider.com/ImagesHandler.ashx?id=2263&w=500&h=500)

Figure 2.1. Structure of berberine

ii) Current applications

The historical application of berberine and berberine containing plants as an antidiabetic therapy, has prompted interest in the potential therapeutic application of berberine in Western medicine for the treatment of metabolic disorders (Affuso et al., 2010; Derosa et al., 2012; Siow et al., 2011a). Berberine salts, including berberine chloride or berberine sulfate, are commercially available and have been investigated in pre-clinical and clinical studies (Derosa et al., 2012).. Over the past decades, berberine has been investigated for its ability to manage and treat a variety of metabolic disorders, and in particular has shown promising effects on the management of glucose and lipid metabolism. A number of studies conducted in animal models of obesity and T2DM have demonstrated that berberine reduces body weight while also improving blood glucose and insulin levels (Affuso et al., 2010). Berberine regulates glucose metabolism in part, by increasing insulin receptor expression, activating adenosine monophosphate kinase (AMPK) and stimulating glycolysis to increases cellular glucose consumption (Affuso et al., 2010). Berberine also regulates glucose metabolism by stimulating insulin secretion, activating GLUT-4 mediated glucose uptake in liver, adipose and muscle cells, as well as regulating insulin receptor substrate-1, which collectively improve insulin sensitivity (Derosa et al., 2012). The glucose lowering effect of berberine that has been observed in pre-clinical studies has also been supported by clinical studies (Derosa et al., 2012). Its hypocholesterolemic and triglyceride lowering effects have been investigated and appear multimodal, and include increased expression and stability of the hepatic LDL receptor (Abidi et al., 2005; Affuso et al., 2010) and activation of AMPK mediated inhibition of *de-novo* lipid synthesis and the liver (Brusq et al., 2006; Wu et al., 2011) and activation of fatty acid beta-oxidation in the liver and skeletal muscle (Kim et al., 2009). Berberine's lipid lowering effects have also been demonstrated in clinical studies (Derosa

et al., 2012; Dong et al., 2013). Berberine has also been shown to alleviate hepatic lipid accumulation and improve liver histology in various animal models of NAFLD (Chang et al., 2010; Kim et al., 2009; Xing et al., 2011). Berberine's ability to inhibit oxidative stress induced liver injury (Hwang et al., 2002; Zhang et al., 2008a) and mitigate pro-inflammatory responses which impair hepatic insulin signaling (Lou et al., 2011) have also supported its potential therapeutic application in NAFLD (Liu et al., 2013; Xing et al., 2011). Although findings from pre-clinical studies are promising, berberine's therapeutic efficacy in NAFLD remains to be confirmed in clinical studies.

Berberine's lipid lowering effects, as well as vasculoprotective properties, have positioned it as a potential therapeutic in CVD (Affuso et al., 2010; Derosa et al., 2012; Siow et al., 2011a; Siow et al., 2011b). Berberine has been shown to maintain endothelial function via activation of endothelial NOS (eNOS) and its lipid lowering, anti-inflammatory and anti-chemotactic properties appear particularly well suited for the prevention and management of atherosclerosis (Affuso et al., 2010). Though berberine is distinguished as having multimodal applications in CVD, more studies are needed to fully characterize berberine's therapeutic properties in the context of this disorder. Specifically, there is a need to investigate berberine's capacity to regulate oxidative stress in CVD, as it is also one of the major mediating factors in atherogenesis.

2.3.2. Folate

Folate is a generic term for a family of water-soluble B9 vitamins (Visentin et al., 2014). Folate is biologically essential, serving as a co-enzyme (co-substrate) in one-carbon transfer reactions (Pietrzik et al., 2010). Folate dependant one-carbon transfer reactions are integral for amino acid

metabolism as well as DNA and RNA biosynthesis (Pietrzik et al., 2010) and thus serve a critical role in supporting cell division, particularly in times of growth. Animal cells lack the synthetic machinery that is needed to synthesize folates, and therefore rely on the acquisition of dietary folates to meet endogenous needs. Folates are taken up by the intestine and are stored predominantly in the liver. The liver is responsible for mobilizing folate to meet the requirement of tissues.

i) Sources of folate

Although folates are widely distributed in foods, liver and dark green leafy vegetables are among the richest sources of naturally occurring folates. Folic acid, a synthetic form of folate, is another dietary source that may be obtained in either supplemental form and/or in fortified foods. Unlike naturally occurring food folates (pteroylpolyglutamate) that are reduced and polyglutamated, folic acid (pteroylmonoglutamate) is an oxidized monoglutamated form. Owing to its high stability, solubility, and efficient absorption, folic acid is the preferred form of folate used for supplementation and fortification purposes (Wright et al., 2007; Zhao et al., 2009). In countries such as Canada and the United States, that introduced mandatory fortification of flour and other grain products in 1998, folic acid constitutes a significant portion of the dietary folates consumed (Zhao et al., 2009). Although the contribution to whole body folate homeostasis is unclear, the symbiotic relationship between intestinal cells and bacteria might serve as an endogenous source of folate since bacteria are capable of synthesizing and metabolizing folates (Visentin et al., 2014).

ii) Dietary folate vs folic acid

Although folic acid is not found in nature, it is often referred to as the parent compound. Folic acid shares the same basic molecular structure as naturally occurring dietary and endogenous folates and is comprised of a pteroic acid and one glutamic acid (Fig. 2.2). Pteroic acid is further composed of a pteridine ring (2-amino-4-hydroxypteridine) that is linked to para-aminobenzoic acid. Dietary folates differ from folic acid on account of two fundamental features. Dietary folates tend to be more reduced that folic acid, and might contain additional hydrogens atoms or various one-carbon moieties on 2-amino-4-hydroxypteridine that influence the reduction/oxidation level of folate (Blom et al., 2006). Dietary folates also contain a polyglutamate chain comprised of at least 3 glutamic acids (Hoffbrand, 1971). Although dietary folates may be comprised of over 100 mixed varieties, the most common are formyl and methyl polyglutamates (Zhao et al., 2009).

iii) Folate absorption

The structural differences between folic acid and natural dietary folates ultimately affect their bioavailability and bioactivity. Whereas only 50-75% of natural dietary folates are bioavailable and only half are biologically active, folic acid approaches 100% bioavailability but it lacks coenzyme activity (DRI, 1998; Pietrzik et al., 2010). The intestine is the major site responsible for regulating the uptake and biotransformation of dietary folates.

Absorption of folates is an active, carrier mediated process that occurs predominantly in the acidic microenvironment of the upper (proximal) small intestine (Visentin et al., 2014). Since

(Source: http://www.chemspider.com/ImagesHandler.ashx?id=5815&w=500&h=500)

Fig. 2.2. Structure of folic acid

intestinal uptake favours folate monoglutamates, dietary polyglutamated folates must first be hydrolysed to their reduced monoglutamates by brush border hydrolases in the intestinal lumen prior to their absorption (Pietrzik et al., 2010; Visentin et al., 2014; Wright et al., 2007). Folic acid, which by comparison is a monoglutamate, bypasses intraluminal hydrolysis and is taken up directly by intestinal cells (Pietrzik et al., 2010). The reduced folate carrier (RFC) and proton coupled folate transporter (PCFT) are responsible for mediating folate uptake by enterocytes (Hamid et al., 2009). The PCFT is largely responsible for folate absorption in the proximal small intestine (Pietrzik et al., 2010).

iv) Folic acid biotransformation/metabolism in enterocyte

The biologically active form of folate is tetrahydrofolate (THF). Although folic acid is efficiently absorbed by the enterocyte, this oxidized monoglutamate lacks co-enzyme activity (Pietrzik et al., 2010). Enterocytes mediate intracellular biotransformation of folic acid to the biologically active THF. Folic acid is first reduced to dihydrofolate (DHF), followed by THF, via dihydrofolate reductase (Pietrzik et al., 2010). Other folate monoglutamates may also undergo biotransformation in the enterocyte to THF (Wright et al., 2007). With the addition of a one-carbon moiety, intracellular THF, is further converted to 5,10-methylene tetrahydrofolate (5,10-methylene-THF) by serine hydroxymethyltransferase and then to 5-methyl-tetrahydrofolate (5-MTHF), by 5,10-methylene-THF reductase. The 5-MTHF, acquired through folate/folic acid biotransformation in the enterocyte or directly from the diet, is the main form of folate transported across the basolateral membrane of the intestinal cell. It is noteworthy however that at elevated intakes (above 260ug), folic acid can traverse the apical and basolateral side of the

enterocyte and enter the blood untransformed (Pietrzik et al., 2010). 5-MTHF (and folic acid) enters the mesenteric veins and is delivered to the liver via the portal vein (Wright et al., 2007).

v) Liver metabolism and storage

During first pass metabolism, up to 20% of the 5-MTHF is assimilated by the liver with the remainder entering the systemic circulation for delivery to extra-hepatic tissues. By comparison, any folic acid delivered to the liver by the portal vein is preferentially retained (Steinberg et al., 1979; Wright et al., 2007). 5-MTHF is the main folate in the circulation of humans and rodents (Zhao et al., 2009). Plasma contains low concentrations of a specific, high affinity folate binding protein. Therefore, folate may be in a free form or may be bound to protein in the plasma. The RFC appears to be ubiquitously expressed, and under normophysiological conditions is the major, if not only, transporter facilitating folate uptake from the circulation and delivery into cells (Visentin et al., 2014). However, PCFT was also found to have residual activity for 5-MTHF transport at physiological pH, and is also speculated to regulate cellular uptake of systemic folate in a number of tissues (Hamid et al., 2009; Qiu et al., 2006). Folate receptor (FR) (folate binding protein), which does not appear to play a role in intestinal absorption, is expressed in kidney and liver and might play a role in folate uptake in these tissues (Hamid et al., 2009; Van Hoozen et al., 1996). Once inside the cell, folate undergoes polyglutamation, as folylpolyglutamate synthase adds glutamate residues to the folate molecule (Visentin et al., 2014; Zhao et al., 2009). Polyglutamated folates are better retained by cells than folate monoglutamates, which ultimately facilitates their accumulation in cells (Blom et al., 2006). Folate polyglutamates they are also preferentially used by enzymes involved in folate

metabolism, including those catalyzing one-carbon donating reactions (Blom et al., 2006; Zhao et al., 2009).

vi) Folate metabolism and function

Cellular folates, in their wide assortment of reduced and oxidized forms, play an essential role in DNA and RNA biosynthesis, methionine metabolism and methylation reactions (s-adenosylmethionine) (Zhao et al., 2009). One-carbon moieties, including methyl and formyl varieties, are derived from amino acids histidine, glycine and serine as well as formate and are covalently linked to intracellular THF (Blom et al., 2006). THF, in turn, not only accepts these one-carbon groups, but donates them to support a number of biosynthetic and regulatory effects. Transfer reactions involving one-carbon moieties are required for de novo synthesis of thymidine and purines which are essential for DNA synthesis (Visentin et al., 2014; Zhao et al., 2009). 5-MTHF also donates a methyl group to vitamin B12 which, in a reaction catalyzed by methionine synthase, remethylates homocysteine (Hcy) to methionine (Blom et al., 2006). Methionine in turn, is converted to s-adenosylmethionine (SAM), the principal methyl donor in cells. Intracellular methylases catalyze the transfer of methyl groups ($-CH_3$) from SAM to a number of acceptors. Methyl groups are used for the methylation of DNA, proteins, lipids and xenobiotic compounds; these modifications ultimately control cellular gene expression, protein function and lipid synthesis (Blom et al., 2006). In light of folate's wide spread cellular functions, its bioavailability is essential for supporting rapid growth.

vii) Folate and the liver

The liver plays a crucial role in folate regulation and storage. The liver expresses RFC, FR and PCFT (Wang et al., 2001a; Van Hoozen et al., 1996). The liver is the major storage site for endogenous folates and a key regulator of whole body folate homeostasis. Total body folate stores in humans approximate 12-28 mg. The human liver contains roughly 6-14mg of folate, which equates to half of total body folate stores (DRI, 1998). The liver is also a major site of folate metabolism, and may also play a significant role in folic acid metabolism (Steinberg et al., 1979). In fact, although folic acid biotransformation is thought to occur primarily in the enterocyte, it has been proposed that the liver might play a more important role in folic acid metabolism, at least in humans (Wright et al., 2007). The liver also plays a central role in regulating whole body folate homeostasis via buffering against perturbations in folate levels in plasma and extraheptic tissues. The liver regulates the enterohepatic circulation of folate, which functions to even out intermittent fluctuations in systemic folate levels (Steinberg et al., 1979; Visentin et al., 2014; Wright et al., 2007). γ-glutamyl hydrolase, located in lysosomes in the liver, is responsible for hydrolysing stored folates to their transportable monoglutamate forms (Visentin et al., 2014). These folate monoglutamates may then be secreted into the bile and returned to the intestine via the bile duct. These hepatic-derived folates may be re-absorbed by the enterocyte, biotransformed and returned back to the circulation as 5-MTHF (Wright et al., 2007). Folate stores are often compromised in response to alcohol induced liver injury (Tamura et al., 1981) as well as in hepatic disorders such as chronic viral hepatitis and biliary cirrhosis (Leevy & Baker, 1970; Leevy et al., 1970).

viii) Folate in NAFLD

Evidence is emerging that raises the possibility that endogenous folate levels could be compromised in NAFLD. Schweiger *et al*, reported that approximately 24% of bariatric surgery candidates present with abnormally low serum folate levels (<5.6ng/ml) (Schweiger et al., 2010), and estimated that patients with a BMI greater than 50 kg/m^2 are 15 times more likely to suffer from folate deficiency. A number of human observational studies have similarly associated increased adiposity with lower folate levels. Among healthy post-menopausal women, serum folate levels were inversely related to BMI, percent total body fat and central and peripheral fat measures (Mahabir et al., 2008). When compared to normal weight individuals, serum folate concentrations in overweight and obese women were 12% and 22% lower (Mahabir et al., 2008). Although in this study, folate levels were still found to be within the normal range (5-16ng/ml) across a range of BMIs, folate levels decreased by 1.7% with each one unit increment in BMI (Mahabir et al., 2008). Serum folate levels in women of childbearing age, also decreased with increasing BMI (Mojtabai, 2004). It is unclear whether this association accounts for the more than 2-fold increase in neural tube defect prevalence among women with a BMI greater than 30 kg/m^2 (Shaw et al., 1996; Werler et al., 1996). Low folate levels in overweight and obese patients does not appear to reflect differences in dietary folate intake (Shaw et al., 1996; Werler et al., 1996). Although several of these aforementioned studies were conducted in populations that were not receiving folate fortification (Mojtabai, 2004; Schweiger et al., 2010) many (Mahabir et al., 2008; Mojtabai, 2004) were conducted in populations after mandatory folate fortification, and the association was retained. Since disparities in serum folate levels have also been observed in control dietary feeding studies, it has been suggested that lower folate levels in overweight/obesity might reflect a disruption in whole body folate homeostasis (Mahabir et al.,

2008). Animal based studies also support the possibility that obesity impairs folate homeostasis. Folate levels were also significantly decreased in obese db/db mice and the rise in circulating folate levels following folic acid supplementation in these mice was significantly blunted compared to lean controls (Seto et al., 2010). There is compelling evidence that obesity is associated with lower folate status; the sum of the evidence suggests that the regulation of folate homeostasis might be impaired in obesity.

Hirsch *et al* reported of a strong positive association between NAFLD and low serum folate levels in obese females, and proposed that the relationship between obesity and the depletion of folate could be a risk factor for NAFLD (Hirsch et al., 2005). Conversely, it is also possible that NAFLD could also contribute to low folate levels in obese patients. In fact, liver injury is associated with the depletion of hepatic folate stores and whole body folate insufficiency. Hepatic folate levels were decreased by 60% in cirrhotic livers (Leevy et al., 1970) and folate deficiency is also one of the most commonly reported micronutrient deficiencies in chronic liver diseases (Leevy & Baker, 1970; Leevy et al., 1970). Although an association between folate deficiency and NAFLD has emerged, a cause/effect relationship has yet to be established. Studies are needed to investigate whether folic acid supplementation might have protective effects in NAFLD.

III. HYPOTHESES AND OBJECTIVES

3.1. Rationale

Oxidative stress is a complex and poorly understood phenomenon associated with the pathogenesis of many diseases. Although oxidative stress is recognized as an important pathogenic mediator in NAFLD, the underlying mechanisms responsible for regulating oxidative stress in NAFLD are not well known. Oxidative stress also contributes to atherogenesis, a cardiovascular related pathology that is strongly associated with NAFLD. The lack of safe and efficacious therapies available for clinical management of NAFLD is a pressing issue given that the incidence of the disorder is increasing worldwide (Loomba & Sanyal, 2013) and the pathology is associated with a growing number of hepatic and extra-hepatic health risks (Anstee et al., 2013). Innovative studies are needed to i) further define the mechanisms involved in the regulation of oxidative stress in NAFLD and to ii) identify novel therapeutics for NAFLD management.

3.2. Hypotheses

Clinical studies have demonstrated that antioxidants such as vitamin E might be efficacious and safe therapies for managing NAFLD. We hypothesize that other NHPs such as berberine which has long history of use in traditional medicines, as well as folic acid, a stable precursor of folate, may have potential for managing NAFLD and its associated cardiometabolic risk through their antioxidant effects. We also hypothesize that the regulation of hepatic oxidative stress is linked to metabolic abnormalities in NAFLD.

3.3. Objectives

The overall objective of my research is to investigate the innate regulation of oxidative stress and the antioxidant actions of NHPs in the context of NAFLD and its associated co-morbidities (namely cardiovascular disease). The main objectives of my research are outlined as follows:

Objective 1: To investigate the effect of berberine on the regulation of oxidative stress in activated macrophages

Objective 2: To investigate the effect of folic acid supplementation on the regulation of hepatic oxidative stress in the HFD mouse model

Objective 3: To identify a novel underlying mechanism that is responsible for regulating oxidative stress and that is also associated with aberrant metabolism in the HFD fed mouse model

IV. MANUSCRIPTS

MANUSCRIPT I

4.1. Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages

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4.1.1. Abstract

Oxidative stress and amplified redox signaling contribute to the pathogenesis of many human diseases including atherosclerosis. The superoxide ($O_2^{\cdot -}$) generating phagocytic NADPH oxidase is a key source of oxidative stress in the developing atheroma. The aim of the present study was to examine the effect of berberine, a plant-derived alkaloid, on NADPH oxidase-mediated $O_2^{\cdot -}$ production in macrophages. Lipopolysaccharide (LPS) treatment activated NADPH oxidase in THP-1 monocyte-derived macrophages and increased the intracellular level of $O_2^{\cdot -}$. Pre-incubation of cells with berberine demonstrated a concentration-dependent (10–50 μmol/L) and time-dependent (6–24 h) inhibition of $O_2^{\cdot -}$ generation in LPS-stimulated macrophages. Cell viability tests confirmed that berberine, at concentrations sufficient for inhibiting NADPH oxidase-mediated $O_2^{\cdot -}$ generation in macrophages, did not affect cell viability. Real-time PCR analysis revealed that addition of berberine to the culture medium was able to reduce $gp91^{phox}$ mRNA expression in LPS-treated cells. Berberine also restored superoxide dismutase (SOD) activity, which was found to be inhibited by LPS treatment. In conclusion, results from the present study demonstrate that berberine can effectively reduce intracellular $O_2^{\cdot -}$ levels in LPS-stimulated macrophages. Such a restoration of cellular redox by berberine is mediated by its selective inhibition of $gp91^{phox}$ expression and enhancement of SOD activity. The therapeutic relevance of berberine in the prevention and management of atherosclerosis remains to be further investigated.

4.1.2. Introduction

The superoxide anion ($O_2^{\cdot -}$) is a redox signalling molecule that plays an important role in physiologic and pathologic processes (Afanas'ev, 2005; Droge, 2002). Perturbations in the redox balance favoring an increase in intracellular $O_2^{\cdot -}$ production can potentiate oxidative stress, disrupt intricate cell signalling pathways and modify biomolecules (Genestra, 2007). Although the direct oxidative capacity of $O_2^{\cdot -}$ is nominal, dismutation of $O_2^{\cdot -}$ generates precarious secondary reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and peroxynitrite ($ONOO^-$), which may also influence signal transduction pathways and induce widespread oxidative injury (Hancock, 1997; Mallozzi et al., 2001; Simon et al., 2004; Stone & Yang, 2006).

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is multi-subunit $O_2^{\cdot -}$ generating enzyme. NADPH oxidase catalyzes the transfer of one electron (e^-) from cytosolic NADPH to reduce extramembranous molecular oxygen (Koshkin et al., 1997). Of the various isoforms (Nox 1-Nox5; Duox 1 and 2) detected in human tissues, $gp91^{phox}$ (analogue of Nox2) is the most extensively studied and understood catalytic subunit of NADPH oxidase (Lambeth, 2004, 2007). The injurious oxidative capacity of NADPH is tightly regulated by the complexity of its construct (Cross & Segal, 2004; Lambeth, 2007). In resting cells, the functional subunits of the enzyme are partitioned in distinct subcellular loci. The catalytic subunit $gp91^{phox}$ and its stabilizing protein $p22^{phox}$ form the catalytic transmembrane heterodimer flavocytochrome b_{558} (Isogai et al., 1991). The regulatory subunit complex in the cytosol has two independent composites, namely $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ (Finan et al., 1994; Ito et al., 1996) as well as Rac 1/2 which is bound to its inhibitory protein guanyl nucleotide dissociation

inhibitors (GDI) (Kwong et al., 1993). Upon exposure of cells to stimuli, $p47^{phox}$ and $p67^{phox}$ undergo phosphorylation and translocate along with $p40^{phox}$ to the membrane (Bouin et al., 1998; Dusi et al., 1993; Siow et al., 2006). Rac dissociates from its inhibitor protein and also localizes in the membrane (Abo et al., 1994; Heyworth et al., 1994). The interaction between the regulatory subunits and the catalytic dimer create stabilizing protein-protein and protein-lipid contacts, facilitating oxidase activation (Heyworth et al., 1994; Kanai et al., 2001; Mizrahi et al., 2006; Sumimoto et al., 1996; Uhlinger et al., 1994). In response to prolonged stimulation, NADPH oxidase gene expression may be upregulated, further enhancing the activity and oxidative potential of the enzyme.

NADPH oxidase is a fundamental antimicrobial system and as such is predominantly expressed in immune cells such as neutrophils and macrophages (Fang, 2004). The upsurge of $O_2^{\cdot -}$ generated by the phagocytic NADPH oxidase during the respiratory burst gives rise to cytotoxic antimicrobial derivatives including hypochlorous acid (HOCl) and $ONOO^-$ (Babior, 1984; Fang, 2004). Although critical for host defence, dysregulation of NADPH oxidase activity appears to play a role in the development of certain diseases, particularly those associated with oxidative stress (Quinn et al., 2006). For instance, oxidative stress is a pathologic feature of atherosclerosis and approximately 60% of the ROS localized in the atheroma of human atherosclerotic plaques have been attributed to NADPH oxidase (Guzik et al., 2006). The hyperactivity of NADPH oxidase in human plaques may be attributable to the increased expression of $gp91^{phox}$ and $p22^{phox}$ (Azumi et al., 2002; Azumi et al., 1999; Guzik et al., 2000; Sorescu et al., 2002). Human atherosclerotic plaques have been shown to develop proportionally to the expression of $gp91^{phox}$

and the population of macrophages (Sorescu et al., 2002). Inhibition of NADPH oxidase-mediated $O_2^{\cdot -}$ generation may effectively reduce oxidative stress in the atheroma.

Berberine is an isoquinoline plant alkaloid traditionally applied in Chinese, Ayurvedic and North American medicines for the treatment of diarrhea (Siow et al., 2011a). Recently, its antioxidant (Hsieh et al., 2007; Ko et al., 2007), anti-inflammatory (Chen et al., 2008; Guo et al., 2008; Ko et al., 2007), anti-proliferative (Lee et al., 2006) and lipid lowering properties (Kong et al., 2004; Zhang et al., 2008b), are supportive of the potential therapeutic application of berberine in the prevention and treatment of atherosclerotic cardiovascular disease (Guo et al., 2008; Siow et al., 2011a). We hypothesized that berberine might also regulate NADPH oxidase activity in macrophages by modulating gene expression of the catalytic flavocytochrome b_{558} subunits. The objective of the present study was to determine the effect of berberine on lipopolysaccharide (LPS)-induced NADPH oxidase activity and the underlying mechanism(s).

4.1.3. Materials and Methods

i) Cell culture

The human monocytic cell line THP-1 was purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium containing 10% (*v*/*v*) fetal bovine serum. To induce cell differentiation into macrophage-like cells (defined as THP-1 macrophages), THP-1 monocytes were cultured in the presence of phorbol 12-myristate 12-acetate (PMA) for 24 h in RPMI 1640 medium (Wang et al., 2001b; Woo et al., 2003). Following differentiation, THP-1 macrophages were washed with HBSS washing buffer and stabilized overnight in RPMI 1640 media. Before treatments, the media was replaced a second time to avert the potential influence of PMA on our results. Cells were preincubated for 20 min with berberine chloride (Sigma Aldrich, St. Louis, USA) or apocynin an NADPH oxidase inhibitor (Stolk et al., 1994), followed by the addition of LPS (10 µg/L) to the culture medium. Berberine and apocynin were both dissolved in dimethyl sulfoxide (DMSO) that was administered at a concentration of 0.1% (*v*/*v*) in all applicable experiments.

ii) Determination of intracellular $O_2^{\cdot -}$

Following incubation with various compounds over different time periods, THP-1 macrophages were washed with HBSS and the nitroblue tetrazolium (NBT) reduction assay was performed to measure intracellular $O_2^{\cdot -}$ levels as previously described (Au-Yeung et al., 2004; Rauen et al., 2000; Woo et al., 2003). In brief, cells were incubated in Krebs–Henseleit buffer in the presence of NBT (1.0 mg/mL) for 45 min. Cells were lysed with a phosphate buffer (80 mmol/L, pH 7.8)

containing 5% sodium dodecyl sulfate and 0.45% gelatin. The cell lysate was centrifuged for 5 min at 13 000*g*. The supernatant was used to measure the absorbance at 540 nm (formazan) and 450 nm. The calculation for the relative concentration of $O_2^{\cdot -}$ was based on the amount of formazan formed, using the following established equation: $E_{540correct} = (E_{540} - E_{450}) / 0.49$ (Rauen et al., 2000). Formazan produced by the reduction of NBT in the presence of $O_2^{\cdot -}$ was proportional to the intracellular generation of $O_2^{\cdot -}$.

iii) Cell viability

Cell viability was assessed using colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). This method is based on the conversion of MTT to MTT-formazan crystals by mitochondrial enzymes. In brief, cells were plated in 96-well plates at a density of 1.0×10^4 cells per well. After treatment, cells were incubated for 4 h in the presence of MTT (0.5 mg/mL). MTT-formazan was extracted by DMSO. The optical density of formazan (540 nm) was then detected. A reduction of measured optical density compared with control cells indicates a loss of cell growth and viability.

iv) Determination of NADPH oxidase activity

NADPH oxidase activity was measured using the lucigenin chemiluminescence assay (Chen et al., 2001; Siow et al., 2006). Following incubation with various treatments, cells were pelleted by centrifugation at 3000*g* for 10 min. Cells were sonicated in a buffer containing 50 mmol/L phosphate buffer (pH 7), 1 mmol/L EDTA, 1 mmol/L PMSF, and 1 mmol/L leupeptin on ice. Cell lysates were incubated for 2 min with 5 μmol/L lucigenin in 50 mmol/L phosphate buffer. NADPH substrate (100 μmol/L) was added to the reaction mixture and the chemiluminescent

signal (photon emission) was measured every 15 s for 3 min using a Lumbat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

v) Measurement of NADPH oxidase subunit mRNA expression

The mRNA expression of $gp91^{phox}$ and $p22^{phox}$ was quantified using real-time polymerase chain reaction (qRT-PCR) and analyzed using the iQ5 real-time PCR detection system (Bio-Rad, Hercules, USA). In brief, total RNAs were isolated from cultured cells with TriZol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed using 2 μg total RNA to convert it to cDNA. The reaction mixture (25 μL) for each PCR sample contained 12.5 μL iQ-SYBR Green Supermix (Bio-Rad), 0.25 μL of each primer (Invitrogen), and 2 μL of cDNA. Primers for each subunit used in the reaction mixtures were as follows: human $gp91^{phox}$, 5′-TGAACTTGGAGACAGGCAAA-3′ (forward) and 5′-TGGTTTTGGTGGAGGAAGTG-3′ (reverse); and human $p22^{phox}$, 5′-GTCCCTGCATTCTGTGCTTT-3′ (forward) and 5′-GAACACCTCTGCACCCTGAT-3′ (reverse). Human GAPDH primers, 5′-ATCATCCCTGCCTCTACTGG-3′ (forward) and 5′-GTCAGGTCCACCACTGACAC-3′ (reverse) were used as a control to normalize threshold values.

vi) Transfection with $gp91^{phox}$ siRNA

Cells were transfected with $gp91^{phox}$ siRNA duplex oligoribonucleotides (Stealth RNAi, Invitrogen). THP-1 cells were seeded in 6-well plates as previous described and transfected with $gp91^{phox}$ siRNA in accordance with the manufacturer's instruction. For a negative control, cells were transfected with Stealth RNAi negative control (Invitrogen) consisting of a scrambled sequence that was unable to inhibit gene expression. At 48 h after transfection, cells were

incubated with or without LPS for 6 h, and then the intracellular $O_2^{\cdot -}$ level was measured by the NBT reduction assay. The mRNA levels of $gp91^{phox}$ in transfected cells were determined by real-time PCR analysis. The primers used for human $gp91^{phox}$ were (forward) 5′-TGAACTTGGAGACAGGCAAA-3′ and (reverse) 5′-TGGTTTTGGTGGAGGAAGTG-3′ (Invitrogen).

vii) Determination of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was determined as previously described (Crapo et al., 1978; Inoue et al., 1996; Wu et al., 2009). In brief, THP-1 cells were homogenized in 50 mmol/L potassium phosphate buffer containing 0.1 mmol/L EDTA (pH 7.8). SOD activity was assayed by monitoring the inhibition rate of xanthine oxidase-mediated reduction of cytochrome *c* in cell lysate (80 μg total proteins). Calibrations were performed using known amounts of purified SOD (Sigma Aldrich). The activity of purified SOD was 30 000 U/6.16 mg protein.

viii) Statistical analysis

Results were analyzed using the 2-tailed independent Student's *t* test or one-way analysis of variance (ANOVA) followed by the Newman–Keuls test using Prism 4 (GraphPad Software, La Jolla, USA). The level of statistical significance was set as $p < 0.05$.

4.1.4. Results

i) Effect of berberine on intracellular $O_2^{\cdot-}$ generation in macrophages

Incubation of THP-1 macrophages with LPS (10 µg/L) resulted in a significant and sustained rise in intracellular $O_2^{\cdot-}$ over a 24 h time course (Fig. 3.1.1). Pretreatment with berberine abolished LPS-induced intracellular $O_2^{\cdot-}$ generation (Fig. 3.1.1). Berberine at concentrations of 25 µmol/L or 50 µmol/L inhibited LPS-induced $O_2^{\cdot-}$ generation. However, berberine alone had no effect on basal $O_2^{\cdot-}$ levels in these cells (Fig. 3.1.2A). Incubation of macrophages with berberine (10–25 µmol/L) for 6 h did not have any considerable effect on cell viability (Fig. 3.1.2B). However, berberine at a concentration of 50 µmol/L resulted in a significant decline in cell viability (Fig. 3.1.2B). In another set of experiments, addition of apocynin, a known NADPH oxidase inhibitor, to the culture medium before LPS administration also completely abolished LPS-induced intracellular $O_2^{\cdot-}$ production (Fig. 3.1.3A), implicating NADPH oxidase as the primary source of $O_2^{\cdot-}$ in these cells. These results suggest that LPS-induced $O_2^{\cdot-}$ generation was mediated via NADPH oxidase and that berberine inhibited such a stimulatory effect in macrophages.

ii) Effect of berberine on NADPH oxidase activity

The lucigenin chemiluminescence assay was conducted to assess the effect of berberine on NADPH oxidase activity. Cells incubated in the presence of LPS showed a significant increase in NADPH oxidase activity (Fig. 3.1.3B). Pretreatment with either berberine or apocynin significantly lowered the activity of NADPH oxidase in LPS-stimulated cells. These results suggest that berberine was able to inhibit NADPH oxidase activity in LPS-stimulated macrophages. To determine whether berberine could affect the NADPH oxidase by directly

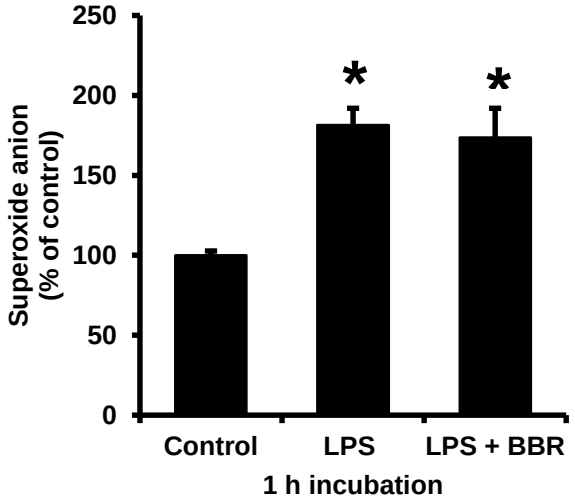
inhibiting the enzyme activity, an aliquot of THP-1 cell lysate was incubated with LPS in the absence or presence of berberine. NADPH oxidase activity was neither stimulated by LPS nor inhibited by berberine, indicating that berberine did not have a direct inhibitory effect on the activity of NADPH oxidase (Fig. 3.1.4).

iii) Effect of berberine on NADPH oxidase gene expression and SOD activity

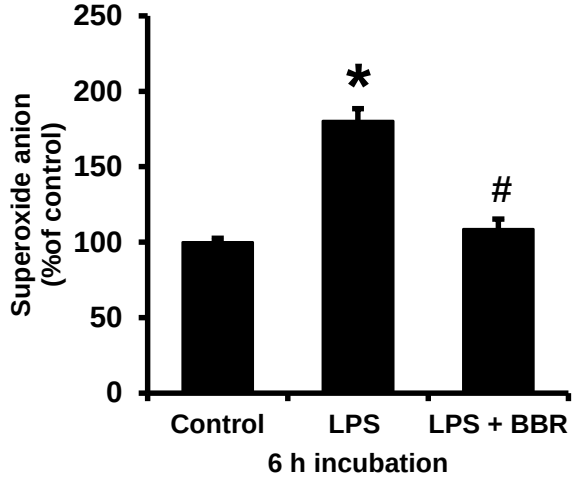
To investigate the molecular mechanism responsible for the regulation of NADPH oxidase activity by berberine, the mRNA levels of NADPH oxidase subunits were determined using real-time PCR analysis. LPS treatment significantly up-regulated the expression of $gp91^{phox}$ and $p22^{phox}$ with approximately a 2.5-fold increase in mRNA levels (Fig. 3.1.5). Pre-treatment with berberine effectively reduced the expression of $gp91^{phox}$ in LPS-stimulated cells (Fig. 3.1.5A) but had no effect on $p22^{phox}$ expression (Fig. 3.1.5B). Gene expression of the other NADPH oxidase subunits, including $p67^{phox}$, $p47^{phox}$, and Rac-1, were also examined in cells after LPS and berberine treatment. Although LPS markedly elevated gene expression of those subunits, berberine failed to restore their expression to basal levels (data not shown). To further examine the role of $gp91^{phox}$ in LPS-induced $O_2^{\cdot -}$ generation, cells were transfected with $gp91^{phox}$ siRNA. Transfection of cells with $gp91^{phox}$ siRNA effectively inhibited $gp91^{phox}$ mRNA expression and reduced LPS-induced elevation of $O_2^{\cdot -}$ levels in macrophages (Fig. 3.1.6). To further investigate the effect of berberine on the regulation of intracellular $O_2^{\cdot -}$ levels, the activity of SOD was measured. There was a significant reduction of SOD activity in cells incubated with LPS (Fig. 3.1.7). Addition of berberine to the culture medium partially restored the SOD activity (Fig. 3.1.7). These results suggest that the inhibitory effect of berberine on LPS-induced elevation of

intracellular $O_2^{\cdot -}$ levels might be mediated via inhibition of NADPH oxidase and activation of SOD.

A.


250
200
150
100
50
0
Superoxide anion
(% of control)
*
*
Control
LPS
LPS + BBR
1 h incubation

B.


250
200
150
100
50
0
Superoxide anion
(%of control)
*
#
Control
LPS
LPS + BBR
6 h incubation

C.

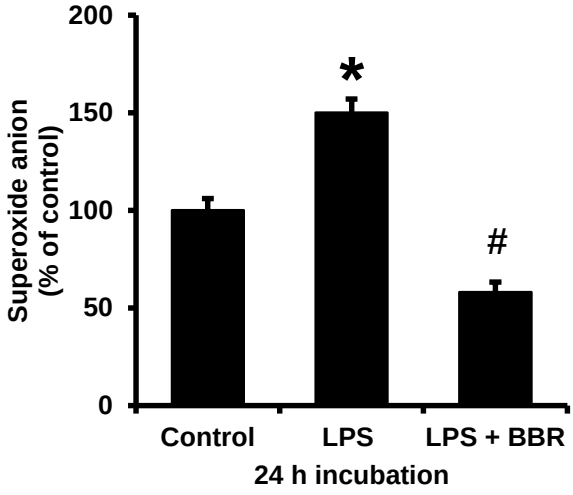
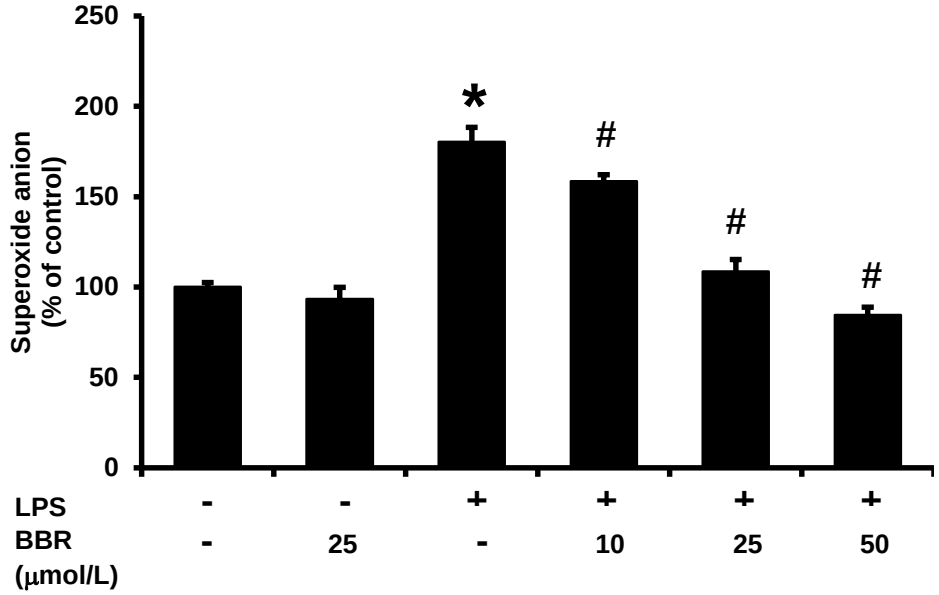

200
150
100
50
0
Superoxide anion
(% of control)
*
#
Control
LPS
LPS + BBR
24 h incubation

Fig. 3.1.1. Superoxide anion ($O_2^{\cdot-}$) production in macrophages

Cells were preincubated with or without berberine (BBR; 25 µmol/L) for 20 min, followed by incubation with lipopoysaccharide (LPS; 10 µg/L) for (A) 1 h, (B) 6 h, or (C) 24 h. Intracellular levels of superoxide anions **(**$O_2^{\cdot-}$) were determined by the nitroblue tetrazolium (NBT) reduction assay. The calculation of the relative concentration of $O_2^{\cdot-}$ was based on the amount of formazan formed in the assay mixture using an established equation ($E_{540correct} = (E_{540} - E_{450}) / 0.49$). The results were as follows: 0.0057 ± 0.0003 (1 h control) vs. 0.0100 ± 0.0004 (1 h LPS treatment), 0.0057 ± 0.0001 (6 h control) vs. 0.0098 ± 0.0007 (6 h LPS treatment), and 0.0071 ± 0.0013 (24 h control) vs. 0.0106 ± 0.0004 (24 h LPS treatment). The level of $O_2^{\cdot-}$ in control cells (at 1 h, 6 h, and 24 h incubation) was arbitrarily expressed as 100. Results are means ± SEM from 4 separate experiments each performed in duplicate.*, Significant at $p < 0.05$ compared with control; $^{\#}$, $p < 0.05$ compared with LPS-treated cells.

A.



B.

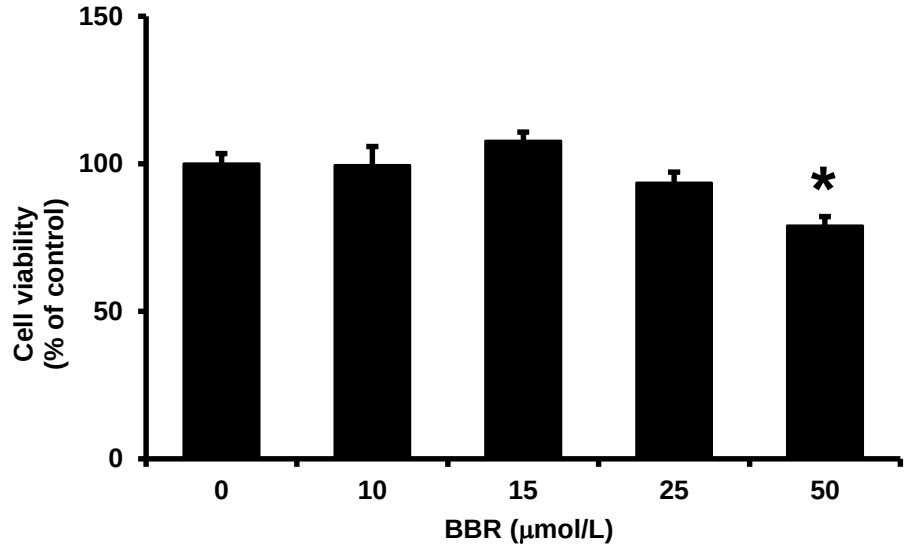
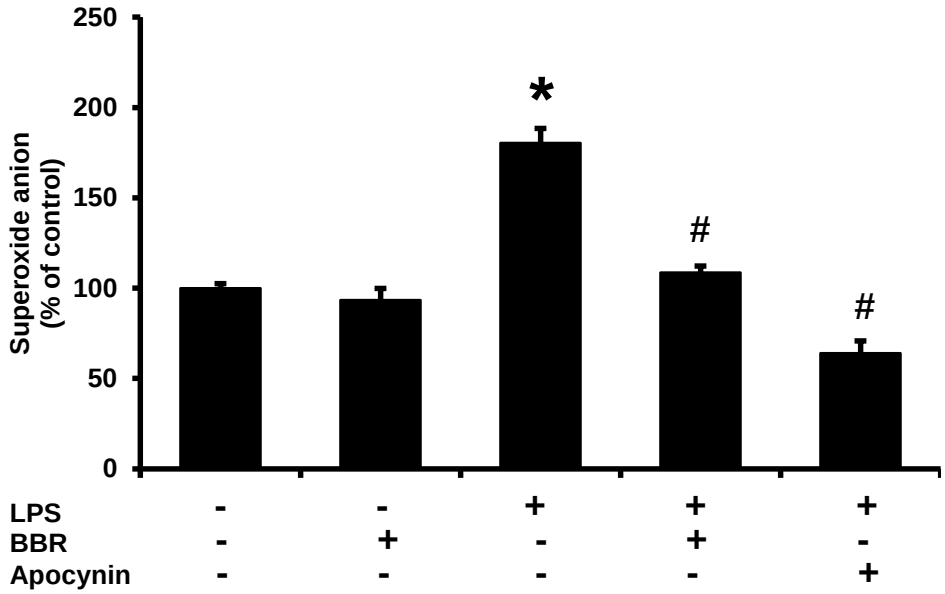


Fig. 3.1.2. Effect of berberine (BBR) on LPS-induced superoxide anion ($O_2^{\cdot-}$) production in macrophages

(A) Cells were preincubated with or without berberine (10–50 μmol/L) for 20 min followed by 6 h incubation with or without LPS (10 μg/L). Results are expressed as the mean ± SEM from 4 separate experiments each performed in duplicate. The level of $O_2^{\cdot-}$ in control cells was arbitrarily expressed as 100. (B) Cells were incubated with berberine (10–50 μmol/L) for 6 h. Cell viability was assessed using the MTT assay. Cell viability for control cells was arbitrarily expressed as 100. Results are means ± SEM from 4 separate experiments each performed in duplicate. *, Significant at $p < 0.05$ compared with control; $^{\#}$, $p < 0.05$ compared with LPS-treated cells.

A.


250
200
150
100
50
0
Superoxide anion
(% of control)
*
#
#
LPS - - + + +
BBR - + - + -
Apocynin - - - - +

B.

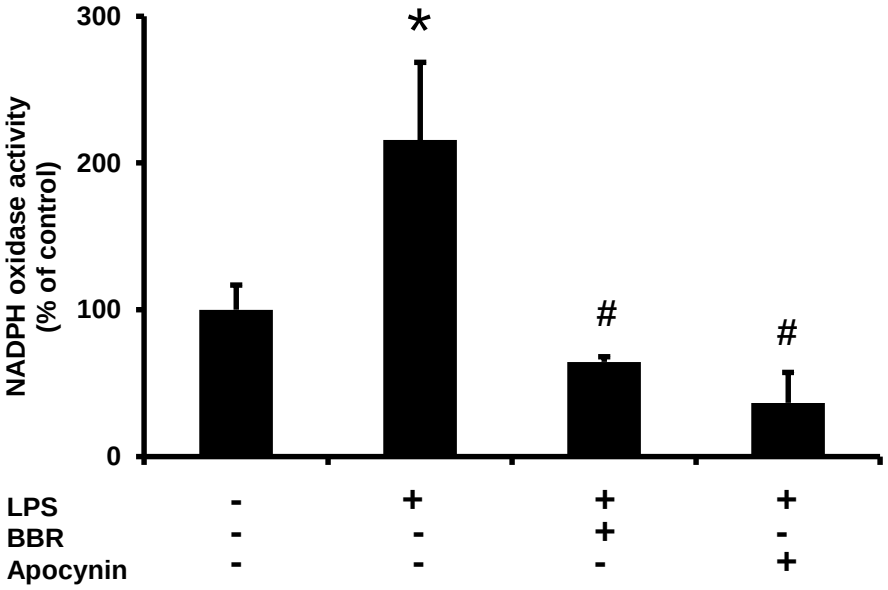

300
200
100
0
NADPH oxidase activity
(% of control)
*
#
#
LPS - + + +
BBR - - + -
Apocynin - - - +

Fig. 3.1.3. Effect of berberine (BBR) and apocynin on superoxide anion ($O_2^{\cdot -}$) production in macrophages.

Cells were preincubated with either berberine (25 μmol/L) or apocynin (300 μmol/L) for 20 min followed by 6 h incubation with or without LPS (10 μg/L). (A) Intracellular levels of $O_2^{\cdot -}$ were determined by the NBT reduction assay. The level of $O_2^{\cdot -}$ in control cells was arbitrarily expressed as 100. (B) NADPH oxidase activity was determined in cell lysate by lucigenin chemiluminescence assay. NADPH oxidase activity in control cells was arbitrarily expressed as 100. Results are means ± SEM from 4 separate experiments each performed in duplicate. *, Significant at $p < 0.05$ compared with control; $^{\#}$, $p < 0.05$ compared with LPS-treated cells.

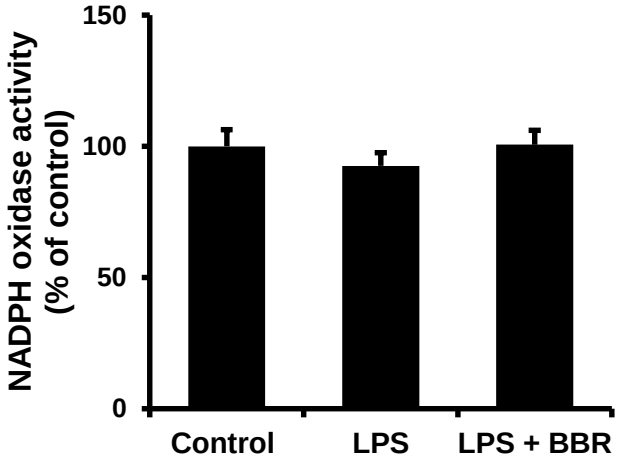
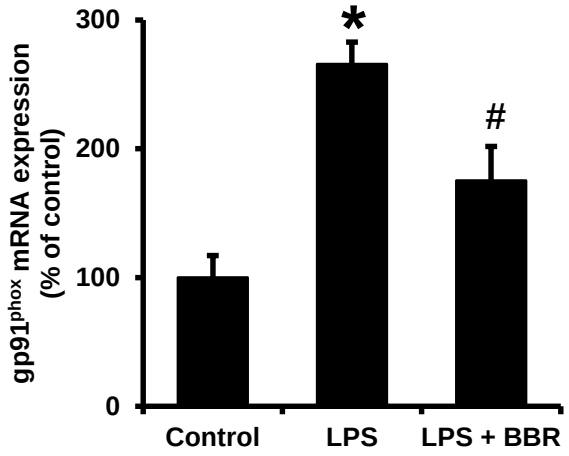


Fig. 3.1.4. Direct effect of berberine (BBR) on NADPH oxidase activity

Cells were collected and sonicated in a phosphate buffer (50 mmol/L, pH 7) containing 1 mmol/L EDTA as described in the methods section. Cell lysates were incubated at 37 °C with or without berberine (25 µmol/L) for 10 min followed by incubation with LPS (10 µg/L) for 5 min. The NADPH oxidase activity was determined by lucigenin chemiluminescence assay. NADPH oxidase activity in control cells was arbitrarily expressed as 100. Results are means ± SEM from 4 separate experiments each performed in duplicate.

A.



B.

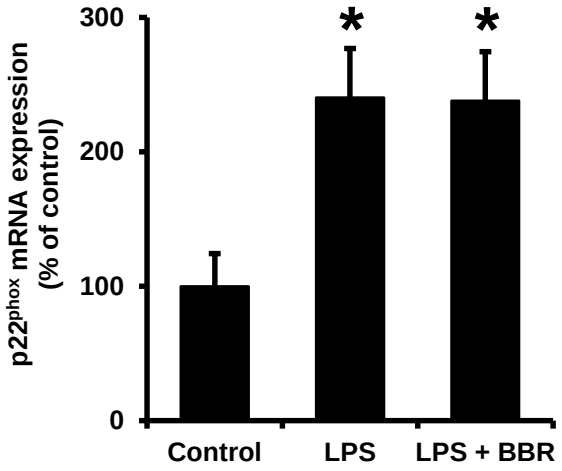
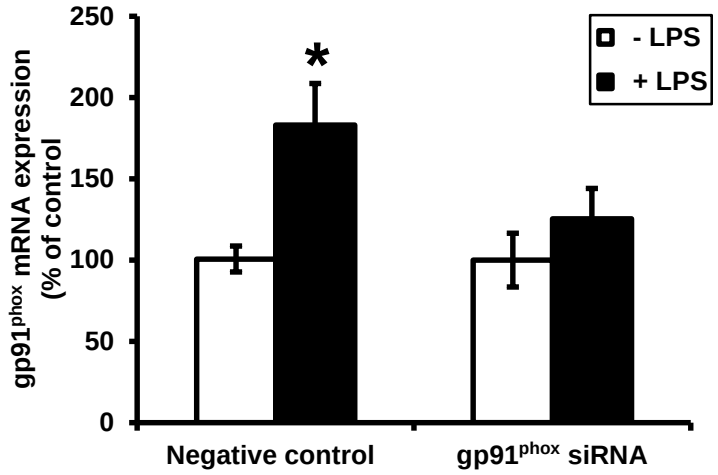


Fig. 3.1.5. Determination of gp91^{phox} and p22^{phox} mRNA in macrophages

Cells were pre-incubated with or without berberine (BBR; 25 µmol/L) for 20 min followed by 6 h incubation with LPS (10 µg/L). (A) gp91^{phox} mRNA and (B) p22^{phox} mRNA were determined by real-time PCR analysis. Results are means ± SEM from 4 separate experiments each performed in duplicate. Crossing threshold values were normalized to GAPDH mRNA expression. The mRNA expression in control cells was arbitrarily expressed as 100. *, Significant at $p < 0.05$ compared with control; $^{\#}$, $p < 0.05$ compared with LPS-treated cells.

A.



B.

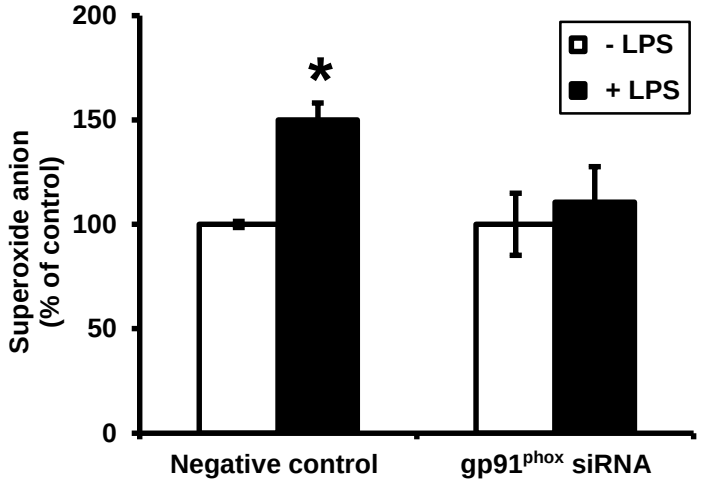


Fig. 3.1.6. Determination of gp91^{phox} mRNA and superoxide anion ($O_2^{\cdot-}$) levels in siRNA transfected macrophages

Cells were transfected with gp91^{phox} siRNA or with a scramble siRNA. Transfected cells were incubated for 6 h in the absence or presence of LPS (10 µg/L). (A) gp91^{phox} mRNA expression was determined by real-time PCR analysis. Crossing threshold values were normalized to GAPDH expression. The mRNA expression in cells without LPS treatment was arbitrarily expressed as 100. (B) Superoxide anion ($O_2^{\cdot-}$) levels were determined by the NBT reduction assay. The level of $O_2^{\cdot-}$ in transfected cells without LPS treatment was arbitrarily expressed as 100. Results are means ± SEM from 4 separate experiments each performed in duplicate. *, Significant at $p < 0.05$ compared with control.

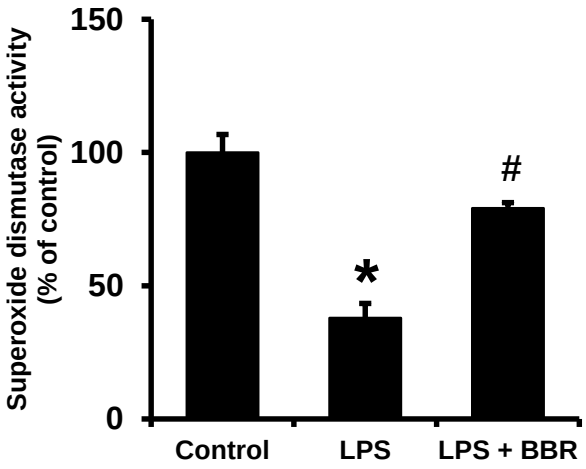


Fig. 3.1.7. Determination of superoxide dismutase (SOD) activity in macrophages

Cells were preincubated with or without berberine (BBR; 25 μmol/L) for 20 min followed by incubation with LPS (10 μg/L) for 6 h. The activity of superoxide dismutase (SOD) was determined. The SOD activity of the control group was 49.47 ± 3.36 U/mg protein. The SOD activity in control cells was arbitrarily expressed as 100. Results are means ± SEM from 4 separate experiments each performed in duplicate. *, Significant at $p < 0.05$ compared with control; $^{\#}$, $p < 0.05$ compared with LPS-treated cells.

4.1.5. Discussion

The novel findings of the present study are that berberine regulates the expression of the catalytic gp91^{phox} subunit of NADPH oxidase in LPS-stimulated macrophages. This, in turn, suppresses the activity of NADPH oxidase and reduces intracellular $O_2^{\cdot -}$ levels.

The pathologic contribution of oxidative stress in human diseases is becoming increasingly appreciated. For instance, atherosclerosis is defined in part by enhanced ROS-mediated signaling at both the initiating and progressive stages of the disease (Madamanchi et al., 2005; Warnholtz et al., 1999). The intensified presence of ROS molecules observed in each facet of the atheroma (Griendling et al., 2000) may be attributable, in part, to the enhanced activity of NADPH oxidase (Guzik et al., 2006; Mohazzab et al., 1994; Vendrov et al., 2007). Another classic feature of atherosclerosis is the permeation and differentiation of monocytes into macrophages within the injured vascular endothelium (Ross, 1993). These macrophages express higher gp91^{phox} and have the capacity to generate $O_2^{\cdot -}$ at levels superior to those of other vascular cells (Griendling et al., 2000; Sorescu et al., 2002). Therefore, targeted inhibition of NADPH oxidase in macrophages can lessen the oxidative burden and may be therapeutically pertinent in atherosclerosis (Cathcart, 2004; Madamanchi et al., 2005; Vendrov et al., 2007).

Recent evidence suggests that the antioxidant capability of berberine is mediated through its ability to upregulate enzymatic and nonenzymatic components of the innate antioxidant defense system including SOD, catalase, glutathione peroxidase, as well as, glutathione, vitamin C, and vitamin E (Tan et al., 2007; Thirupurasundari et al., 2009; Zhou et al., 2009). In the present

study, berberine was shown to alleviate the oxidative burden by inhibiting NADPH oxidase through downregulation of $gp91^{phox}$ expression. Inhibition of $gp91^{phox}$ expression is significant given its obligatory role in oxidase activation. The $gp91^{phox}$ subunit is imperative for the membrane-localized assembly of oxidase constituents (Leusen et al., 1994), and it possesses all intermediary cofactors crucial for the intramembrane transfer of electrons including NADPH and flavin adenine dinucleotide (FAD) docking sites and 2 flavin-binding elements (Cross et al., 1995; Hashida et al., 2004; Lambeth, 2007; Segal et al., 1992). Consequently, in line with previous reports (Banerjee et al., 2000; Champelovier et al., 1993), the impaired expression of $gp91^{phox}$ coincided with a reduction in NADPH oxidase activity in the present study. Furthermore, berberine was shown to upregulate the SOD activity, hence contributing to the lowering of intracellular $O_2^{\cdot -}$ levels in LPS-treated cells.

As an intriguing aside, it appears that berberine has conditional redox regulating properties. Previously, berberine has been shown to have devastating pro-oxidant capabilities in human prostate cancer cells (Meeran et al., 2008), whereas conversely in our study, berberine displayed antioxidant effects in stimulated macrophages using similar concentration (25–50 μmol/L) and treatment (1–24 h) conditions. Moreover, berberine treatment did not affect basal $O_2^{\cdot -}$ levels in non-LPS-treated THP-1 macrophages. Such contextual redox control is attractive given the emerging role of ROS-mediated cellular signaling under physiologic and pathologic situations. In macrophages, NADPH oxidase-derived ROS are fundamental since they intercede various cellular events including facilitation of the respiratory burst and the induction of inflammatory and survival pathways (Forman & Torres, 2001; Iles & Forman, 2002; Wang et al., 2007). Therefore, a reduction of $O_2^{\cdot -}$ beyond basal levels could have unfavorable outcomes on host

defense and cellular homeostasis. In the present study, 6 h incubation with 50 µmol/L of berberine reduced intracellular $O_2^{\cdot -}$ below control levels. Such conditions also resulted in a significant decline in cell viability suggesting that berberine, at higher concentrations, could mediate toxicity via a redox sensitive mechanism.

In conclusion, the present study clearly demonstrates that berberine can effectively regulate NADPH oxidase-mediated $O_2^{\cdot -}$ generation through the selective downregulation of $gp91^{phox}$ expression in macrophages. Our results indicate a novel mechanism through which berberine may exert a beneficial effect against oxidative stress. Further investigation is needed to evaluate the therapeutic efficacy of berberine in the prevention and treatment of diseases that are associated with oxidative stress including atherosclerosis.

MANUSCRIPT II

4.2. Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase

Can. J. Physiol Pharmacol. 90: 155-165 (2012)

4.2.1. Abstract

Diets high in saturated fat and cholesterol facilitate weight gain, a predisposing factor that contributes to the onset of obesity and metabolic disorders. Hepatic oxidative stress is commonly reported in various animal models of obesity and has been associated with enhanced expression of NADPH oxidase. We have previously reported several antioxidant mechanisms through which folic acid confers protection during hyperhomocysteinemia-induced oxidative stress. The objective of the present study was to investigate whether folic acid supplementation ameliorates high fat diet (HFD) induced oxidative stress in the liver, and to identify the underlying mechanisms. Male C57BL/6J mice were fed a control diet, a HFD, or a HFD supplemented with folic acid for 12 weeks. A HFD led to increased body mass, hepatic lipid peroxidation, and liver injury. There was a significant increase in hepatic NADPH oxidase activity, which was associated with enhanced expression of several NADPH-oxidase subunits. Folic acid supplementation had a protective effect against HFD induced hepatic oxidative stress and liver injury. Further analysis revealed that the antioxidant effect of folic acid was attributed, in part, to transcriptional regulation of NADPH oxidase. These results suggested that folic acid supplementation may be hepatoprotective from liver injury associated with a HFD.

4.2.2. Introduction

Regular consumption of saturated fats and cholesterol promote weight gain and exacerbate the development of obesity; an antecedent condition from which other metabolic disorders including Type 2 diabetes mellitus (T2DM), metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD) arise (Gaemers et al., 2011; Golay & Bobbioni, 1997; Hariri et al., 2010). Oxidative stress is a common feature in those chronic diseases. Oxidative stress is a biological phenomenon characterized by the endogenous accumulation of unstable reactive oxygen species (ROS), arising when enhanced ROS generation is not appropriately compensated for by an increase in antioxidant defense mechanisms. Chronic exposure to elevated levels of these highly reactive molecules potentiates the chemical modification of proteins, lipids, and DNA, while also upsetting redox sensitive signaling events (Droge, 2002).

It has been suggested that hepatic oxidative stress following the consumption of a high-fat diet (HFD) is a response that occurs prior to the onset of weight gain, insulin resistance, and glucose intolerance (Matsuzawa-Nagata et al., 2008). Hepatic oxidative stress also persists with a long-term HFD, and intensifies proportionally to diet-induced weight gain (Matsuzawa et al., 2007; Milagro et al., 2006). Hepatic oxidative stress is associated with a striking increase in the expression of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in animal models of obesity and metabolic syndrome (Gao et al., 2010; Gupte et al., 2009; Matsunami et al., 2010). The classical NADPH oxidase is a multi-subunit superoxide anion ($O_2^{\cdot -}$) generating enzyme. The catalytic subunit of the enzyme, $gp91^{phox}$, is associated with the $p22^{phox}$ subunit to form a membrane-bound dimer termed cytochrome b_{558}. Several regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac 1/2) are sequestered in the cytosol. The translocation of cytosolic

subunits to the membrane followed by their interaction with the membrane subunits is essential for the assembly and activation of NADPH oxidase. The NADPH oxidase is regulated at the transcriptional, translational, and post-translational levels (Lambeth, 2007). Although reports have identified enhanced mRNA or protein expression of nearly all NADPH oxidase subunits in the liver of genetically or diet-induced obesity animal models, those consistently mentioned include $gp91^{phox}$ and $p22^{phox}$, as well as regulatory subunits $p47^{phox}$and $p67^{phox}$ (Furukawa et al., 2004; Gupte et al., 2009; Matsunami et al., 2010; Sohet et al., 2009). Nuclear factor kappa B (NF-κB) is one of the transcription factors for NADPH oxidase subunits in various cell types (Anrather et al., 2006; Edderkaoui et al., 2011; Manea et al., 2007). Activation of NF-κB has also been observed in the liver of obese mice (Sajan et al., 2009).

Folate is an essential water-soluble vitamin (B_9) that participates in DNA/RNA biosynthesis as well as amino-acid interconversions. An inverse association between serum folate levels and body-mass index has been reported, and folate deficiency is notable in patients with established hepatic diseases (Gallistl et al., 2000; Hirsch et al., 2005; Leevy & Baker, 1970). Folic acid is a synthetic form of folate, with higher bioavailability than its natural counterpart (Iyer & Tomar, 2009). Recent findings from our laboratory have demonstrated that folic acid is a potent modulator of oxidative stress, protecting against oxidative injury *in vitro*, as well as *in vivo* (Au-Yeung et al., 2006; Hwang et al., 2011; Woo et al., 2006). In the present study, we have hypothesized that folic acid protects against hepatic oxidative injury, induced by the chronic consumption of a HFD, by regulation of NADPH oxidase at the transcriptional level. The objectives of the present study were to investigate the effect of folic acid supplementation on oxidative stress in the liver, and to identify the mechanisms responsible for such action.

4.2.3. Materials and methods

i) Animal model

Male C57BL/6J mice (6 weeks old; The Jackson Laboratory, Bar Harbor, Maine, USA) were fed for 12 weeks with (*i*) a control diet (10% kcal fat) (*ii*) a high-fat diet (60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$ (HFD), or (*iii*) high-fat diet (60% kcal fat) plus 0.025% folic acid (252.6 mg folic acid·(kg chow)$^{-1}$) (The Jackson Laboratory) (HFD + FA). The composition of the control and HFD formulas have been outlined (*Appendix I-III*)*. Mice were kept on a 12 h (light) – 12 h (dark) cycle, and had access to water, ad libitum. Mice were weighed prior to the commencement, as well as at the end of the 12 week feeding period. Liver function was assessed by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Genzyme Diagnostics, Charlottetown, Prince Edward Island, Canada) (Woo et al., 2006). Customized diets were approved by the Jackson Laboratory Animal Care and Use Committee. All procedures were performed in accordance with the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care, and approved by the University of Manitoba Protocol Management and Review Committee.

ii) Determination of hepatic lipid peroxidation

Malondialdehyde (MDA), a stable product of lipid peroxidation, was assessed in liver tissue using the thiobarbituric acid reactive substances (TBARS) method as previously reported (Woo et al., 2006). In brief, a portion of liver was homogenized with 1.14% KCl solution containing 50 mmol·L^{-1}deferoxamine. Homogenate was centrifuged at 3000*g* for 10 min at 4 °C, and an

* Variance to that published as requested by examining committee

aliquot of the supernatant was incubated for 1 h at 95 °C in a reaction mixture consisting of 0.45% SDS, 8.3% acetic acid, and 0.33% thiobarbituric acid *v*/*v*. The TBARS present in the reaction mixture were extracted with butanol, and quantified spectrophotometrically at absorbance 535 nm with the aid of an MDA standard curve. Protein concentrations in sample supernatants were quantified using a commercially available detection reagent (Bio-Rad, Hercules, California, USA) and used to normalize sample MDA concentrations.

iii) Determination of NADPH oxidase activity

The activity of NADPH oxidase was measured in liver tissue as previously described, using the lucigenin chemiluminescence assay with modification (Sarna et al., 2010; Woo et al., 2006). Liver tissue was homogenized in a 50 mmol·L^{-1} phosphate buffer (pH 7.0) containing 1 mmol·L^{-1} EDTA and 12.5 µmol/L of both PMSF and leupeptin protease inhibitors. The homogenate was centrifuged at 3000*g* for 10 min at 4 °C, and the supernatant collected for analysis. In the presence of NADPH substrate, liver tissue homogenate will emit photons relative to the interaction of $O_2^{\cdot -}$with lucigenin. This luminescent signal may be detected using a luminometer, and provides a direct measurement of NADPH-oxidase-mediated $O_2^{\cdot -}$ production in biological samples (Li et al., 1998). An aliquot of the supernatant was added to a reaction mixture consisting of 50 mmol·L^{-1}phosphate buffer and 5 µmol/L lucigenin. Following the addition of 0.2 mmol·L^{-1} NADPH substrate, the luminescent signal of the reaction mixture was measured every 12 s for 3 min (1000 ms integration) using a Spectra Max M5 Multi-Detection Reader (Molecular Devices, Sunnyvale, Calif.). Mean photon emission from the reaction mixture was calculated using SoftMax Pro-5 software (Molecular Devices). A standard curve was prepared using known dilutions of xanthine oxidase (Sigma–Aldrich, St. Louis, Missouri, USA)

and xanthine (100 μmol/L) as substrate, and was used to quantify the concentration of $O_2^{\cdot-}$ generated in the reaction mixture. Protein concentrations of the sample supernatants were measured and were used to correct for NADPH oxidase activity.

iv) Measurement of NADPH oxidase subunit mRNA expression

The mRNA expression of NADPH oxidase subunits $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ was determined using the iQ5 real-time PCR detection system (Bio-Rad). Briefly, total RNAs were isolated from liver tissue using TriZol reagent (Invitrogen, Carlsbad, Calif.) and template cDNA was converted from RNA (2 μg) by reverse transcription. The reaction mixture (25 μL) for each PCR sample contained 2 μL of cDNA, 0.4 μmol/L of 5′ and 3′ primers (Invitrogen) and iQ-SYBR Green Supermix (Bio-Rad). Primers used for each subunit were as follows: mouse $p22^{phox}$, 5′-AAAGAGGAAAAAGGGGTCCA-3′ (forward) and 5′-TAGGCTCAATGGGAGTC CAC-3′ (reverse); mouse $gp91^{phox}$, 5′-ACTGCGGAGAGTTTGGAAGA-3′ (forward) and 5′-GGTGATGACCACCTTTTGCT-3′ (reverse); mouse $p47^{phox}$, 5′-ATACTTCAACGGCCTCAT GG-3′ (forward) and 5′-CTGTTCCCGAACTCTTCTCG-3′ (reverse); and mouse $p67^{phox}$, 5′-GCAGTGGCCTACTTCCAGAG-3′ (forward) and 5′-CTTCATGTTGGTTGCCAATG-3′ (reverse); Mouse GAPDH primers, 5′-GCACAGTCAAGGCCGAGAAT-3′ (forward) and 5′-G CCTTCTCCATGGTGGTGAA-3′ (reverse). GAPDH was used as the internal control to normalize threshold values.

v) Electrophoretic mobility shift assay (EMSA)

To determine whether NADPH oxidase subunit expression was regulated by NF-κB, the binding activity of NF-κB with DNA was measured by EMSA. In brief, nuclear proteins were prepared

from mouse liver as previously described (Woo et al., 2008; Wu et al., 2009). Nuclear proteins (10 μg) were incubated with excess ^{32}P-end-labeled oligonucleotides containing a consensus sequence specific for the NF-κB DNA binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, Wisconsin, USA). The reaction mixture was then separated in 6% nondenaturing polyacrylamide gel and dried on a piece of filter paper followed by autoradiography. The cold competition experiment was performed by adding a 100-fold excess unlabelled NF-κB probe prior to the addition of the ^{32}P-end-labeled oligonucleotide in the reaction mixture to confirm the specificity of binding of ^{32}P-end-labeled oligonucleotide with NF-κB.

vi) Determination of superoxide dismutase (SOD) and catalase activities and glutathione levels

Hepatic SOD activity was determined in liver tissue homogenate by measuring the inhibition rate of xanthine-oxidase-mediated reduction of cytochrome *c* (Crapo et al., 1978; Hwang et al., 2011). Catalase activity in liver was assessed by measuring the rate of H_2O_2 breakdown as previously reported (Beers & Sizer, 1952; Woo et al., 2006). The liver tissue was also assessed for reduced (GSH) and oxidized (GSSG) glutathione concentrations as previously described (Anderson, 1985). For GSH measurement, an aliquot of the sample was added to 0.2 mol/L sodium phosphate buffer and incubated for 10 min at room temperature with 0.1 mmol·L^{-1} of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Sample absorbance was measured at 412 nm using a spectrophotometer (DU800; Beckman Coulter, USA). For GSSG quantification, an aliquot of the sample was pre-incubated with 10% 2-vinylpyridine for 45 min, which made GSH unavailable for reaction with DTNB. Following incubation, the sample was added to a reaction

mixture consisting of 0.2 mol/L sodium phosphate buffer, 0.2 mmol·L^{-1} NADPH, 0.1 mmol·L^{-1} DTNB and 1.25 units of glutathione reductase. After 10 min incubation at room temperature, the sample absorbance was measured at 412 nm using a spectrophotometer (DU800).

vii) Liver histology and lipid analysis

A portion of mouse liver was fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Paraffin-embedded sections (5 µm) were prepared and stained with hematoxylin and eosin (H&E) to evaluate the morphological changes (Woo et al., 2005). Images of H&E sections (5 per liver) were captured using a Zeiss Axtoskop 2 light microscope equipped with an Axiovision digital camera (Carl Zeiss Microimaging, Thornwood, New York, USA) and were analyzed at ×200. Images presented in the figure are representative of each dietary group and were selected by an independent observer. Hepatic lipids were extracted from liver tissue using the Folch method (Folch et al., 1957). Hepatic cholesterol and triglyceride levels in the extract were determined using commercial kits (Wako Diagnostics, USA).

viii) Statistical analysis

Results obtained were analyzed by one-way ANOVA followed by the Newman–Keuls test using Prism 4 (GraphPad Software, La Jolla, San Diego, Calif.). Data are presented as the mean ± SEM. Values for $P < 0.05$ were considered statistically significant.

4.2.4. Results

i) Effect of high-fat diet (HFD) and folic acid supplementation on body mass and liver injury

Feeding mice with a HFD for 12 weeks led to a significant increase in body mass in these animals compared with those maintained on the control diet (Fig. 3.2.1A). Folic acid supplementation had no effect on body mass gain induced by a HFD (Fig. 3.2.1A). Consumption of a HFD induced liver injury, as indicated by a significant elevation of serum ALT (Fig. 3.2.1B) and AST (Fig. 3.2.1C) levels. Supplementation of the HFD with 0.025% folic acid was hepatoprotective, and resulted in a significant decline in both serum ALT (Fig. 3.2.1B) and AST (Fig. 3.2.1C). These results suggested that folic acid supplementation could curtail HFD induced liver injury, although it did not have a significant effect on body mass.

ii) Effect of a high-fat diet (HFD) and folic acid supplementation on hepatic lipid peroxidation and NADPH oxidase activation

The degree of lipid peroxidation in the liver was examined by measuring the level of MDA, a stable by-product of lipid oxidation that served as an indicator of hepatic oxidative stress. Mice fed a HFD for 12 weeks displayed a significant elevation in MDA levels in the liver tissue (Fig. 3.2.2). Folic acid supplementation completely abolished the rise in hepatic MDA levels in mice fed a HFD (Fig. 3.2.2). Administration of apocynin, an NADPH oxidase inhibitor, to mice fed a HFD significantly reduced the MDA level in the liver (Fig. 3.2.2). Similarly, the elevation in serum ALT levels, which was indicative of injury in the liver of mice consuming the HFD, was also lowered by apocynin treatment (data not shown). These results suggested that NADPH

oxidase might be involved in increased free radical generation in the liver, leading to lipid peroxidation and liver injury in mice fed a HFD. To further examine the involvement of NADPH oxidase in hepatic oxidative stress, the lucigenin chemiluminescence assay was performed to determine NADPH oxidase activity in liver tissue. The activity of NADPH oxidase was markedly increased in the liver tissue of mice fed a HFD (Fig. 3.2.3). However, mice receiving a HFD supplemented with folic acid showed a significant reduction in hepatic NADPH oxidase activity (Fig. 3.2.3). Taken together, these results suggest that NADPH oxidase might be a key mediator of hepatic lipid peroxidation in the liver of mice fed a HFD. The antioxidant effect of folic acid supplementation was likely attributable to the inhibition of NADPH oxidase activity.

iii) Effect of a high-fat diet (HFD) and folic acid supplementation on hepatic NADPH oxidase mRNA expression

To investigate the mechanism by which folic acid supplementation inhibited NADPH oxidase activity, the mRNA expression of NADPH oxidase subunits were analyzed. Relative to mice maintained on the control diet, there was a significant increase in the mRNA levels of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ in the liver of mice consuming a HFD for 12 weeks (Fig. 3.2.4). Folic acid supplementation resulted in a significant decrease in mRNA levels of $gp91^{phox}$ (Fig. 3.2.4A), $p22^{phox}$ (Fig. 3.2.4B), and $p47^{phox}$ (Fig. 3.2.4C) in the liver of mice fed a HFD. However, folic acid supplementation did not affect HFD-induced $p67^{phox}$ expression in the liver (Fig. 3.2.4D). These findings demonstrate that folic acid likely inhibited NADPH oxidase activity via interfering with the gene expression of several essential subunits of the oxidase enzyme.

iv) Effect of a high-fat diet (HFD) and folic acid supplementation on NF-κB activation

To further investigate the regulation of NADPH oxidase gene expression in the liver, the involvement of NF-κB in NADPH oxidase activation was examined. In our study, the binding activity of NF-κB with a consensus DNA sequence was determined by EMSA. The DNA binding activity of NF-κB was significantly increased in the liver of mice fed a HFD (Fig. 3.2.5), while contrastingly, folic acid supplementation significantly inhibited NF-κB activation in mice fed a HFD (Fig. 3.2.5). These results indicated that NF-κB activation might be involved in the regulation of NADPH oxidase subunit gene expression in mouse liver.

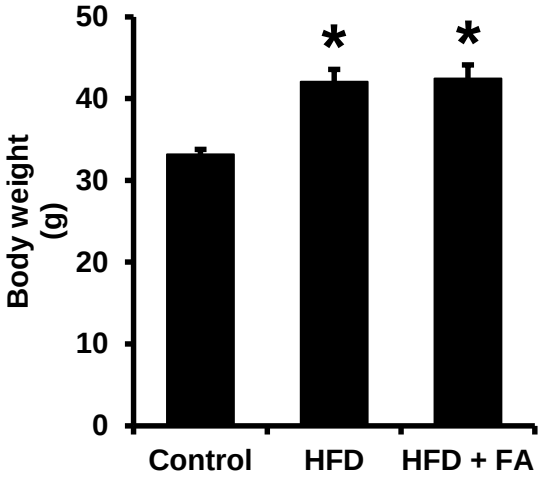
v) Effect of a high-fat diet (HFD) and folic acid supplementation on enzymatic antioxidant activity and glutathione concentration

Oxidative stress might also present as a result of a decrease in endogenous antioxidant defenses. Therefore, the activity of the antioxidant enzymes SOD and catalase were determined. The activity of both SOD (Fig. 3.2.6A) and catalase (Fig. 3.2.6B) was significantly decreased in the liver of mice fed a HFD. Such inhibitory effects were reversed in mice consuming a HFD supplemented with folic acid (Fig. 3.2.6A and 3.2.6B). Glutathione is the major endogenous non-enzymatic antioxidant in the liver. The equilibrium between GSH and GSSG reflects the redox potential of a given tissue, with a lower GSH:GSSG ratio indicative of oxidative stress. The relative ratio of GSH to GSSG was severely decreased in the liver of mice consuming a HFD (Fig. 3.2.6C). Folic acid supplementation effectively corrected the ratio of GSH:GSSG in the liver of mice fed a HFD (Fig. 3.2.6C). These results suggested that folic acid was able to prevent the redox perturbations that occurred in the liver in response to a HFD by maintaining the integrity of antioxidant defenses in the liver.

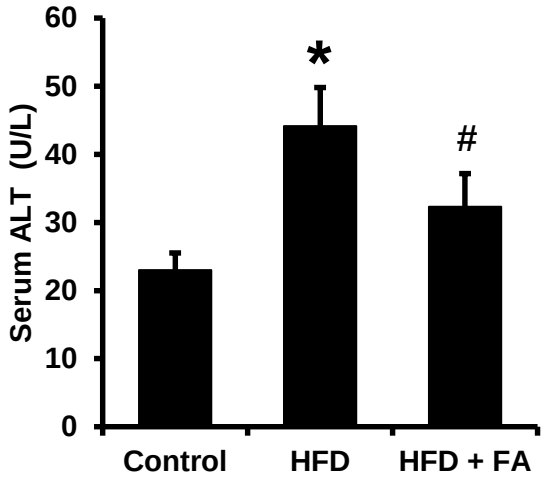
vi) Effect of a high-fat diet (HFD) and folic acid supplementation on hepatic morphology and lipid accumulation

Liver tissue was further examined with H&E staining for morphological changes. The H&E staining revealed notable lipid vacuoles in the liver of mice receiving the HFD (Fig. 3.2.7A). Such morphologies were consistent with significantly higher hepatic cholesterol (Fig. 3.2.7B) and triglyceride (Fig. 3.2.7C) levels in the liver of mice maintained on the HFD. Supplementation with folic acid protected the structural integrity of the liver, with fewer and strikingly smaller lipid droplets present in the liver of mice consuming the same HFD (Fig. 3.2.7A). Such morphological features were also associated with a partial reduction in hepatic cholesterol (Fig. 3.2.7B) and triglyceride (Fig. 3.2.7C) levels in those mice maintained on the HFD supplemented with folic acid.

A.


Body weight (g)
50
40
30
20
10
0
*
*
Control
HFD
HFD + FA

B.


Serum ALT (U/L)
60
50
40
30
20
10
0
*
#
Control
HFD
HFD + FA

C.

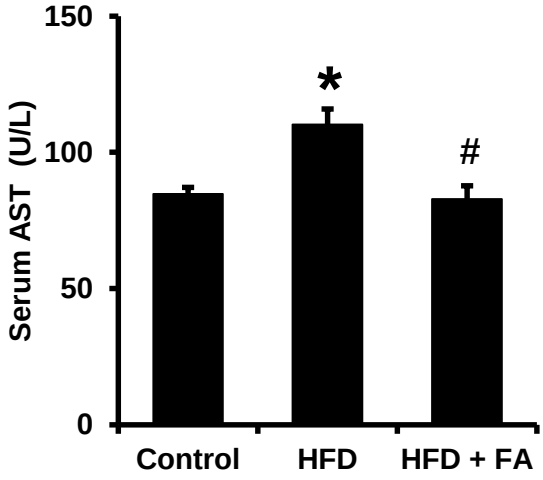

Serum AST (U/L)
150
100
50
0
*
#
Control
HFD
HFD + FA

Fig. 3.2.1. Body mass and liver function

Mice were fed one of the following diets for 12 weeks: (*i*) a control diet (10% kcal fat); (*ii*) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; (*iii*) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ *w*/*w* (HFD+FA). (A) Body mass measured at the end of the 12 week feeding period (in grams). (B) Serum alanine aminotransferase (ALT) and (C) aspartate aminotransferase (AST) were determined at the end of the 12 week feeding period. Results are expressed as the mean ± SEM (n = 10); *, $P < 0.05$ compared with the control group; $^{\#}$, $P < 0.05$ compared with the high fat diet groups.

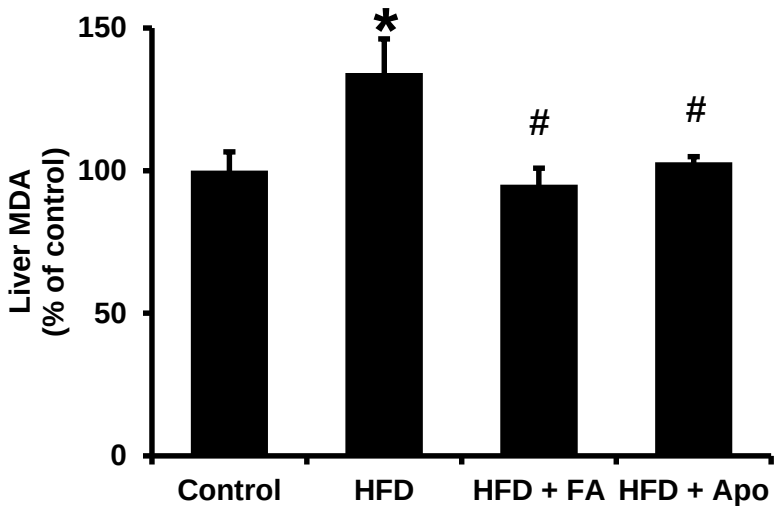


Fig. 3.2.2. Liver malondialdehyde (MDA) levels

Mice were fed one of the following diets for 12 weeks: (*i*) a control diet (10% kcal fat); (*ii*) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; (*iii*) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ *w*/*w* (HFD+FA); or (*iv*) a high-fat diet with an intraperitoneal injection of apocynin (4 mg·(kg body mass)$^{-1}$, daily) for 5 days prior to euthanasia (HFD+Apo). The levels of MDA in the liver tissue were determined by using the thiobarbituric acid reactive substances (TBARS) method. Results are expressed as the mean ± SEM (n = 10); *, $P < 0.05$ compared with the control group;$^{\#}$, $P < 0.05$ compared with the high fat diet groups.

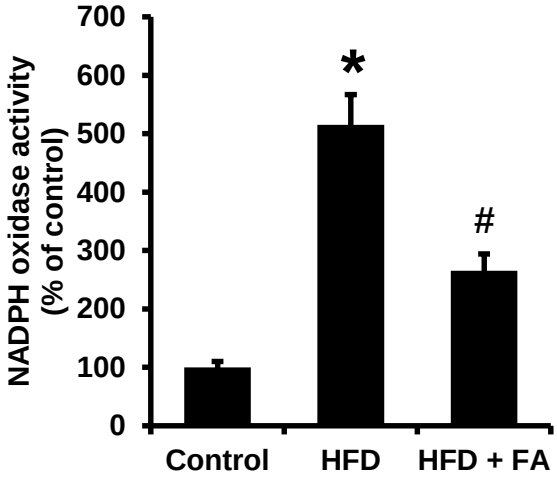
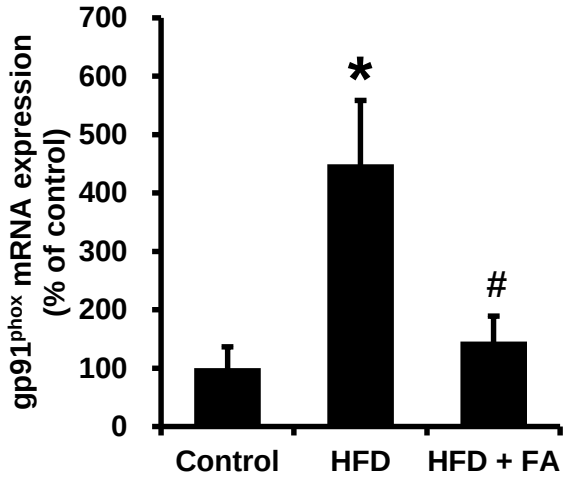


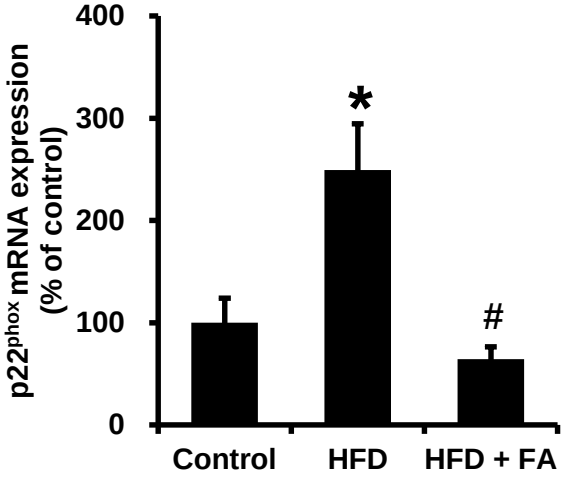
Fig. 3.2.3. Liver NADPH oxidase activity

Mice were fed one of the following diets for 12 weeks: (*i*) a control diet (10% kcal fat); (*ii*) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; or (*iii*) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ *w*/*w*(HFD+FA). The activity of NADPH oxidase was measured in liver tissue homogenate using the lucigenin chemiluminescence assay. The NADPH oxidase activity of the control group was 111.4 ± 11.40 nmol·min^{-1}·(mg protein)$^{-1}$, which was arbitrarily expressed as 100%. Results are expressed as the mean ± SEM (n = 10); *, $P < 0.05$ compared with the control group; $^{\#}$, $P < 0.05$ compared with the high fat diet groups.

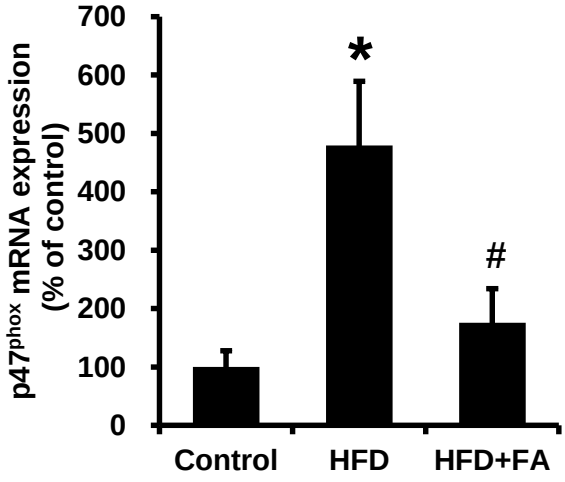
A.


gp91phox mRNA expression
(% of control)
700
600
500
400
300
200
100
0
*
#
Control
HFD
HFD + FA

B.


p22phox mRNA expression
(% of control)
400
300
200
100
0
*
#
Control
HFD
HFD + FA

C.


p47phox mRNA expression
(% of control)
700
600
500
400
300
200
100
0
*
#
Control
HFD
HFD+FA

D.

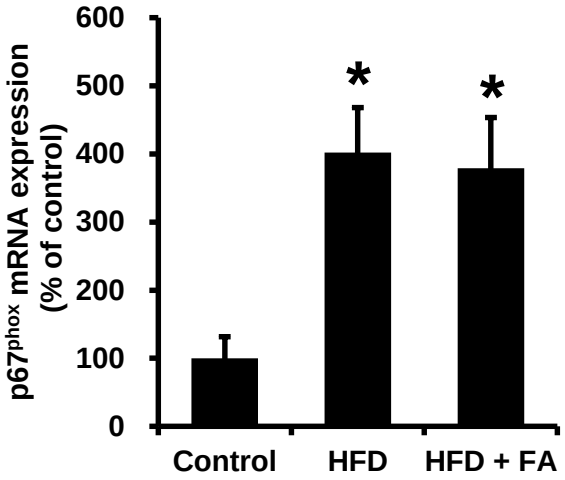


Fig. 3.2.4. Liver NADPH oxidase mRNA expression

Mice were fed one of the following diets for 12 weeks: (i) a control diet (10% kcal fat); (ii) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; or (iii) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ w/w (HFD+FA). The mRNA level of liver NADPH oxidase subunits (A) gp91^{phox}, (B) p22^{phox}, (C) p47^{phox}, and (D) p67^{phox} was determined by real-time PCR analysis. Results are expressed as the mean ± SEM (n = 10); *,P < 0.05 compared with the control group; $^{\#}$, P < 0.05 compared the high fat diet groups.

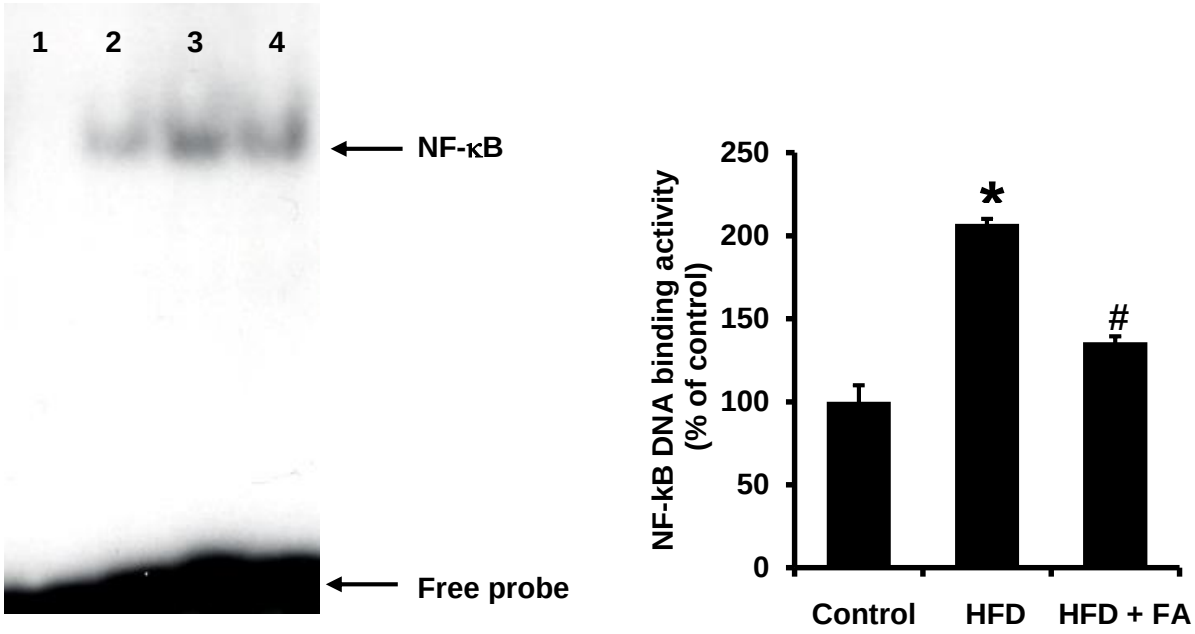
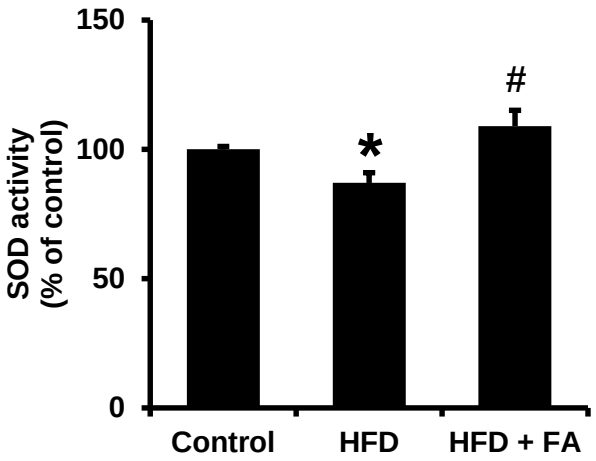


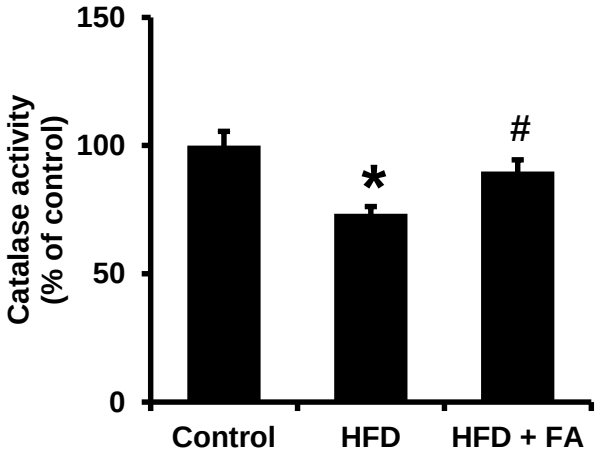
Fig. 3.2.5. NF-κB DNA binding activity

Mice were fed one of the following diets for 12 weeks: (*i*) a control diet (10% kcal fat); (*ii*) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; or (*iii*) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ *w*/*w*(HFD+FA). The NF-κB DNA binding activity in the liver was determined by electrophoretic mobility shift assay. Lane 1, cold competition; Lane 2, control; Lane 3, HFD; Lane 4, HFD+FA. Results are expressed as the mean ± SEM (n = 5); *, $P < 0.05$ compared with the control group;$^{\#}$, $P < 0.05$ compared with the high fat diet groups.

A.


150
100
50
0
SOD activity
(% of control)
*
#
Control
HFD
HFD + FA

B.


150
100
50
0
Catalase activity
(% of control)
*
#
Control
HFD
HFD + FA

C.

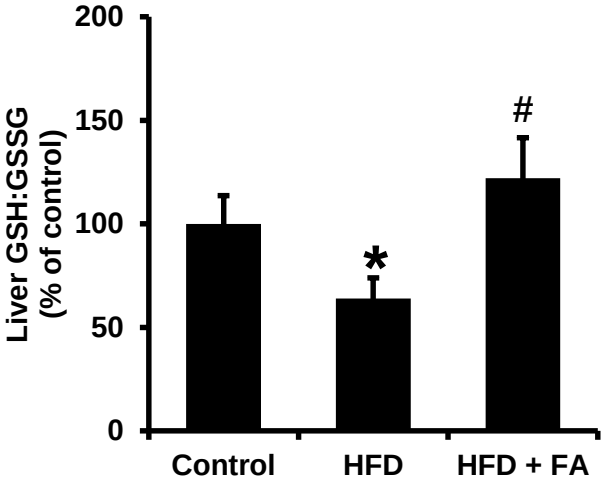
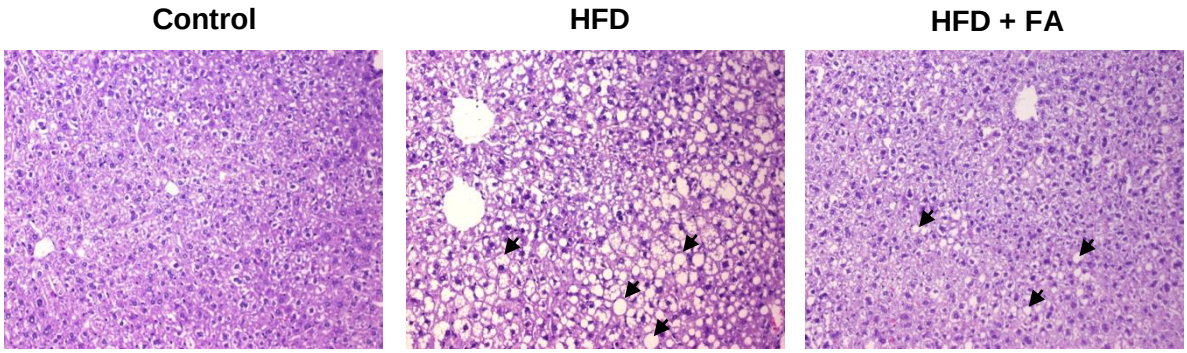

200
150
100
50
0
Liver GSH:GSSG
(% of control)
*
#
Control
HFD
HFD + FA

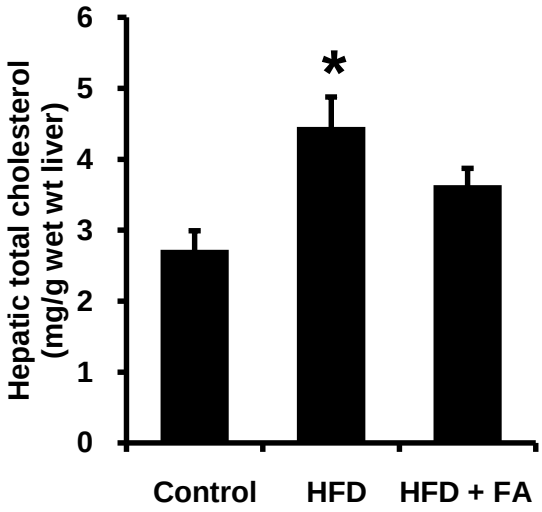
Fig. 3.2.6. Liver enzymatic antioxidants and redox potential

Mice were fed one of the following diets for 12 weeks: (*i*) a control diet (10% kcal fat); (*ii*) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; or (*iii*) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ *w*/*w* (HFD+FA). (A) Hepatic superoxide dismutase (SOD) activity was determined by the cytochrome *c* reduction method. (B) Hepatic catalase activity was determined by measuring the decomposition rate of hydrogen peroxide. (C) The ratio of reduced to oxidized glutathione (GSH:GSSG) was measured in the liver tissue as a measure of hepatic redox potential. The SOD activity of the control group was 81.56 ± 3.29 U·(mg protein)$^{-1}$ and the hepatic catalase activity of the control group was 285.86 ± 24.91 U·mg^{-1} protein. The reduced glutathione (GSH) level of the control group was 11.65 µmol·(g tissue)$^{-1}$, and the oxidized glutathione (GSSG) level of the control group was 347.99 nmol·(g tissue)$^{-1}$. Control values were arbitrarily expressed as 100%. Results were expressed as the mean ± SEM (n = 10); *, $P < 0.05$ compared with the control group; $^{\#}$, $P < 0.05$ compared with the high fat diet groups.

A.


Control
HFD
HFD + FA

B.


Hepatic total cholesterol (mg/g wet wt liver)
6
5
4
3
2
1
0
*
Control
HFD
HFD + FA

C.

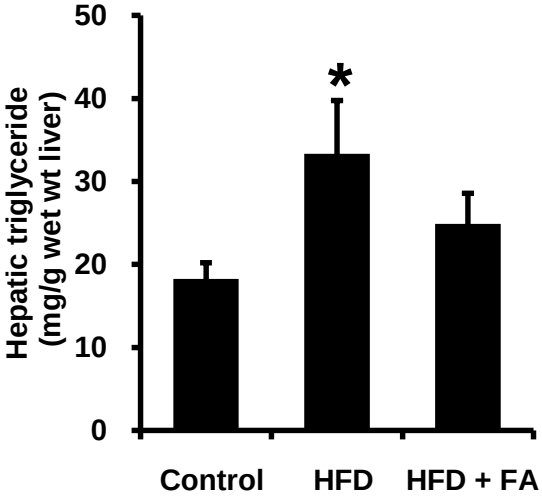

Hepatic triglyceride (mg/g wet wt liver)
50
40
30
20
10
0
*
Control
HFD
HFD + FA

Fig. 3.2.7. Liver morphology, total cholesterol, and triglyceride levels

Mice were fed on one of the following diets for 12 weeks: (*i*) a control diet (10% kcal fat); (*ii*) a high-fat diet (HFD; 60% kcal fat) with 2.6 mg folic acid·(kg chow)$^{-1}$; or (*iii*) a high-fat diet supplemented with 252.6 mg folic acid·(kg chow)$^{-1}$ *w*/*w* (HFD+FA). (A) Five livers from each dietary group were examined for morphological changes by hematoxylin and eosin (H&E) staining (magnification ×200). A total of 10 images were taken per liver. The images are representative of each dietary group. Arrowheads point to lipid vacuoles. (B) Total cholesterol, and (C) triglyceride levels in the liver were determined by commercial kits. Results are expressed as the mean ± SEM (n = 10); *, $P < 0.05$ compared with the control group.

4.2.5. Discussion

The novel findings of the present study were that (i) folic acid supplementation has a protective effect against HFD induced hepatic oxidative injury; (ii) the antioxidant effects of folic acid are likely mediated, in part, by the inhibition of NADPH oxidase; (iii) the reduction of NADPH oxidase gene expression in response to folic acid supplementation may be mediated via inhibition of NF-κB pathway. The beneficial effects of folic acid were independent of weight loss.

The consistent rise in obesity rates have been attributed to the overconsumption of foods rich in fats, namely saturated fats and cholesterol (Hariri et al., 2010; Piers et al., 2003). Similarly, the prevalence of NAFLD and non-alcoholic steatohepatitis (NASH) are on the rise, and such a trend has been linked to dietary fat induced hepatotoxicity and adiposity (Gaemers et al., 2011). In the present study, we have chosen a 12 week high-fat (60% kcal fat) feeding regimen in mice to replicate the human response to a HFD. Consumption of a HFD led to a marked increase in body mass in mice. In addition to weight gain, a significant increase in serum ALT and AST levels, markers of hepatic injury, were also observed in mice consuming a HFD.

Although the folic acid supplied by a Western diet is sufficient to meet the requirements of a generally healthy population, its levels may not be adequate for those afflicted by certain disorders. An inverse association between serum folate levels and body mass index has been reported, and folate deficiency is a common occurrence in various hepatic diseases (Gallistl et al., 2000; Hirsch et al., 2005; Leevy et al., 1970). Folate levels are also significantly lower in animal models of obesity and the metabolic syndrome. Strikingly lower plasma folate levels

were observed in +db/+db mice relative to control animals (Lam et al., 2009). Although folic acid supplementation raised folate levels in the circulation of both mouse strains, the effect was blunted in the obese/diabetic mice. Such findings suggest that folate metabolism is impaired during obesity, and may lead to folate deficiency even in the event of adequate intake. Taken together, in light of the obesity phenotype and the presence of liver injury in the HFD model, the application of folic acid is justified as a treatment strategy in our study (Lam et al., 2009).

Although mice receiving folic acid supplementation gained comparable weight to those fed a HFD, folic acid supplementation offered significant protection against HFD induced liver injury. Circulating levels of ALT are associated with the accumulation of toxic lipid peroxides in the liver (Yamada et al., 2006). In the present study, an increase in hepatic lipid peroxidation was observed in mice consuming a HFD. Folic acid supplementation was shown to abolish HFD induced oxidative stress in the liver, which suggested that the hepatoprotective effect of folic acid supplementation might be mediated by its antioxidant effect.

The NADPH oxidase has gained considerable attention as one of the major sources of oxidative stress in metabolic disorders (Furukawa et al., 2004; Roberts et al., 2006). Abnormal activation of NADPH oxidase can lead to over-production of $O_2^{\cdot -}$, which is linked to oxidative stress induced tissue injury (Guzik et al., 2002; Hwang et al., 2011; Woo et al., 2006). In the present study, NADPH oxidase activity was markedly increased in the liver of mice fed a HFD. Such an increase in NADPH oxidase activity coincided with a significant increase in the mRNA expression of NADPH oxidase subunits including $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ in the liver of mice consuming a HFD. Administration of the known NADPH oxidase inhibitor,

apocynin, not only effectively reduced hepatic lipid peroxidation, but also improved liver function in mice fed a HFD (data not shown). These results suggested that NADPH oxidase mediated oxidative stress contributed to liver injury during HFD feeding. Further investigation revealed that inhibition of NADPH oxidase by folic acid was due to a significant reduction in the mRNA levels of several essential NADPH oxidase subunits including $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$. We have previously reported that a reduction in $gp91^{phox}$ expression or loss of $p47^{phox}$ function inhibits NADPH oxidase activity *in vitro* (Au-Yeung et al., 2006; Sarna et al., 2010; Siow et al., 2006). Similarly, others have demonstrated that $p22^{phox}$ expression is also essential for the activation of NADPH oxidase (DeLeo et al., 2000; Zhu et al., 2006). In the present study, the inhibitory effect of folic acid on NADPH oxidase activation in the liver of mice consuming a HFD was likely through transcriptional down-regulation of these critical NADPH oxidase subunits.

It has been reported that the transcription factor NF-κB plays an essential role in the regulation of NADPH oxidase expression (Anrather et al., 2006; Edderkaoui et al., 2011; Manea et al., 2007). In the present study, NF-κB was found to be activated in the liver of mice fed a HFD that were over-expressing $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$. Folic acid supplementation, however, not only inhibited NF-κB activation, but also abolished the elevation of mRNA expression of NADPH oxidase subunits $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$ in the liver of mice fed a HFD. In another study using LPS/IFN-γ cell models, $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$, but not $p67^{phox}$, were shown to be regulated via the NF-κB pathway (Anrather et al., 2006). The present study provides the evidence for such an association in an animal model as inhibition of NF-κB by folic acid also affected NADPH oxidase subunit $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$, but not $p67^{phox}$.

One intriguing finding in the present study that deserves additional consideration was that folic acid completely abolished the increase in hepatic lipid peroxides, while only partially inhibiting NADPH oxidase activity. Such findings suggest that folic acid may have other antioxidant effects besides reducing NADPH oxidase mediated $O_2^{\cdot -}$ production. Further analysis demonstrated that while HFD feeding suppressed the activity of both SOD and catalase, 2 important enzymatic antioxidants in the liver, folic acid supplementation preserved the antioxidant action of both enzymes. We have previously reported similar antioxidant effects of folic acid supplementation in the kidney tissue of hyperhomocysteinemic rats (Hwang et al., 2011) using higher levels of folic acid (0.25% wt/wt) as well as in the liver of these animals using comparable supplemental levels (0.025% wt/wt) in the diet (Woo et al., 2006)*. Moreover, in the present study the redox potential of the liver, as measured by the GSH:GSSG ratio, was found to be severely compromised in the liver of mice consuming a HFD. Consistent with the antioxidant effects presented, supplemental folic acid effectively maintained the redox potential in the liver of mice consuming the same HFD. In addition to the antioxidant effects, folic acid supplementation was also found to preserve the morphological integrity of the liver, and to blunt cholesterol and triglyceride accumulation in the liver of mice consuming a HFD. Future mechanistic studies are warranted to investigate the effect of folic acid on HFD induced hepatic lipid metabolism.

The present study has demonstrated for the first time that folic acid supplementation can protect against hepatic oxidative injury induced by the chronic consumption of a HFD. Such

* Variance to that published as requested by examining committee

hepatoprotective antioxidant effects are mediated in part via transcriptional regulation of NADPH oxidase. Folic acid supplementation may be therapeutically advantageous for the management of hepatic oxidative injury during consumption of a HFD.

MANUSCRIPT III

4.3. High-fat diet stimulates hepatic cystathionine β-synthase and cystathionine γ-lyase expression

Can. J. Physiol. Pharmacol. 91: 913-919 (2013)

4.3.1. Abstract

Cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) catalyze homocysteine (Hcy) metabolism via the trans-sulfuration pathway. They are also responsible for hydrogen sulfide (H_2S) production via desulfuration reactions. The liver contributes significantly to the regulation of Hcy and H_2S homeostasis, which might participate in many physiological and pathological processes. The aim of this study was to investigate the effect of a high-fat diet (HFD) on hepatic CBS and CSE expression and its impact on Hcy and H_2S metabolism. Mice (C57BL/6) fed a HFD (60% kcal fat) for 5 weeks developed fatty liver. The mRNA and protein levels of CBS and CSE in the liver were significantly elevated in mice fed a HFD. Subsequently the metabolism of Hcy by CBS and CSE was increased in the liver, and its level decreased in the circulation. Increased CBS and CSE expression also caused a significant elevation in H_2S production in the liver. The level of lipid peroxides was elevated, indicating oxidative stress, while the level of total glutathione remained unchanged in the liver of HFD-fed mice. Up-regulation of the trans-sulfuration pathway might play an adaptive role against oxidative stress by maintaining total glutathione levels in the liver.

4.3.2. Introduction

Homocysteine (Hcy) and hydrogen sulfide (H_2S) are 2 important molecules produced in the body during the metabolism of sulfur amino acids. Hcy is an intermediate amino acid formed in the methionine cycle. Hcy has important roles in essential cellular functions including its participation in sulfur amino acid metabolism and methyl group generation. Aberrant regulation of Hcy metabolism is associated with several vascular and cognitive abnormalities (Lentz, 2005; Zhuo et al., 2011). The liver is an important organ for Hcy metabolism and contributes significantly to the regulation of plasma Hcy levels (Stead et al., 2000). In the liver, Hcy is metabolized via the remethylation pathway to regenerate methionine and via the transsulfuration pathway to form cysteine (Finkelstein, 1998). In the transsulfuration pathway, cystathionine β-synthase (CBS, EC 4.2.1.22), an enzyme dependent on pyridoxal 5′-phosphate (PLP), catalyzes the rate-limiting step by condensing Hcy with serine to form cystathionine. In turn, another enzyme cystathionine γ-lyase (CSE, EC 4.4.1.1) metabolizes cystathionine to form cysteine, α-ketobutyrate, and ammonia (Dominy & Stipanuk, 2004). In addition to their role in Hcy metabolism via the transsulfuration pathway, both CBS and CSE are responsible for alternative desulfuration reactions that significantly contribute to the endogenous synthesis of H_2S (Chen et al., 2004; Chiku et al., 2009; Stipanuk & Beck, 1982). H_2S has been identified as an important molecule involved in biological functions. The physiological and pathophysiological actions of H_2S have been extensively reviewed (Wang, 2012). Tight regulation of Hcy and H_2S metabolism is of physiological importance.

The liver is fundamental to macronutrient metabolism and is sensitive to nutrient imbalances. It appears particularly susceptible to the injurious effects that are associated with the intake of

high-fat diets (HFD). Diets that are rich in saturated fats and cholesterol are known to contribute to the development of obesity and are also associated with the development of nonalcoholic fatty liver disease (NAFLD) (Golay & Bobbioni, 1997; Hariri et al., 2010; Larter & Yeh, 2008). NAFLD is considered to be a hepatic manifestation of the metabolic syndrome. It covers a broad spectrum of disorders, ranging from simple lipid accumulation in the liver, defined as steatosis, to nonalcoholic steatohepatitis (NASH), which is further characterized by the added complexity of cellular oxidative stress, tissue inflammation, and fibrosis. A recent study has reported that serum Hcy levels are lower in patients with NASH than in patients with steatosis (Polyzos et al., 2012b). However, the mechanism responsible for reduced serum Hcy levels is not clear. On the contrary, other studies have revealed no change or an elevation in circulating Hcy levels in NAFLD patients (Gulsen et al., 2005; Hirsch et al., 2005). In animal models, HFD feeding induces liver injury, disrupts hepatic antioxidant defenses, and produces histopathological features characteristic of human NAFLD (Gaemers et al., 2011; Larter & Yeh, 2008; Sarna et al., 2012). Although HFDs are associated with the development of NAFLD, little information is available concerning the impact of high-fat feeding on CBS- and CSE-mediated Hcy and H_2S homeostasis.

The aim of the present study was to investigate the effects of a HFD on the regulation of the transsulfuration enzymes CBS and CSE in the liver and to identify their association with Hcy metabolism and H_2S biosynthesis.

4.3.3. Materials and Methods

i) Animals and diet

Male C57BL/6 mice (Charles River Laboratories) aged 8 weeks were fed for 5 weeks with either a control diet (10% kcal fat) or a high-fat diet (HFD) (60% kcal fat) (Research Diets D12450B and D12492, respectively). The composition of the diets have been outlined (*Appendix I-III*)*. Mice had free access to food and water and were kept on a 12 h (light): 12 h (dark) cycle. The high-fat-fed mouse model has been shown to develop obesity and hepatic manifestations that are characteristic of NAFLD (Collins et al., 2004; Ito et al., 2007; Larter & Yeh, 2008). All procedures were performed in accordance with the *Guide to the Care and Use of Experimental Animals* as set forth by Canadian Council on Animal Care and were approved by the University of Manitoba Protocol Management and Review Committee. Total Hcy concentrations in the serum were measured with the IMx Hcy assay, which was based on fluorescence polarization immunoassay technology (Abbott Diagnostics, Abbott Park, Illinois) (Prathapasinghe et al., 2007; Woo et al., 2008). Hepatic levels of malondialdehyde (MDA) were determined by using the thiobarbituric acid reactive substances (TBARS) method as a measure of lipid peroxides (Sarna et al., 2012). The relative H_2S production in the liver was measured spectrophotometrically by using sodium hydrosulfide as a standard (Stipanuk & Beck, 1982; Xu et al., 2009), and total glutathione levels were determined (Anderson, 1985; Srisook et al., 2005).

ii) Histological staining

A portion of the liver was fixed in 3.6% formaldehyde in phosphate-buffered saline overnight

* Variance to that published as requested by examining committee

and embedded in paraffin. Paraffin-embedded sections (5 μm) were prepared and stained with hematoxylin and eosin (H&E) to examine morphological changes in the liver (Sarna et al., 2012). Images of H&E sections were captured using an Olympus BX43 light microscope equipped with a Q-Color3 camera and were analyzed at × 200 magnification.

iii) Measurement of CBS and CSE mRNA expression

Total RNAs were isolated from the liver tissue with QIAzol reagent (Qiagen) according to the manufacturer's instructions. The mRNA expression of CBS and CSE was determined by real-time PCR analysis using the iQ5 real-time PCR detection system (Bio-Rad). In brief, total RNA (4 μg) was converted to cDNA by reverse transcription. The real-time PCR reaction mixture contained 0.4 $\mu mol \cdot L^{-1}$ (each) 5′ and 3′ primers and 1 μL of cDNA product in iQ-SYBR green supermix reagent (Bio-Rad). The relative changes in mRNA expression were determined by the fold change analysis. The primers (Life Technologies) used in this study were mouse CBS, 5′-CTGCGTGTTCAAGAGCTGAG-3′(forward), 5′-TTGCAGACTTCGTCTGATGG-3′ (reverse); mouse GAPDH, 5′- GCACAGTCAAGGCCGAGAAT-3′ (forward), 5′-GCCTTCTCCATGGTGGTGAA-3′ (reverse); and mouse CSE, 5′-TGCTAAGGCCTTCCTCAAA-3′ (forward), 5′-AAGCTCGATCCAGGTCTTCA-3′ (reverse).

iv) Western immunoblotting analysis

Liver CBS and CSE protein expression was analyzed by Western Immunoblotting. Liver proteins (20μg) were separated by electrophoresis in 8% or 10% polyacrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane. The membrane was probed with mouse anti-CBS monoclonal antibodies (1:3000, Abnova Corporation) or anti-CSE monoclonal

antibodies (1:3000, GeneTex). Secondary antibodies used were HRP-conjugated anti-mouse or anti-rabbit IgG antiobodies (1:5000, Cell Signaling Technology). Corresponding protein bands were visualized by enhanced chemiluminescence reagents and optical density was determined with the Bio-Rad Gel Doc1000 gel documentation system. Equal loading of proteins in each sample was confirmed by re-probing the membrane with mouse anti-β-actin monoclonal antibodies (1;5000, Cell Signaling Technology).

v) Measurement of CBS and CSE enzyme activities

Liver tissue was homogenized in 50 mmol·L^{-1} potassium phosphate buffer (pH 6.9) followed by centrifugation at 18 000*g* for 30 min at 4 °C (Mudd et al., 1965; Taoka et al., 1998). The supernatant was collected and the CBS reaction was carried out in a reaction mixture containing 125 mmol·L^{-1} Tris–HCl (pH 8.5), 2.1 mmol·L^{-1} EDTA, 0.146 mmol·L^{-1} L-cystathionine, 41.7 mmol·L^{-1} DL-Hcy, 0.316 mmol·L^{-1} *S*-adenosylmethionine, 2.1 mmol·L^{-1} propargylglycine, and 0.42 mmol·L^{-1} PLP. The reaction was initiated by adding 30 mmol·L^{-1} [^{14}C] serine (PerkinElmer) to the reaction mixture. After 1 h of incubation at 37 °C, 15% trichloroacetic acid was added to stop the reaction. An aliquot of the reaction mixture was applied to a Bio-Rad AG 50W-X8 anion exchange column containing hydrogen form resin. The column was washed with a series of water and 1 mol·L^{-1} HCl and the cystathionine was eluted with 3 mol·L^{-1} NH_4OH. The radioactivity associated with cystathionine was determined by using a Beckman liquid scintillation counter. The assay for CSE activity was based on a method described by (Stipanuk, 1979). Briefly, liver homogenates were prepared in 50 mmol·L^{-1} potassium phosphate buffer (pH 6.9) followed by centrifugation at 18 000*g* for 30 min at 4 °C. The resulting supernatant was used for the CSE activity assay performed in a 96-well microplate. The reaction mixture contained

100 mmol·L^{-1} potassium phosphate buffer (pH 7.4), 4 mmol·L^{-1} L-cystathionine, 0.125 mmol·L^{-1} PLP, 0.32 mmol·L^{-1} NADH, 1.5 units of lactate dehydrogenase, and 5 μL of liver homogenate in a total volume of 200 μL per well. The decrease in optical density at absorbance of 340 nm was kinetically monitored with a microplate reader (SpectraMax-5, Molecular Devices) at 37 °C for 30 min. Blank reactions were performed using the same reaction mixture with the omission of L-cystathionine (Stipanuk, 1979).

vi) Statistical analysis

Results obtained were analyzed by a 2-tailed Student's *t* test. *P* values of <0.05 were considered statistically significant.

4.3.4. Results

i) Effect of HFD on body mass, hepatic lipids, and morphology

Mice fed a HFD for 5 weeks gained more mass than those fed a control diet (Fig. 3.3.1A). Morphological examination by H&E staining revealed the formation of lipid vacuoles in the liver of HFD-fed mice (Fig. 3.3.1B). Total hepatic cholesterol and triglyceride levels in these mice were elevated (data not shown). Serum total Hcy levels in mice fed a HFD were significantly lower compared with that of the control group (Fig. 3.3.1C). These results suggested that high-fat feeding for 5 weeks induced fatty liver and altered Hcy metabolism in mice.

ii) Effect of HFD on CBS and CSE expression in the liver

To investigate whether the reduction in Hcy levels was due to up-regulation of CBS and CSE expression, liver CBS and CSE mRNA levels were measured by real-time PCR analysis. The mRNA levels of both CBS and CSE were significantly increased in the liver of mice fed a HFD as compared with that of the control group (Fig. 3.3.2A and 3.3.2B). To determine whether the elevation of both CBS and CSE mRNA expression led to an increase in the protein expression, Western immunoblotting analysis was performed. In accordance with mRNA expression, the protein levels of both CBS and CSE were also significantly increased in the liver of HFD-fed mice compared with that of the control group (Fig. 3.3.2A and 3.3.2B). These results suggested that HFD feeding for 5 weeks stimulated CBS and CSE expression in the liver.

iii) Effect of HFD on CBS and CSE enzyme activities and H_2S production in the liver

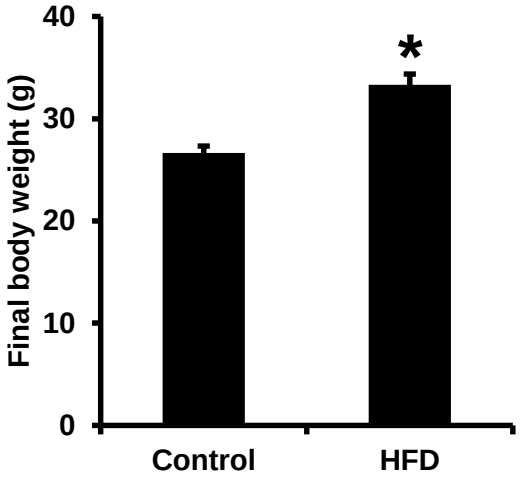
CBS and CSE participate in the transsulfuration pathway and are the major enzymes responsible for the catabolism of Hcy in the liver. Both CBS and CSE enzyme activities via the

transsulfuration pathway were significantly increased in the liver of mice fed a HFD for 5 weeks (Figs. 3.3.2A and 3.3.2B). Apart from roles in the catabolism of Hcy via the transsulfuration pathway, both CBS and CSE are also responsible for endogenous synthesis of H_2S via desulfuration reactions. There was also a significant elevation in H_2S production in the liver tissue of mice consuming a HFD (Fig. 3.3.2C). These results suggested that both transsulfuration and desulfuration reactions in the liver might be increased as a result of the elevated expression of CBS and CSE in mice fed a HFD.

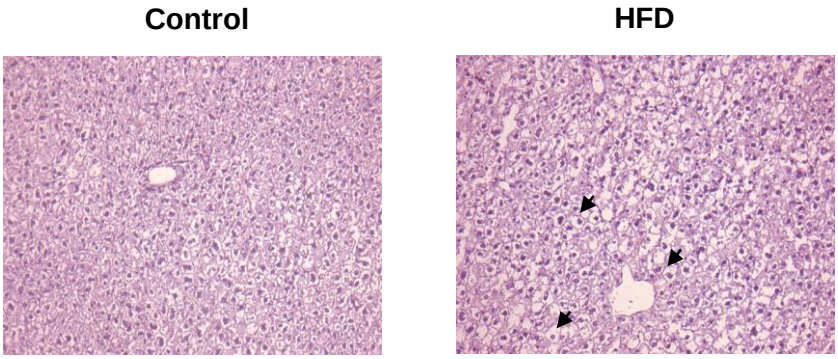
iv) Effect of HFD on lipid peroxidation and hepatic total glutathione levels

The degree of lipid peroxidation in the liver tissue was determined by measuring the level of MDA. The MDA level was significantly elevated in the liver of HFD-fed mice (Fig. 3.3.3A), suggesting an increased lipid peroxidation in the liver. Glutathione is a tripeptide that lies downstream of the transsulfuration pathway. It is regarded as a major antioxidant buffer in the liver. There was no significant difference in total glutathione levels in the liver between the control and HFD-fed mice (Fig. 3.3.3B).

A.


40
30
20
10
0
Final body weight (g)
*
Control
HFD

B.


Control
HFD

C.

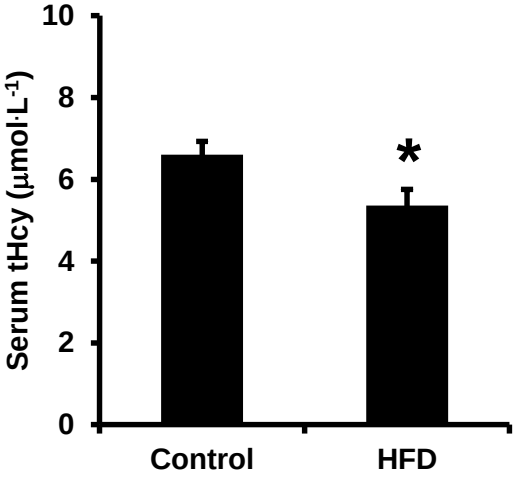
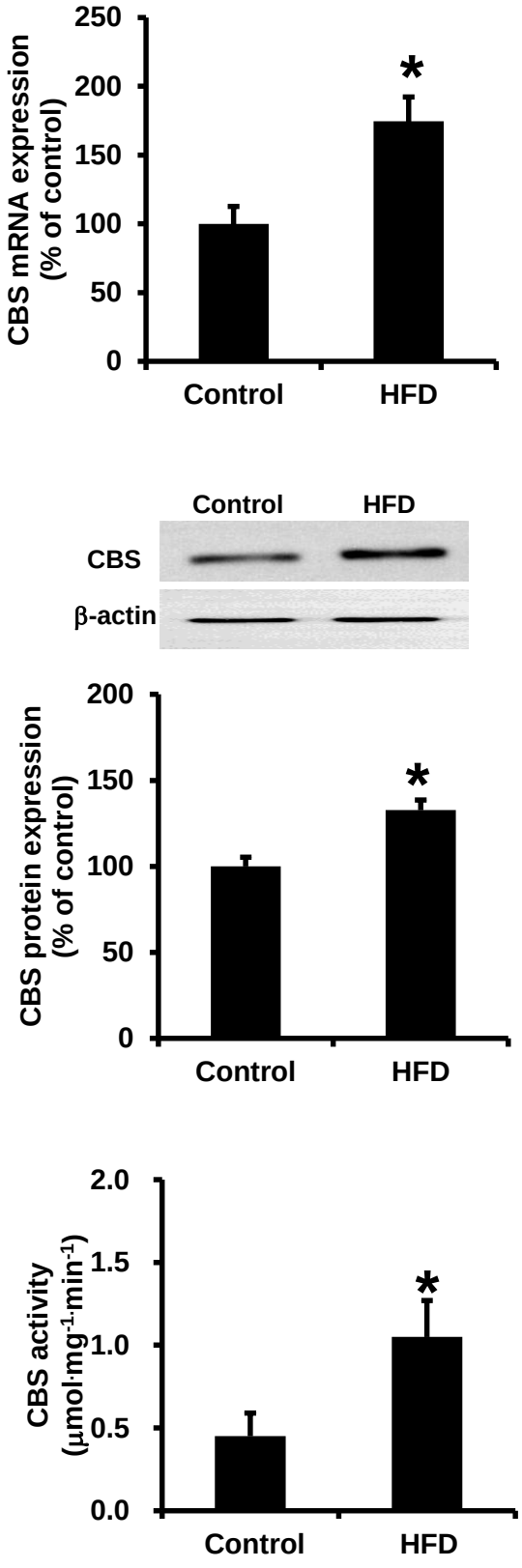

10
8
6
4
2
0
Serum tHcy (μmol·L-1)
*
Control
HFD

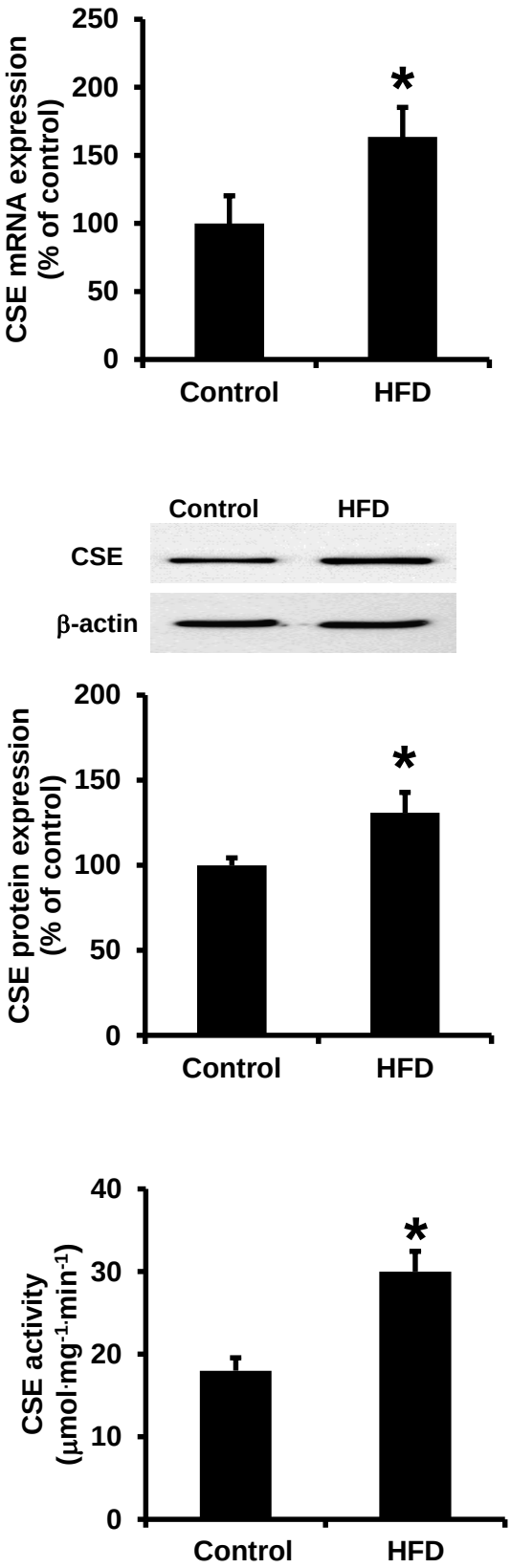
Fig. 3.3.1. Body mass, liver morphology, serum total homocysteine level

Mice were fed a control diet or a high-fat diet (HFD) for 5 weeks. (A) Body mass was measured at the end of the 5-week feeding period; final body mass is shown in grams. (B) Liver morphology was examined by hematoxylin and eosin (H&E) staining. A total of 5 images were taken per liver ($n = 4$ per group). The representative photos were shown with a magnification of $\times 200$. Arrows indicate lipid vacuoles. The images shown are representative of each group. (C) Serum total Hcy (tHcy) levels were determined. Results are expressed as the mean ± SEM ($n = 8$). *, indicates a significant difference from the control group at $P < 0.05$.

A.


CBS mRNA expression (% of control)
250
200
150
100
50
0
*
Control
HFD
Control
HFD
CBS
β-actin
CBS protein expression (% of control)
200
150
100
50
0
*
Control
HFD
CBS activity (μmol·mg-1·min-1)
2.0
1.5
1.0
0.5
0.0
*
Control
HFD

B.


CSE mRNA expression (% of control)
250
200
150
100
50
0
*
Control
HFD
Control
HFD
CSE
β-actin
CSE protein expression (% of control)
200
150
100
50
0
*
Control
HFD
CSE activity (μmol·mg-1·min-1)
40
30
20
10
0
*
Control
HFD

C.

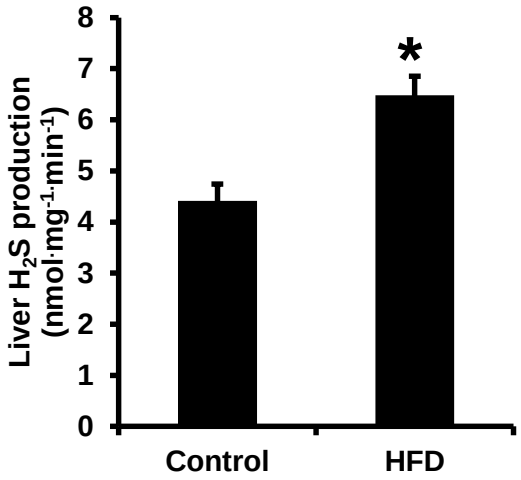
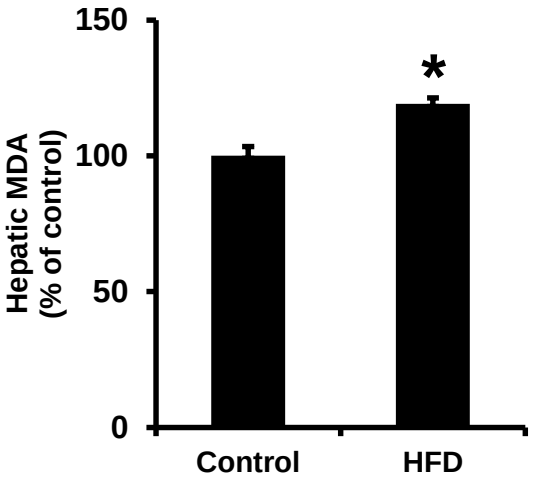


Fig. 3.3.2. Cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and hydrogen sulfide (H_2S) production in the liver

Mice were fed a control diet or a high-fat diet (HFD) for 5 weeks, and livers were isolated. (A) The CBS mRNA levels were determined by real-time PCR analysis, proteins were measured by Western immunoblotting analysis, and CBS enzyme activities were measured. (B) The CSE mRNA levels were determined by real-time PCR analysis, proteins were measured by Western immunoblotting analysis, and CSE enzyme activities were measured. (C) H_2S production was determined. Results are expressed as the mean ± SEM (n = 6 to 8). *, indicates a significant difference from the control group at $P < 0.05$.

A.


150
100
50
0
Hepatic MDA
(% of control)
*
Control
HFD

B.

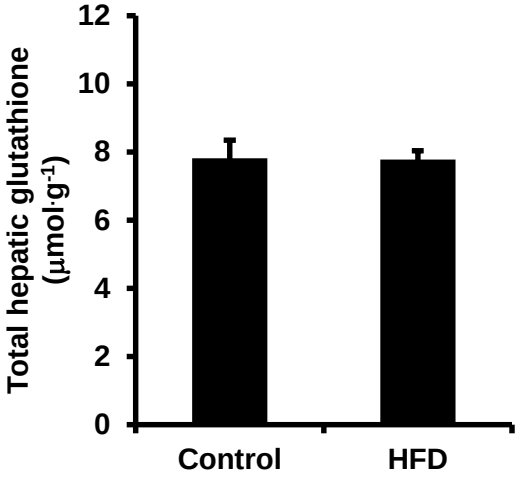

12
10
8
6
4
2
0
Total hepatic glutathione
(μmol·g-1)
Control
HFD

Fig. 3.3.3. Malondialdehyde (MDA) and total glutathione levels in the liver

Mice were fed a control diet or a high-fat diet (HFD) for 5 weeks and livers were isolated. (A) The levels of MDA in the liver tissue were determined. (B) Total glutathione levels were measured in the liver tissue. Results are expressed as the mean ± SEM (n = 8). *, indicates a significant difference from the control group at $P < 0.05$.

4.3.5. Discussion

The novel findings of the present study were that consumption of a HFD for 5 weeks upregulated the transsulfuration pathway in the liver. The mRNA expression of both CBS and CSE was significantly increased. In accordance, the protein levels and enzyme activities of CBS and CSE were also elevated. The up-regulation of CBS and CSE expression led to increased metabolism of Hcy in the liver with a concomitant decrease of total Hcy levels in the circulation. Increased CBS and CSE expression also caused a significant increase in hepatic H_2S production.

In the liver, both CBS and CSE are responsible for Hcy catabolism via the transsulfuration pathway, which in turn regulates Hcy levels in the circulation (Dominy & Stipanuk, 2004; Stead et al., 2000). In the present study, we observed a significant reduction in serum total Hcy levels in mice fed a HFD for 5 weeks. Though a decrease in methionine intake could lead to lower Hcy levels by limiting it synthesis, no significant differences in the dietary intake of methionine was observed between control and HFD fed mice in our study (*Appendix IV*)*. Such a reduction in systemic Hcy levels was however accompanied by a significant increase in both the expression as well as the activity of CBS and CSE in the liver of HFD-fed mice. While we observed an activation of both CBS and CSE activities in the 5-week HFD model, increased CSE activity was reported in rats fed a HFD for 3 weeks (Kwon do et al., 2009). An association between low plasma Hcy levels and increased activities of hepatic CBS and CSE was found in obese diabetic rats (Wijekoon et al., 2005). A similar association between low plasma Hcy levels and increased hepatic CBS activity was also reported in non-obese diabetic rats (Noll et al., 2011). Targeted

* Variance to that published as requested by examining committee

deletion of the CSE gene has been shown to dramatically elevate plasma Hcy levels (Yang et al., 2008). In 10-week-old mice with CSE deficiency, plasma Hcy levels were shown to increase approximately 2- and 18-fold in $CSE^{-/+}$ and $CSE^{-/-}$ mice, respectively (Yang et al., 2008). In addition, CBS-deficient mice also display significantly higher levels of plasma Hcy (Robert et al., 2005). However, other studies revealed that consumption of a HFD for longer periods of time might increase circulating Hcy levels in rodents (Bravo et al., 2011; Yun et al., 2013). The activity of CBS was found to be reduced in the liver of mice consuming a HFD for 12 weeks, although no change in protein expression was observed (Yun et al., 2013). The activities of both CBS and CSE were also down-regulated in the liver of rats fed a HFD for 18 weeks (Bravo et al., 2011). The discrepancies between our current findings and those reported by others may be attributed to species differences, the composition of the HFD, and the duration of the study period. Since fatty acids have been reported to differentially regulate hepatic CBS and CSE expression in rats as well as in cultured hepatocytes (Huang et al., 2012; Huang et al., 2013), the fatty acid profile of our lard based HFD could be a potential source of variability*. It is also plausible that CBS and CSE activation in the liver could be transiently responsive to the hepatic burden imposed on by shorter periods of HFD consumption.

Aside from participating in the transsulfuration pathway, both CBS and CSE also contribute to the desulfuration of cysteine, leading to synthesis of H_2S (Dominy & Stipanuk, 2004; Fiorucci et al., 2006; Stipanuk & Ueki, 2011). The liver appears to be one of the major sites for the regulation of endogenous H_2S synthesis (Jensen et al., 2011; Norris et al., 2011). In accordance with the elevation of CBS and CSE expression, hepatic H_2S production was also significantly

* Variance to that published as requested by examining committee

increased in the liver of mice fed a HFD for 5 weeks. Our findings are in line with the results of a recent study in which overexpression of human CBS in mouse liver led not only to a striking reduction in plasma Hcy concentrations but also to an increase in circulating H_2S levels (Jensen et al., 2011). These results suggest that HFD may modulate H_2S production via activation of CBS and CSE in the liver.

In the present study, 2 consequences were found to be associated with increased expression of hepatic CBS and CSE, namely, increased metabolism of Hcy and increased production of H_2S. The implication of our findings remains open for interpretation, as it is difficult to decipher whether CBS and CSE activation in the liver is a causative mechanism or an adaptive response to the high-fat feeding. It is possible that activation of the transsulfuration pathway could be an early adaptive response to hepatic oxidative stress in HFD-fed mice. The significant reduction in total Hcy levels in the present study likely reflected the enhanced catabolism of Hcy via the transsulfuration pathway in the liver of HFD-fed mice. These findings might be rather significant in terms of regulating oxidative stress, since glutathione, the major endogenous direct antioxidant in the liver, lies downstream of this pathway. In fact, 50% of glutathione pool in the liver is derived from the transsulfuration pathway (Mosharov et al., 2000; Vitvitsky et al., 2003). It is unclear why the up-regulation of the transsulfuration pathway does not translate directly to increased glutathione levels in our model. We can only speculate that an increased use of glutathione as a direct antioxidant or perhaps detoxifying agent, may limit its accumulation in the liver. Certain factors such as oxidative stress or ER stress could also influence regulatory mechanisms that control glutathione metabolism. A recent study has also demonstrated that CSE mediated an adaptive coping mechanism to overcome cellular stress via maintaining hepatic glutathione synthesis (Dickhout et al., 2012). Since Hcy has been shown to induce oxidative

stress at elevated levels (Edirimanne et al., 2007; Woo et al., 2006), one could rationalize that lower levels of Hcy could reduce oxidative stress. However, given that fatty liver is a multifaceted disorder, it is likely that multiple factors contribute to oxidative stress. The effect of low Hcy on oxidative stress may then be outweighed by other pro-oxidative mechanisms. Furthermore, H_2S, which is generated via alternative CBS and CSE desulfuration reactions, has also been shown to have hepatoprotective properties, including antioxidant, antiapoptotic, antifibrotic, and antihypertensive effects, in the liver (Distrutti et al., 2008; Jha et al., 2008; Kang et al., 2009; Tan et al., 2011). However, such beneficial associations should be interpreted with caution, as excessive production of H_2S also plays a pathological role in inflammatory disorders (Wang, 2012; Whiteman & Winyard, 2011). The pathophysiological implication of imbalanced Hcy–H_2S homeostasis remains to be addressed in future studies.

The present study is the first known demonstration that CBS and CSE expression is increased in the liver of mice fed a HFD for 5 weeks. Activation of both CBS and CSE enzymes is associated with a significant increase in Hcy metabolism in the liver via the transsulfuration pathway and a reduction of Hcy level in the circulation. There was a concomitant increase in H_2S production in the liver. Our findings clearly indicate that activation of CBS and CSE may serve as one of the important regulatory mechanisms contributing to imbalanced Hcy–H_2S homeostasis in response to high-fat feeding. Future studies are warranted to investigate whether targeted regulation of CBS and CSE expression would be of therapeutic value.

V. GENERAL DISCUSSION

5.1. Overview

Oxidative stress is a complex, and potentially cytotoxic biological phenomenon. Its relevancy to health is underscored by its implication in over one-hundred pathologies (Djordjevic, 2004; Genestra, 2007). Oxidative stress is an important underlying factor mediating NAFLD pathogenesis (Rolo et al., 2012) and oxidative stress induced vascular injury also contributes to the mediation of atherogenesis (Stocker & Keaney, 2004), a cardiovascular related pathology that is strongly associated with NAFLD. The lack of safe and efficacious therapies available for clinical management of NAFLD is a pressing issue given that the incidence of the disorder is increasing worldwide (Loomba & Sanyal, 2013) and the pathology is associated with a growing number of hepatic and extra-hepatic health risks (Anstee et al., 2013). Innovative studies are needed to i) further define the mechanisms involved in the regulation of oxidative stress in NAFLD and to ii) identify novel therapeutics for NAFLD management. Through the use of both cell and animal based model systems, our studies investigated the regulation of oxidative stress and its modulation by NHPs in the context of NAFLD and its associated co-morbidities (CVD). Our findings demonstrated that i) berberine effectively regulates NADPH oxidase mediated oxidative stress in activated macrophages by inhibiting the gene expression of the catalytic $gp91^{phox}$ subunit of the oxidase and enhancing the $O_2^{\cdot -}$ scavenging activity of SOD; ii) folic acid supplementation abolishes hepatic oxidative stress in HFD fed mice by down-regulating NADPH oxidase gene expression and upregulating antioxidant defences in the liver; and iii) up-regulation of the CBS/CSE system in the liver of HFD fed mice leads to imbalanced Hcy–H_2S homeostasis but maintained glutathione levels. NHPs such as berberine and folate, and Hcy catabolising enzymes CBS and CSE might have therapeutic potential for managing oxidative stress in NAFLD and its associated co-morbidities.

5.1.1. Manuscript 1 - Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages

CVD is the major cause of death among NAFLD patients (Loomba & Sanyal, 2013). Macrophages, which are recognized for playing a central role in atherogenesis (Bieghs et al., 2012), contribute to vascular injury via NADPH oxidase mediated oxidative stress (Cathcart, 2004). Therefore, in the first study the effect of berberine, a plant derived isoquinoline alkaloid, was investigated for its effects on NADPH oxidase mediated $O_2^{\cdot -}$ production in macrophages. Our results in LPS stimulated THP-1 macrophages revealed that berberine effectively decreased NADPH oxidase mediated $O_2^{\cdot -}$ production in a time and concentration dependant manner. Although berberine could down-regulate $O_2^{\cdot -}$ in stimulated cells, it did not affect $O_2^{\cdot -}$ levels in unstimulated macrophages. These findings suggested that berberine's regulatory effects might be contextual in nature. Berberine did not affect cell viability at the concentration (25μmol/L) sufficient for inhibiting NADPH oxidase mediated oxidative stress. Further mechanistic study revealed that berberine inhibited NADPH oxidase mediated $O_2^{\cdot -}$ production in stimulated macrophages via specifically down-regulating the expression of the catalytic $gp91^{phox}$ subunit of the oxidase. Berberine also regulated $O_2^{\cdot -}$ clearance in these cells by preventing the decrease in SOD activity that was induced with LPS stimulation. Taken together, this study demonstrates for the first time, that berberine effectively regulates oxidative stress in macrophages. Our novel findings demonstrate that berberine i) inhibits NADPH oxidase mediated $O_2^{\cdot -}$ production via selectively inhibiting $gp91^{phox}$ expression and ii) restores SOD activity in these cells.

Our study was among the first to characterize berberine as having antioxidant properties (Tan et al., 2007; Thirupurasundari et al., 2009; Zhou et al., 2009) and is the first to demonstrate that

berberine regulates NADPH oxidase mediated $O_2^{\cdot -}$ production in macrophages (Sarna et al., 2010). Previously, studies evaluating the biological activity of berberine in the context of CVD have focused primarily on its lipid lowering and glucose regulatory properties and its effects on the vascular endothelium (Affuso et al., 2010). Our results suggest that berberine's antioxidant effects in activated macrophages could also contribute to its potential therapeutic application in CVD (Siow et al., 2011a), particularly since NADPH oxidase mediated $O_2^{\cdot -}$ production has been identified as an important therapeutic target in atherosclerosis (Cathcart, 2004). Intriguingly, Bieghs *et al* have suggested that atherosclerosis and NASH might be manifestations of the same disease which is mediated by the localization of activated macrophages (Bieghs et al., 2012). Therefore the ability for berberine to regulate NADPH oxidase and reduce $O_2^{\cdot -}$ levels in macrophages could have important implication in NAFLD and its associated CVD risk.

5.1.2. Manuscript 2 - Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase

The liver is a major storage site for folate. Studies conducted in our laboratory have demonstrated that folic acid is protective against hyperhomocysteinemia induced oxidative stress in various model systems (Au-Yeung et al., 2006; Hwang et al., 2011; Woo et al., 2006). Since hepatic oxidative stress is an important pathogenic mediator in NAFLD, the second study investigated the effect of folic acid supplementation on HFD induced hepatic oxidative stress. Mice maintained on a HFD for 12 weeks were heavier than control mice. Hepatic oxidative stress manifested in HFD fed mice and was associated with liver injury. NADPH oxidase, which was markedly activated in the liver of HFD fed mice due to its increased expression, contributed to hepatic oxidative stress in these animals. The activity of several antioxidant enzymes were

also impaired by HFD feeding and the hepatic GSH:GSSG ratio was markedly decreased. Histological analysis revealed marked lipid vacuole formations in the parenchymal tissue and was consistent with the significant accumulation of cholesterol and triglyceride in the liver. Intriguingly, although folic acid supplementation did not affect HFD induced weight gain, it was hepatoprotective and completely abolished hepatic oxidative stress in these animals. Further analysis revealed that folic acid supplementation significantly reduced the activity of NADPH oxidase by down-regulating $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$ gene expression. Moreover, folic acid supplementation maintained the activity of enzymatic antioxidants SOD and catalase in the liver of HFD fed mice. These multifaceted antioxidant effects of folic acid were also associated with the preservation of the hepatic GSH:GSSG ratio, demonstrating that folic acid could effectively correct HFD induced perturbations in hepatic redox. In addition to these multifaceted antioxidant effects, histological analysis revealed that folic acid supplementation improved the morphological integrity of the liver and biochemical analysis identified a modest improvement in hepatic lipid accumulation. Taken together, results from this study have demonstrated for the first time that folic acid is hepatoprotective, and mitigates HFD induced hepatic oxidative stress via inhibiting NADPH oxidase and activating enzymatic antioxidants.

Our investigations in the 12 week HFD model confirm that oxidative stress is an important therapeutic target in NAFLD. Our study is among the first to identify NADPH oxidase as a major source of hepatic oxidative stress in the HFD mouse model (Meng et al., 2011; Sarna et al., 2012). These novel findings suggest that NADPH oxidase could be a rather significant source of hepatic oxidative injury in NAFLD. Results from this study also suggests that folic acid may be therapeutically advantageous for managing hepatic oxidative stress in NAFLD. Folates are

not traditionally known for their antioxidant properties. However, folic acid, as well as naturally occurring folates have been reported to directly scavenge ROS (Gliszczynska-Swiglo & Muzolf, 2007; Joshi et al., 2001). In our study folic acid was characterized as having multifaceted indirect antioxidant effects in the liver of HFD fed mice. Previous studies conducted in our lab have similarly shown that folic acid could reduce hepatic oxidative stress in hyperhomocysteinemic rats (Woo et al., 2006), while conversely, others have reported that dietary folate depletion induces hepatic oxidative stress in rats (R. F. Huang et al., 2001). Recently, Fan *et al* demonstrated that a novel biological function of folate metabolism may be the production of NADPH, which in turn serves to buffer against oxidative stress by generating reducing power in cells (Fan et al., 2014). Although further mechanistic studies are needed to confirm whether a similar interdependent relationship manifested in our study, it is interesting that folic acid also restored the GSH:GSSG ratio in the liver of HFD fed mice, a parameter that is dependent on the bioavailability of NADPH. Taken together, the sum of the evidence suggests that folate may be an important and multifaceted biological antioxidant.

Finally, one of the particularly thought provoking findings in this study was the ability for folic acid to improve multiple liver parameters including liver enzymes, hepatic oxidative stress, lipid accumulation, and liver histology in HFD fed mice without affecting the body weight of these animals. Currently, gradual and sustained weight loss is the only acceptable treatment strategy used to manage NAFLD clinically. Our findings might therefore have a significant implication since not only do they identify a potential therapeutic benefit of folic acid supplementation, but they also suggest that NAFLD could be managed independent of weight loss.

5.1.3. Manuscript 3 - High-fat diet stimulates hepatic cystathionine β-synthase and cystathionine γ-lyase expression

Although a number of studies have reported alterations in circulating Hcy levels in NAFLD patients (Gulsen et al., 2005; Hirsch et al., 2005; Polyzos et al., 2012b), the mechanisms responsible for these observations remain unclear. The liver is one of the major sites regulating systemic Hcy levels (Stead et al., 2000). In the liver, Hcy may be remethylated to methionine via the remethylation pathway, or it may be irreversibly consumed by the canonical transsulfuration pathway to generate cysteine, the rate limiting amino acid in glutathione biosynthesis. Hcy may also serve as a substrate for alternative desulfuration reactions catalyzed by transsulfuration enzymes CBS and CSE that lead to the endogenous synthesis of H_2S, a multifaceted gasotransmitter that is becoming increasingly recognized for its redox regulating properties (Kabil et al., 2014). In light of its potential implication to oxidative stress, the third study investigated the regulation of the transsulfuration enzymes CBS and CSE in the liver of HFD fed mice and their association with Hcy metabolism and H_2S biosynthesis. In this study, 5 week HFD feeding significantly decreased serum Hcy levels in mice. The expression of CBS and CSE were significantly elevated in the liver of these animals. Concomitantly, the transsulfuration activities of these enzymes were significantly increased and hepatic H_2S biosynthesis was also markedly elevated. Total glutathione levels were maintained in the liver of these animals, despite increased hepatic oxidative stress. For the first time, this study demonstrates that the CBS/CSE system is activated in the liver of HFD fed mice and may contribute to imbalanced Hcy-H_2S homeostasis.

Although homocysteine levels are perturbed in NAFLD, there are inconsistencies in the literature regarding the relative change in Hcy levels among these patients (Gulsen et al., 2005; Hirsch et al., 2005; Polyzos et al., 2012b). Previous studies conducted in other HFD models have reported that increased Hcy levels are associated, in part, with deficiencies in the hepatic CBS/CSE system (Bravo et al., 2011; Yun et al., 2013). Our study is the first to provide insight into one of the potential mechanisms that may be responsible for lowering Hcy levels in NAFLD (Polyzos et al., 2012b). Another novel finding obtained through this study was that up regulation of the CBS/CSE system was associated with increased H_2S biosynthesis in the liver of HFD fed mice. These original findings (Hwang et al., 2013) are among the first to show that H_2S metabolism is altered in animal models of NAFLD (Luo et al., 2014; Sarna et al., 2015). Though a relationship between H_2S and NAFLD remains to be clinically investigated, the perturbations in H_2S levels that have been reported in both obese (Whiteman et al., 2010) and T2DM (Jain et al., 2010) patients suggests that our findings may be clinically relevant.

Currently, the pathophysiological implication of our findings remain open for interpretation. We speculate that the activation of CBS and CSE enzymes may play an important role in regulating hepatic oxidative stress in the HFD model. There is compelling evidence that the CBS/CSE system is inherently integrated in the innate adaptation to oxidative stress and that the cytoprotective antioxidant properties of this system are reliant on both the transsulfuration and desulfuration capacities of CBS and CSE enzymes (Sarna et al., 2015). It is also possible that the implication of our findings may also extend beyond the regulation of hepatic oxidative stress. The biological importance of the CBS and CSE enzymes in the liver are evidenced by their prominent expression in the parenchyma and by the overt abnormalities that manifest in response

to their functional disruption. In fact, a number of the metabolic and stress related molecular mediators that underly NAFLD pathogenesis appear to be closely integrated with the CBS/CSE system. Such associations have led us to propose that the CBS/CSE system may be a potential therapeutic target in NAFLD (Sarna et al., 2015).

5.2. Connecting concepts and themes

Although these three studies differ in their outlined objectives, and model systems, there are several connecting concepts that have emerged. A major commonality in all three studies, emphasizes the underlying complexity by which oxidative stress is regulated in cells and tissues. Our studies demonstrate that in many cases, the manifestation of oxidative stress is attributed to the activation of pro-oxidant enzymes such as NADPH oxidase as well as deficiencies in antioxidant defense systems. The innate regulation of oxidative stress also appears to be closely linked to aberrations in metabolism. It is important to consider that oxidative stress is regulated via multifaceted mechanisms when designing therapies for its management. Antioxidant therapies have often been chosen on the basis of their efficiency to react with ROS. However, antioxidants, just like ROS, are not generic (Winterbourn, 2008), and so it is important that when trying to regulate oxidative stress through antioxidant based therapies one does not take a "one size fits all" approach. In support, two NHPs with differential direct scavenging properties (Joshi et al., 2001; Shirwaikar et al., 2006), were shown to effectively regulate oxidative stress in these studies. The ability for both berberine and folic acid to regulate oxidative stress indirectly by restoring balance between pro-oxidants and antioxidant systems, was likely an important property that facilitated their effectiveness in these studies. Another important consideration is that the mechanisms responsible for regulating oxidative stress in a complex and progressive

disorder such as NAFLD may also evolve and change overtime. Our studies in the 5 week and 12 week HFD mouse model attest to this, and have not only shown that hepatic oxidative stress intensifies over time, but that the relative contribution of certain pro-oxidant and antioxidant systems also markedly fluctuates. For instance, while deficiencies in enzymatic antioxidants SOD and catalase were observed in the 12 week HFD model, our preliminary findings in the 5 week model suggested that the activity of these systems were maintained (data not shown). Finally, another important commonality that emerged through these studies was that NADPH oxidase is an important ROS generating system, that is responsible for increasing $O_2^{\cdot -}$ production in both stimulated macrophages and in the liver of HFD fed mice. The activation of NADPH oxidase in these different model systems suggests that NADPH oxidase might play a rather important role in regulating oxidative stress. Further studies are warranted to better characterize the role of NADPH oxidase in the regulation of cellular redox and the mediation of oxidative stress under different pathophysiological states.

VI. CONCLUSIONS & FUTURE PERSPECTIVES

6.1. Oxidative stress

Investigating the regulation of oxidative stress has been a central theme throughout my studies. It is clear that oxidative stress is a complex and dynamic biological phenomenon. The perception of ROS, and their biological role has shifted tremendously from the early theories that focused primarily on their cytotoxicity. It is now understood that ROS are regulated and play a fundamental role in many aspects of cellular physiology. It is also evident that their dysregulation can contribute to pathology. Still, there remains much to learn about the early events that are responsible for tipping normal cellular redox responses to oxidative stress. With the development of new technologies and sensitive analytical methods, it is anticipated that a clearer picture will emerge in the future. New redox sensors and probes that are designed to measure ROS and their subcellular compartmentalization are under development for use in both intact cells and *in-vivo* (Kaludercic et al., 2014). Mass spectrometry and redox proteomic approaches (Winterbourn, 2008) will also provide valuable insight into the functional changes associated with these signals. Applying these new and emerging methods in future studies, will help to identify and characterize site specific targets that are responsible for mediating oxidative stress in complex diseases like NAFLD. These emerging techniques will also be essential for designing and evaluating targeted antioxidant based therapies to manage oxidative stress in multisystem pathologies as well as in broad spectrum disorders such as NAFLD.

6.2. Manuscript 1 - Berberine inhibits NADPH oxidase mediated superoxide anion production in macrophages

The novel findings in this study were that berberine inhibits NADPH oxidase mediated $O_2^{\cdot -}$ production via selectively inhibiting $gp91^{phox}$ expression and maintaining SOD activity in

macrophages. Since our investigations, more information regarding berberine and its metabolism have emerged. It is now evident that the liver might be the main target of berberine (Liu et al., 2013). Though berberine is widely circulated to tissues upon oral administration in rats, the liver appears to be the major site of its distribution, with levels exceeding plasma nearly 70 fold (Liu et al., 2010). Future studies evaluating berberine's capacity to manage hepatic oxidative stress are therefore warranted. Since NADPH oxidase is up-regulated in the liver of 12 week HFD fed mice, and SOD activity is impaired in these animals, this model might be an attractive option for validating our findings *in-vivo* and also for characterizing berberine's antioxidant properties in the liver. Moreover, we have also reported that berberine has strong lipid lowering effects in hyperhomocysteinemic rats, which protect these animals against both hyperlipidemia and hepatic steatosis (Wu et al., 2011). Additional studies investigating the multimodal hepatoprotective effects of berberine, and its ability to regulate cardiometabolic risk factors are also justified in the HFD model. These studies might provide valuable information regarding the therapeutic potential of berberine in the management of NAFLD and its associated morbidities (CVD).

6.3. Manuscript 2 - Folic acid supplementation attenuates high fat diet induced hepatic oxidative stress via regulation of NADPH oxidase

The novel findings presented in this study were that folic acid is hepatoprotective, and mitigates HFD induced hepatic oxidative stress via inhibiting NADPH oxidase and activating enzymatic antioxidants. Although our study suggests that NADPH oxidase may play a significant role in mediating hepatic oxidative stress in HFD fed mice, it is also noteworthy that in a recent study, Nox2-knockout mice exhibited preferential storage of lipids in the liver and marked hepatic inflammation compared to wild-type animals (Costford et al., 2014). Though results from these

studies are seemingly opposing, they echo one of the important paradigms in the field of free radical biology: that is that ROS are inherently involved in, and are fundamental to, both physiological and pathophysiological processes. These findings necessitate the need to fully characterize the role(s) of NADPH oxidase mediated $O_2^{\cdot -}$ production in the liver under normophysiological states as well as in hepatic disorders like NAFLD in future studies. The ability for folic acid to preserve the functional integrity a number of antioxidant defences in the liver of HFD fed mice also warrants further investigation into the mechanism responsible. Nuclear factor erythroid 2–related factor 2 (NRF2) regulates various antioxidant response elements that lead to the transcriptional up-regulation of cytoprotective and endogenous antioxidant defense systems. It would be interesting to investigate whether folic acid's multifaceted antioxidant effects in HFD fed mice are mediated through the regulation of NRF2 in follow-up studies.

Lastly, a number of studies have reported that obesity is associated with perturbed folate homeostasis (Hirsch et al., 2005; Mahabir et al., 2008; Mojtabai, 2004). Liver injury is also associated with the depletion of folate stores (Leevy et al., 1970). In our study, the hepatoprotective effect of folic acid supplementation in HFD fed mice suggests that folate requirements may also be increased in these animals. Future studies are needed to investigate whether folate deficiency manifests in the HFD model, and if so, the mechanisms responsible should be delineated by comprehensively evaluating folate absorption, metabolism, storage and excretion in the HFD model. The level of folic acid used to supplement the diet in this study (0.025% w/w) was originally selected for its potential to lower Hcy, improve liver function and regulate hepatic oxidative stress in hyperhomocysteinemic rats (Woo et al., 2006). The

nutritional relevance of using this level of folic acid for supplementation in the HFD model remains open for debate. On one hand, this level exceeds the recommended dietary concentration of folic acid in mice (0.0002% w/w) (Reeves et al., 1993) and the levels provided in the study diets (control, 0.0002% w/w; HFD, 0.00026% w/w) by approximately 100 fold. By comparison, the tolerable upper intake level of folic acid in humans (1000μg/d) only exceeds the recommended dietary allowance of folate (400μg/d) by 2.5 fold (DRI, 1998). On the other hand, it is also important to consider that the recommendations for mice aim to support the general growth and maintenance of these animals (Reeves et al., 1993) (NRC, 1995), and in humans, the recommendations apply only to the needs of an apparently healthy population (DRI, 2003). Therefore, it is unknown what the actual requirements of folic acid are in the HFD mouse model, as well as in those individuals with NAFLD. Since no evidence of toxicity was observed in our study (weight gain in the folic acid supplemented group was comparable to HFD group and liver enzymes were improved to levels similar to controls), it is conceivable that a high level of supplemental folic acid might be appropriate in the context of the extreme 60% kcal fat HFD model. It is also possible that lower levels of folic acid could confer similar hepatoprotective effects. Lowering the fat content of the diet to a level that is also considered more nutritionally relevant (45% kcal fat) (Choi et al., 2015) might help to better interpret our findings from a nutritional perspective. Further investigations using lower levels of folic acid supplementation in the HFD mouse model are therefore required to fully ascertain the nutritional relevance of the folic acid level chosen in this study. Taken together, these proposed studies could provide valuable insight into potential alterations in micronutrient requirements in NAFLD.

6.4. Manuscript 3 - High-fat diet stimulates hepatic cystathionine β-synthase and cystathionine γ-lyase expression

The novel findings in study 3 are that the CBS/CSE system is activated in the liver of HFD fed mice and is associated with a reduction in circulating Hcy levels and increased hepatic H_2S biosynthesis in these animals. These results raise many new and exciting questions. While the health implications associated with hyperhomocysteinemia have received much attention, by comparison, the pathophysiological significance of decreased homocysteine is less clear. Since low Hcy levels have been correlated with the histological severity of NAFLD (Polyzos et al., 2012b), further studies are warranted to investigate the biological implication of decreased Hcy in the HFD model. The biological functions of H_2S in the liver are beginning to emerge (Mani et al., 2014), but its role(s) in hepatic disorders like NAFLD is unclear. Since H_2S is a multifaceted gasotransmitter (Wang, 2012), further studies investigating the consequences associated with increased H_2S synthesis in the liver of HFD fed mice are also needed.

Finally, it is important to consider that through catalysis of the transsulfuration pathway and alternative desulfuration reactions, the CBS/CSE system converges homocysteine and cysteine metabolism with endogenous H_2S biosynthesis (Sarna et al., 2015). Though our study suggests concomitant activation of these pathways in the liver of HFD fed mice, their potential contribution to hepatic redox control requires further study in the model. It is evident that both homocysteine catabolism by the transsulfuration pathway (Mosharov et al., 2000; Vitvitsky et al., 2003), as well as H_2S (Kimura et al., 2010; Lee et al., 2014) can regulate glutathione homeostasis in hepatocytes. However, in general, the relative contribution of the transsulfuration versus desulfuration functions of the CBS/CSE system are unknown (Kabil & Banerjee, 2014).

Therefore, it is unclear whether the transsulfuration and desulfuration capacities of these enzymes are working in concert to regulate glutathione or whether they are functioning independently, competing for substrates and partitioning their products into different metabolic fates. Metabolomic analysis and in-depth mechanistic studies in the both the HFD model and in isolated hepatocytes are needed to interpret our findings and gain insight into the metabolic and redox-related consequences associated with our findings.

VIII. REFERENCES

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VIII. APPENDIX

Appendix I

Composition of purified diets: energy, macronutrient and other primary ingredients

	Control (D12450B)	High Fat Diet (D12492)
kcal/g	3.85	5.24

	Control (D12450B)	High Fat Diet (D12492)
Distribution of Energy	%kcal	% kcal
Protein	20	20
Carbohydrate	70	20
Fat	10	60

	Control	(D12450B)	High Fat Diet	(D12492)
Purified Ingredient	g	kcal	g	Kcal
Casein, 30 mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	315	1260	0	0
Maltodextrin 10	35	140	125	500
Sucrose	350	1400	68.8	275.2
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Cellulose, BW200	50	0	50	0
Mineral Mix, S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Dye #5 or #1	0.05	0	0.05	0
TOTAL	**1055.05**	**4057**	**773.85**	**4057**

Based on product data sheets for the DIO series diets obtained from Research Diets, Inc.

Diets are formulated on the basis of their nutrient to calorie ratios. Such formulation ensures that when the diet is consumed for calories, discrepancies in intake relate only to the carbohydrate and fat contents of the diet and not to other primary ingredients, including protein, micronutrients and fiber.

Appendix II

Composition of purified diets: typical profile of dietary fats

	Control	(D12450B)	High Fat Diet	(D12492)
Fatty Acid Composition	**g/1055.05g**	**% of Total Lipid**	**g/773.85g**	**% of Total Lipid**
Saturated	9.9	22.7	81.5	32.0
Monounsaturated	13.0	29.9	91.5	35.9
Polyunsaturated	20.7	47.4	81.5	32.0
Total	**43.7**		**254.5**	

	Control	(D12450B)	High Fat Diet	(D12492)
Lipids	**mg/4057kcal**	**% Diet Weight**	**mg/4057kcal**	**% Diet Weight**
Cholesterol	54.4	0.0052	216.4	0.0280
Fatty Acid Profile	**g/4057kcal**	**% Diet Weight**	**g/4057kcal**	**% Diet Weight**
C10 Capric	0.0	0.00	0.1	0.01
C12 Lauric	0.0	0.00	0.2	0.03
C14 Myristic	0.2	0.02	2.8	0.36
C15	0.0	0.00	0.2	0.03
C16 Palmitic	6.5	0.62	49.9	6.45
C16:1 Palmitoleic	0.3	0.03	3.4	0.44
C17	0.1	0.01	0.9	0.12
C18 Stearic	3.1	0.29	26.9	3.48
C18:1 Oleic	12.6	1.19	86.6	11.19
C18:2 Linoleic	18.3	1.74	73.1	9.45
C18:3 Linolenic	2.2	0.21	5.2	0.67
C20 Arachidic	0.0	0.00	0.4	0.05
C20:1	0.1	0.01	1.5	0.19
C20:2	0.2	0.02	2.0	0.26
C20:3	0.0	0.00	0.3	0.04
C20:4 Arachidonic	0.1	0.01	0.7	0.09
C22:5 Docosapentaenoic	0.0	0.00	0.2	0.03
Total lipid	**43.7**	**4.14**	**254.7**	**32.91**

Fatty acid profile determined 4th quarter of 2011

Based on product data sheets for the DIO series diets obtained from Research Diets, Inc.

Appendix III

Composition of purified diets: typical amino acid profile

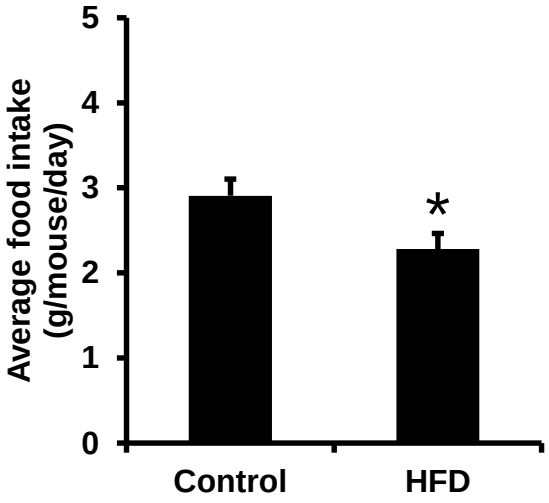
	Control	(D12450B)	High Fat Diet	(D12492)
Amino Acid Profile	**g/4057kcal**	**% Diet Weight**	**g/4057kcal**	**% Diet Weight**
Arginine	6.0	0.57	6.0	0.78
Histidine	4.6	0.44	4.6	0.59
Isoleucine	7.6	0.72	7.6	0.98
Leucine	15.8	1.50	15.8	2.04
Lysine	13.2	1.25	13.2	1.71
Methionine	5.1	0.48	5.1	0.66
Phenylalanine	8.4	0.80	8.4	1.09
Threonine	7.2	0.68	7.2	0.93
Tryptophan	2.1	0.20	2.1	0.27
Valine	9.3	0.88	9.3	1.20
Alanine	5.1	0.48	5.1	0.66
Cystine	4.2	0.40	4.2	0.54
Aspartic acid	12.1	1.15	12.1	1.56
Glutamic acid	38.2	3.62	38.2	4.94
Glycine	3.0	0.28	3.0	0.39
Proline	17.8	1.69	17.8	2.30
Serine	10.0	0.95	10.0	1.29
Tyrosine	9.2	0.87	9.2	1.19
Total Essential AA	**79.3**	7.52	**79.3**	10.25
Total Non-essential AA	**99.6**	9.44	**99.6**	12.87
Total AA	**178.9**	16.96	**178.9**	23.12

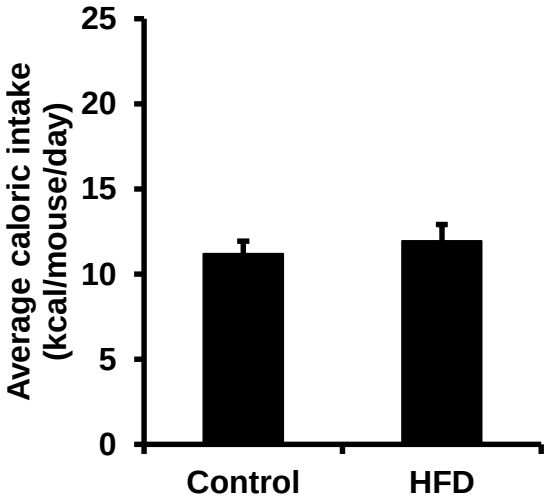
Amino acid profile per 200g casein and 3g of L-Cystine

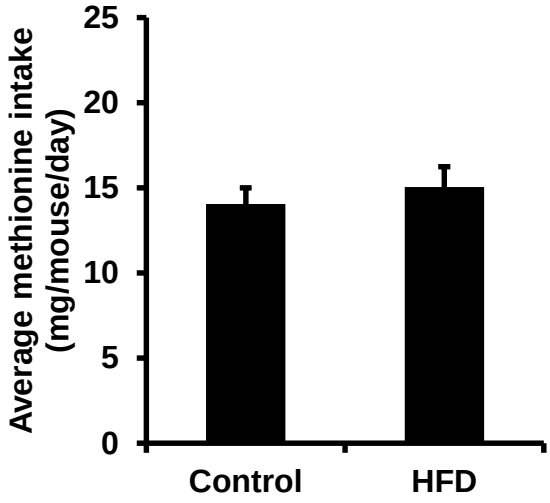
Based on product data sheets for the DIO series diets obtained from Research Diets, Inc.

Appendix IV

Approximate dietary intake of control and high fat diet (HFD) mice over a 5 week period*







* values provided are based on approximate food intakes in mice fed *ad libitum* over a 5 week period. Food intake was determined after a period of acclimation by calculating the difference between the initial weight of the diet provided and its residual weight after 3 days.