The importance of thiol availability to transsulfuration, antioxidant systems and mitochondrial H_2S formation in the rat

By

Nahid Tamanna

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Department of Biological Sciences

University of Manitoba

Winnipeg, Manitoba

Canada

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Abstract

Sulphur containing amino acids play numerous roles in cellular system including protein synthesis, cell replication, a cellular defence mechanism and energy metabolism. Of the two-major sulphur containing amino acids, methionine is indispensable whereas cysteine is semi-essential considering cysteine can be synthesized from dietary methionine. During the metabolism of methionine, homocysteine is formed as an intermediate which holds a critical position between transsulfuration and transmethylation pathways. Metabolism of homocysteine through transsulfuration leads to the formation of cysteine which serves as a precursor for the major antioxidant glutathione (GSH) or hydrogen sulphide (H_2S). A diet low in sulphur amino acids, known as methionine restriction (MR), increases lifespan in diverse species. Unconventional to lifespan extension mechanism, methionine restriction causes hyperhomocysteinemia and decreases tissue GSH levels in rodents. Linking the significance of sulphur amino acids, longevity and H_2S, previously it has been proposed that increased H_2S production delays the onset of aging and age-related aspects in nutritional restriction diets. Additionally, it has been suggested that the upregulation of transsulfuration pathway produces H_2S in MR mice while hyperhomocysteinemia and low tissue GSH in MR animals indicate the opposite. Despite a declination of a major antioxidant GSH, how MR protects the cellular environment from pro-oxidants is not clear. Apart from transsulfuration, H_2S can also be produced by the mercaptopyruvate sulfurtransferase (MPST). Along with other functions, H_2S is reported to increase mitochondrial respiration rate. To generate H_2S in *in vitro*, MPST require accessory reductant(s) and it is not known whether mitochondria also needs reductant(s) to release H_2S which in turn enhances respiration rate. To

investigate the link between homocysteine, H_2S and GSH, we fed Fischer 344 rats MR and control diets. We found that MR impedes transsulfuration by inhibiting the cystathionine-β-synthase and simultaneously maintains H_2S production capacity by enhancing alternate pathways. Methionine restriction increases the activity of thioredoxin reductase and glutathione reductase which can compensate for the low GSH level in cytosol and parallelly reduces reactive oxygen species production in mitochondria presumably by enhancing proton leak. Liver mitochondria assays from Sprague-Dawley rat further suggested that MPST require thioredoxin to enhance mitochondrial bioenergetics.

Keywords: Methionine restriction, sulphur amino acid, aging, antioxidant, oxidative stress, mitochondria, H_2S

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Dedication

To my parents, Shahjahan Mia and Sufia Begum

To my brother, Hasan Mahmud

To my sisters, Taslima and Tahera Tamanna

Preface

This thesis is structured in the "sandwich thesis" format approved by the advisory committee. Chapter one provides a general introduction for the thesis. Chapter two is published in 'Mechanisms of Ageing and Development Journal' and chapter three is published in the 'Experimental Gerontology' journal. Chapter four represents a manuscript soon to be submitted to 'Mitochondrion' journal. Chapter five provides a summarizing discussion of the findings of the respective chapters.

Thesis, the publication status of chapters, and the role of each co-author in each chapter

Chapter	Author	Contribution of authors	Publication status
1	Nahid Tamanna	NT searched the literature, organized and wrote the chapter	Not to be published
2	Nahid Tamanna, Shyamchand Mayengbam, James D. House, Jason R. Treberg	NT and JRT designed the experiments, JDH and SM helped in designing biochemical assays, NT did the experiments and analyzed the data, JRT and NT prepared the manuscript, JDH and SM edited the manuscript	Published in the 'Mechanisms of Ageing and Development' journal, publication date 1st December, 2018 Vol 176, p-9-18
3	Nahid Tamanna, Kathryn Kroeker, Kristen Braun, Sheena Banh, Jason R. Treberg	NT and JRT designed the experiments, NT did most of the experiments, KK conducted the respirometry, KB did the protein carbonyls and SB did the mitochondrial GSH assay. NT analyzed the data. NT and JRT prepared the manuscript.	Published in 'Experimental Gerontology' journal, publication date 28th August, 2019, Vol 127, article 110712
4	Nahid Tamanna and Jason R. Treberg	NT and JRT designed the experiments, NT did the experiments and analyzed the data, NT and JRT wrote the manuscript	Soon to be submitted in a peer reviewed journal
5	Nahid Tamanna	NT summarized the research, reviewed the literature and wrote the chapter.	Not to be published

Abbreviations

αKG- α-ketogluterate

BCA-Bicinchoninic acid

BHMT-Betaine-homocysteine-methyltransferase

CAT- Cysteine aminotransferase

CBS- Cystathionine-β-synthase

CGL-Cystathionine-γ-lyase

Cys-Cysteine

DHLA-Dihydrolipoic acid

DTT-Dithiothreitol

GR-Glutathione reductase

GPx-Glutathione Peroxidase

GSH- Glutathione (reduced)

GSSG-Glutathione (oxidized)

H_2O_2-Hydrogen peroxide

H_2S-Hydrogen sulphide

Hcys-Homocysteine

HPLC-High performance liquid chromatography

LDH- Lactate dehydrogenase

CCCP-*m*-chlorophenyl hydrazone

MAT-Methionine adenosyl transferase

MDA- Malondialdehyde

MTHFR-Methionine-tetrahydrofolate-reductase

MR-Methionine restriction

MICA-Methoxy indole carboxylic acid

MPST-Mecaptopyruvate-sulfurtransferase

NaMP-Sodium Mercaptopyruvate

NaHS- Sodium hydrosulfide

Ox-Oxidized

PC-Protein carbonyls

PCA- perchloric acid

PLP-Pyridoxal phosphate

PPG- Propargylglycine

Pyr-Pyruvate

Q- Ubiquinone

Red-Reduced

ROS-Reactive oxygen species

RBC-red blood cells

RCR-Respiratory control ratio

SAM- S-adenosyl methionine

SAH-S-adenosyl homocysteine

SBDF-7-Fluorobenzofurazan-4-sulfonic acid ammonium salt

Suc-Succinate

SQR- Sulphur Quinone oxidoreductase

TCA-Trichloro acetic acid

TCEP- tris-carboxyl-ethyl-phosphine

TMRM-Tetramethylrhodamine methyl ester

Trx-Thioredoxin

TrxR-Thioredoxin reductase

3-MP- 3- Mercaptopyruvate

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Chapter One

General Introduction

This thesis focuses on three core aspects: sulphur amino acid metabolism, oxidative stress and H_2S production, all of which have substantial importance in aging research. Elevated levels of homocysteine are considered detrimental whereas increased H_2S production by tissues is beneficial in delaying aging processes. The first research chapter investigates how a lifespan extension dietary regimen termed 'methionine restriction' (MR) regulates the transsulfuration pathway to metabolize homocysteine and to produce H_2S. Methionine restriction decreases tissue glutathione (GSH) levels which is a major antioxidant scavenging oxidants. The second chapter explores how MR deals with oxidative damage while GSH levels are low. The third research chapter investigates the predominant H_2S producing enzyme in liver mitochondria and also characterizes its' role on respiration. In advance of the research chapters, this introductory chapter provides a general review of aspects relevant to the research chapters that follow.

Aging theories

Aging can be characterized by the progressive decline of various physiological responses which is experienced by almost all multicellular organisms [1]. The trade-off between longevity and energy metabolism has been a matter of attention since the late 1800s [2, 3]. In 1908, Max Rubner proposed the idea that metabolic rate (oxygen consumption rate) and longevity are inversely related [4]. This idea was further expanded in 1928 by Pearl and is now known as 'rate-of-living-theory' where it has been postulated that lifetime metabolic energy expenditure is finite for an organism and higher energy expenditure is related to a shorter lifespan [5]. However, the rate-of-

living theory is no longer as widely accepted because the relationship between mass-specific energy expenditure and longevity do not meet expectations among taxa i.e., between birds and mammals or even within the same Class interspecific variability is seen [6]. Although the rate-of-living theory is no longer widely accepted it directs aging research towards oxygen metabolism [7]. Since then a number of theories have been proposed and developed [8, 9]; among them the 'free radical theory of aging' is a prominent one which hypothesizes that aging processes are caused by oxygen free radical reactions presumably driven by superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and protonated superoxide or hydroperoxyl ($HO_2^{\bullet}$) [10]. This theory of aging has been refined over the years and also added other non-radical reactive species such as H_2O_2, hypochlorus acid and ozone. This modification of Harman's original theory is collectively termed as 'oxidative stress theory' which hypothesizes that the imbalance between the pro-oxidants and antioxidants within tissues triggers oxidative stress, as a result aging occurs [1]. Therefore, this theory suggests that a decrease in oxidative stress will delay the onset of aging as well increase lifespan. While this current version of the oxidative theory of aging is accepted by many, there is an ongoing debate whether low oxidative stress can actually delay the aging process (reviewed in [9, 11]). Lowering mitochondrial reactive oxygen species(ROS) formation will not necessarily always increase lifespan. On that note, a recent study demonstrated that mitochondrial oxidative damage does not explain lifespan in *Caenorhabditis elegans* [12]. Nevertheless, low oxidative stress per se has been shown in several cases to be beneficial either by increasing lifespan or by improving health in different species. For instance, oxidative stress is associated with several neurological disorders [13] and lowering oxidative stress by different therapeutic approaches showed beneficial effects on neurological diseases [14, 15].

A brief history of the influence of diet restriction on lifespan extension

In 1935, McCay and colleagues reported that lifespan can be increased in rodents by restricting calorie intake [16]. Since then, researchers have conducted a number of experiments and reported that calorie restriction increases lifespan in diverse taxa including yeast, nematodes, fish, hamsters and dogs (reviewed in [17]). Inspired by the response to calorie restriction, protein, carbohydrates and lipids have been limited in the diet to study the underpinning role of specific macronutrients in determining lifespan [18-20]. Comparisons among different dietary regimens can be difficult because of inconsistencies among the study designs. On that note, limiting any component in the diet might not always lead to the same response as calorie restriction, because in some studies to compensate the limiting components, other constituents in the diet are increased to keep the total calorie density of the diet the same and the study subjects are often fed *ad libitum*. For instance, to investigate the impact of differing the amount of dietary protein on longevity in rats lipid and carbohydrate percent contributions were also varied to maintain the energy density across different treatment groups [21]. Nonetheless, specific restriction of carbohydrates or lipids did not increase lifespan in rodents, rather some studies reported that increased carbohydrate content in the diet either extends or did not alter lifespan (reviewed in [22]). In contrast, protein restriction itself extends lifespan by approximately 15-20 % in rodents which is about half the extension compared to calorie restriction diets ([22, 23]). It has been suggested that the protein restriction follows a different mechanism than calorie restriction , because calorie restricted animals are provided less calories by experimental design whereas the lifespan of rodents only increases by protein restriction while they are fed at *ad libitum* [23]. During the 1980s, there was a growing attention on dietary tryptophan restriction in mostly reversing age-associated brain pathology as well as on lifespan extension and reproduction [24-26]. It has been demonstrated that 30 or 40% restriction

of tryptophan increases the mortality rate; however when the surviving rats were switched to control diet after 24 months they live longer than the control rats [24, 26]. Therefore, the impact of tryptophan on longevity is inconclusive.

In the 1990s, it was reported that methionine restriction, where methionine is the sole source of dietary sulphur amino acids, increases lifespan in Fischer-344 rats [27, 28]. Since then a number of studies have been conducted and consistently reported that methionine restriction increases lifespan in diverse species including yeast, fruit flies and rodents [29-32]. Compared to calorie restriction, methionine restriction is a fairly new model in aging research and because of this a lot is unknown about methionine restriction. There are both similar and contrasting phenotypes that emerge when comparing between calorie and methionine restriction in rodents. For instance, both calorie and methionine restriction improve insulin sensitivity [33, 34], reduce adiposity in rodents [35, 36] and decrease growth rates. But calorie and methionine restriction reduce growth rates by different means. While calorie restriction decreases growth rate relative to *ad libitum* fed 'control' animals [37], methionine restriction leads to lower growth rates compared to both *ad libitum* and pair-fed methionine sufficient rodents, the latter of which consume the same mass of food as those getting the methionine restricted diet [28, 35]. By contrast, methionine restricted animals consume more food (~30%) on a mass-specific basis than control fed rats [38], whereas calorie restricted animals are experimentally provided less food than controls [16]. This hyperphagia in methionine restricted rodents coincides with increased total mass-specific energy expenditure and core body temperature in methionine restricted animals [38], whereas these traits are reduced in calorie restricted animals [39]. Therefore, the underlying mechanism (s) of extending lifespan necessarily may not be same in these diets. Although methionine is a component in protein, dietary compositions varied between protein restriction and methionine restriction. In protein restricted

diets, carbohydrate and fat content are increased to make the diet isocaloric, whereas in methionine restriction, glutamic acid content is increased to make the diet isonitrogenous and isocaloric while the carbohydrate and fat content are same. Nevertheless, protein restriction resembles at some extent to methionine restriction, because limiting the protein will also limit methionine in the diet.

Oxidative stress of theory and dietary restriction

Several studies reported that calorie restriction decreases oxidative damage to lipids, proteins and DNA [1, 40-42] suggesting calorie restriction may provide some support for the oxidative theory of aging; however, this is not the only aging hypothesis that describes the mechanism induced by calorie restriction in lifespan extension (reviewed [43]) rather this is an explanation accepted by a wide number of gerontologists. Thiols, particularly glutathione and thioredoxin play key roles in regulating oxidative stress through their antioxidant properties [44, 45]. Evidence suggests that antioxidant enzymes might decline with age in different tissues including human erythrocytes, and rodent lung and liver tissues [46-48]. Calorie restriction prevents the age associated decline of GSH and thioredoxin dependant enzymes in rat kidney tissue [49] and thus, the antioxidants might retain the redox environment with aging as a lifespan increasing phenotype. However, in contrast to caloric restriction, methionine restriction decreases GSH levels in certain tissues including liver, kidney, spleen and pancreas [28, 50] which raises the questions whether methionine restriction leads to a tissue environment that is more susceptible to oxidative stress. Moreover, methionine restriction causes hyperhomocysteinemia which is considered as a marker of cardiovascular and neurological disease [51, 52] as well as they are well recognized diseases of aging in mammals [53, 54]. Although in recent years it has been questioned whether homocysteine is a causal factor for developing cardiovascular disease, nevertheless, hyperhomocysteinemia is considered as a

determinant of an unhealthy lifestyle which can trigger cardiovascular disease [55]. It is established that a methionine enriched diet elevates homocysteine level [56]; thereby while Elshorbagy *et al*. reported that a methionine restricted diet causes hyperhomocysteinemia [57], the accuracy of this finding was questioned [58]. Despite a reduction of a major antioxidant and an elevation of the pro-oxidant homocysteine, methionine restricted animals have an extended lifespan which is paradoxical to increased longevity. If oxidative damage is not prevalent, then there is a possibility that methionine restriction might prevent the oxidative damage by other means. For example, increased activity of other antioxidants might be one of the key strategies to protect the cellular environment. However, it is not known whether less oxidative stress is one of the key mechanisms to extend the lifespan in methionine restriction.

Integrating concepts of energy metabolism in dietary restriction

It is often assumed that calorie restriction delays the aging process by reducing energy flux and metabolism and hence, supports 'the-rate-of-living-theory' [59, 60]. Lower food consumption, which will be accompanied by lower thermic energy loss to process food and low growth rate together presumably contribute for the reduced energy expenditure in calorie restricted animals. However, the reduction of energy expenditure by calorie restricted animals is still a matter of debate because normalization of energy expenditure to body weight or body composition instead of on a whole animal basis gives an inconsistent pattern. For instance, calorie restriction decreases energy expenditure at the whole animal level, however while the energy expenditure is adjusted to body size or body composition (fat mass, fat free mass) a decrease in energy expenditure was only observed in severely calorie restricted animals [60] and one study reported that calorie restriction might enhance energy expenditure while energy expenditure is normalized to body composition

of F-344 rats [61]. In contrast, methionine restriction does not fit the ‘rate-of living-theory’ because mass-specific energy expenditure increases in response to methionine restriction [38].

To understand the regulation of energy expenditure by an animal, under calorie or methionine restriction, it is valuable to consider the constituent components of energy metabolism. The total energy available to animals for reproduction and survival largely depends on the energy available after digestion and absorption processes [62]. During the processing of digestion, energy is expended which is defined as specific dynamic action of food or diet induced thermogenesis. To understand the overall energy metabolism, an animal can be considered as a system where the energy budget for certain processes will depend on the physiology and an assumed conservation of energy and mass. For instance, the chemical potential energy which is obtained from the diet will be transformed into different forms and not all of the consumed energy can be used by an individual because a portion of the energy is lost as heat or as fecal, urinary or gaseous energy. Although an organism loses energy in urine or feces, these mechanisms are necessary to remove excess nitrogenous waste or undigested food from the system. The remaining energy can be used for locomotor work, growth and reproduction [63], the details are provided in figure 1.1.

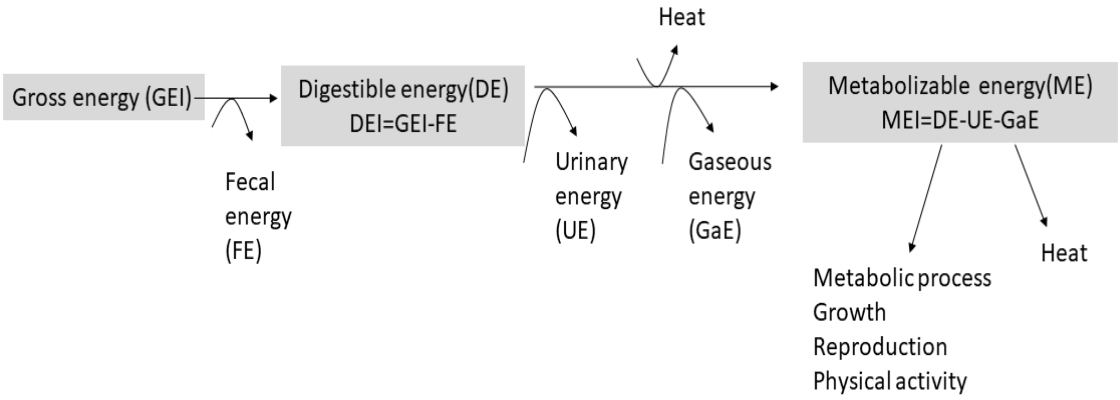

Fig. 1.1. Flow diagram of energy metabolism. Following the ingestion of food which is defined as gross energy intake, energy is transformed into various forms of which some are lost, and the remaining energy is used up by the organism for various processes. Total energy intake of an individual organism can be lost in the form of urinary energy, fecal energy and gaseous energy; then the retained metabolizable energy intake is used for growth and reproduction, lactation as well as physical activity. Energy is lost to heat through various processes i.e., basal or fasting metabolism, but also included in this term are metabolic costs of digestion, absorption and assimilation processes (Specific dynamic action); as well as heat lost due to voluntary activity and thermoregulation.

Methionine restricted rodents consume more food on a mass specific basis than the control rodents [28, 64], this suggests that their gross energy intake is higher than controls. Even though methionine restricted animals maintain a stable weight througout their adult lifespan their body weight is very low compared to control animals [27, 28]. This suggests methionine restricted animals either obtain less digestible energy, due to poor digestion efficiency, or they are converting available energy for other functions, i.e metabolic processes and heat generation rather than using it on growth. On that note, uncoupling protein 1(UCP1) expression was higher in both brown and white adipose tissues in MR animals and it has been reported that UCP1 is required for methionine restriction induced elevaiton of energy expenditure [38, 65].

A pilot study was conducted on the food intake of methionine restricted rats relative to ad libitum fed control rats (see Chapter 2 for the diet composition and husbandry details). While gross energy intake was measured via weighing the food consumed, it was found that methionine restricted animals obtain more energy, on a mass-specific basis, than controls by 3 weeks on methionine restriction (Fig. 1.2A). For digestible energy intake, fecal dry mass was calculated and consistent with gross energy intake, digestible energy intake was also higher in methionine restricted animals after 3 weeks (Fig. 1.2B). Although direct energy content was not estimated by bomb calorimeter, weighing the food amount or fecal dry mass can be used as proxy measures for energy content . In this study, gasesous energy or urinary energy was not estimated, however; it can be assumed that these will not probably differ between controls and methionine restricted animals, because gasesous energy mostly depends on the sources of food such as whether it is obtained from plants or animals and for the urinary energy it costs negligible amount of energy in herbivorous mammals [66]. The microbial consumption of materials may also lead to an inaccuracy if the microbiome is altered by the diet, however this might not be the case because the data obtained from our pilot

study fits results using indirect calorimetry [38]. In addition, previous studies reported that methionine restriction increases core body temperature as well as increases daily energy expenditure while not changing their overall daily activity relative to control animals [38]. Despite appearing to obtain higher gross energy intake or digestible energy intake, the body weight of methionine restricted animals was significantly lower than controls (Fig. 1.2C). These data and the literature together suggest that energy is preferentially diverted to other purposes like heat generation or other metabolic ineffcienct processes rather than growth during methionine restriction.

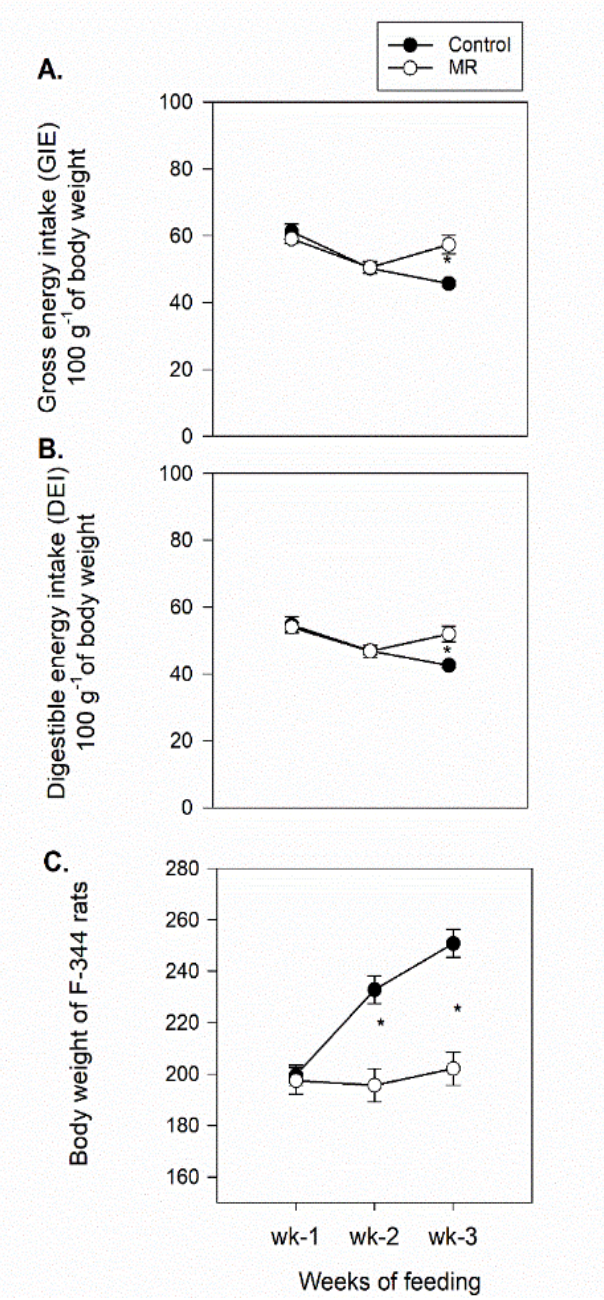

Fig. 1.2. Energy metabolism in MR animals. A) For gross energy intake the weight of the left over food was subtracted from the weight of the ration and then it was adjusted with per 100 g of body weight of rats **B)** Digestible energy intake was calculated by subtracting the fecal dry mass from gross energy intake and it was also adjusted to per 100 g of body weight **C)** Body weight of F-344 rats are shown at different weeks, n=8 for controls and n=9 for animals, (* $p < 0.05$; One-way ANOVA)

Lifespan extension by methionine restriction in rodents

In a regular chow diet, casein is the protein source where both cystine (two molecules of cysteine are joined via disulfide bonds) and methionine are present. In amino acid restriction studies, semi-purified diets are used meaning that instead of whole protein (casein), specific free amino acids are added. When developing a diet is devoid of cystine and using methionine as the sole source of sulphur amino acids, growing rats require approximately 0.50% (w/w) of methionine which is equivalent to 0.0035 moles of sulphur per 100 g of diet [67, 68]. In the AIN-76 A diet 20% (w/w) casein is the sole source of protein. Casein is comprised of ≥ 85% of protein and therefore 20% (w/w) casein will provide 17% of protein in the diet. Since, casein is low in sulphur amino acid an additional 0.3% methionine was added in AIN-76A diet [69, 70]. The first two studies which reported that methionine restriction extend mean (42%) and maximum lifespan (44%) in Fischer-344 rats used a formulated diet based on AIN-76A with 0.17% and 0.86% methionine in the methionine restricted and control diet respectively [27, 28]. Zimmerman *et al*. similarly reported that 0.17% methionine restricted diet increases lifespan in Brown Norway, Sprague Dawley and Wistar Hanover rats suggesting the diet's effect is not limited to one strain of rat [32]. In a lifespan extension study of C57BL/6J mice, a different amount of methionine (0.43%) was chosen for the control diet because in the chow diet (Purina Test Diets, Inc. (Richmond, IN, USA)) this amount of methionine was available at that time for mouse (Richard Miller, personal communication) and it was found that 0.15% of methionine containing diet extend the lifespan [31, 64]. In a more recent study, diets comprised of 0.16%, 0.43% and 1.3% methionine were defined as severe methionine restriction, moderate methionine restriction and enriched methionine diets [71]. To investigate the necessity of growth hormone signalling in lifespan extension by methionine restriction, these three levels of methionine (0.16%, 0.43% and 1.3%) containing diets were provided to Ames dwarf/wild

type, growth hormone receptor knock-out mouse and growth hormone over expressing transgenic mouse [71]. None of the methionine levels extended lifespan or altered food consumption or growth retardation in any of the growth hormone lacking or loss of function mutant strains, whereas 0.16% of methionine extended lifespan, increased food consumption and retarded growth in all of the sufficient wildtype strains compared to two other (0.43% and 1.3% of methionine) groups within genotypes [71]. Therefore, to execute the effects of methionine restriction on lifespan extension functional growth hormone signalling is required [71]. A summary of lifespan extension by methionine restriction has been added in Table 1.1.

Different strains or methionine amount used in the control and methionine restricted diets also alter methionine metabolites. Despite the inconsistency reported in metabolite levels, several studies reported that methionine restriction decreases hepatic GSH [28, 50, 72-76] and increases plasma homocysteine [57, 77, 78]. Although, hyperhomocysteinemia can trigger atherosclerosis it has been found that methionine restriction do not change cardiac function despite elevation of homocysteine levels [78]. Altogether, it is hypothesized methionine restriction offsets the low GSH and high homocysteine by triggering alternative cellular protective response(s) and that presumably includes pathways relevant to methionine metabolism and switching alternate antioxidant pathways.

Table 1.1: Effects of methionine restriction on lifespan extension in rodents.

Percentage of methionine in diet		Species	Strain	Lifespan change	References
Control	**MR**				
0.86	0.17	Rat	F-344	↑	[28]
0.86	0.17	Rat	Sprague-Dawley	↑	[32]
0.86	0.17	Rat	Brown-Norway	↑	[32]
0.86	0.17	Rat	Wistar Hanover	↑	[32]
0.43	0.15	Mouse	C57BL/6J	↑	[31]
0.43	0.15	Mouse	C57BL/6J	↑	[64]
1.3 and 0.43	0.16	Mouse	Ames Dwarf	No change	[71]
1.3 and 0.43	0.16	Mouse	Ames wildtype	↑	[71]
1.3 and 0.43	0.16	Mouse	GHRKO	No change	[71]
1.3 and 0.43	0.16	Mouse	GHRKO wildtype	↑	[71]
1.3 and 0.43	0.16	Mouse	GH Tg	↑	[71]
1.3 and 0.43	0.16	Mouse	GH Tg wildtype	↑	[71]
0.86	0.12	Mouse	$Lmna^{G609G/G609G}$	↑	[79]
0.86	0.12	Mouse	$Zmpste24^{-/-}$	↑	[79]

GHRKO-growth hormone receptor knock out, GH Tg- growth hormone transgenic, $Lmna^{G609G/G609G}$ (LMNA gene encodes Lamin A and C protein) and $Zmpste24^{-/-}$ (ZMPSTE24 gene encodes metallopeptidase which is involved in the processing of Lamin A), Hutchinson-Gilford progeria syndrome (HGPS) mice model from C57BL/6J background**.** Up arrows represent increased lifespan to methionine restriction compared to controls.

Responses of sulfur amino acids, H_2S production and oxidative stress to MR

Metabolism of sulphur amino acids

Methionine and cysteine are the major sulphur amino acids and are involved in a number of cellular functions. Between the two, methionine is indispensable meaning that it must be obtained from diet and it can be a precursor for other sulphur amino acids. Although, cysteine is not considered as indispensable or essential, the availability of cysteine can be limited when methionine is limited in the diet. Apart from being the first codon in polyamine synthesis, methionine has versatile functions that include but are not limited to regulating the immune system, digestive functions, lipid metabolism and antioxidant activation process [80]. While the typical methionine restricted diet is comprised of limited methionine and devoid of cysteine, it is assumed that pathways relevant to methionine metabolism will be affected and cells might prioritize the cellular processes which are dependant on sulfur amino acids.

Methionine reacts with S-adenosyl methionine transferase (MAT) and forms S-adenosyl-methionine (SAM) which is known as a global methylation agent. Through methyltransferase reactions, SAM is converted to S-adenosyl-homocysteine (SAH), which subsequently forms homocysteine. Homocysteine can be metabolised in two ways, either it can be reconverted to methionine by transmethylation/remethylation pathways or it can be degraded to cysteine via the transsulfuration pathway. Cysteine has multiple functions, i.e. it can be incorporated into proteins, glutathione synthesis or can be oxidized to serve as precursors for other pathways. The specific cysteine degradation pathway depends on dietary protein or sulfur amino acid content [81-83]. For instance, the cysteine oxidation pathway is barely detectable in liver when rats are fed a low protein diet and conversely is elevated in the presence of increased dietary protein or higher sulphur

containing amino acid content [81-83]. On the other hand, GSH synthesis increases in rat liver fed low protein diets and declines with high protein diets or enriched sulphur containing diets [81-83]. γ-glutamylcysteine-synthetase adds a γ-glutamyl moiety on cysteine and thus regulates the first step in glutathione (GSH) synthesis. Since, methionine restricted diet has limited methionine and no cystine, there is a possibility that cysteine availability as well GSH synthesis will be affected by methionine restriction.

Regulation of methionine metabolism pathways

Homocysteine is a branch point of methionine metabolism and whether homocysteine can be metabolised via transmethylation or transsulfuration, the favoured fate being directed by the cellular methylation potential. The ratio of SAM to SAH is an indicator of cellular methylation potential. An increased SAM:SAH ratio is associated with hypermethylation potential whereas a lower ratio indicates lower methylation potential [84]. To determine how cells preferentially shuttle flux to transmethylation over transsulfuration or vice versa, a mathematical model was proposed based on methionine metabolism in liver cells [85]. The proposed kinetic model is mostly predicted by the production and consumption of SAM at equllibrium and it was suggested that lower methionine decreases the SAM level and enhances the remeythlation process whereas higher methionine favours enhances SAM synthesis as well transsulfuration [85]. Cystathionine-β-synthase (CBS) is the regulatory enzyme of the transsulfuration pathway. At high methionine concentrations, SAM acts as a positive modulator of CBS whereas it inhibits the enzymes involved in the transmethylation pathways, i.e. methylenetetrahydrofolate reductase (MTHFR) and in low methionine concentration inverse events are observed [86, 87]. Therefore, it is evident that SAM plays a key role in both transmethylation and transsulfuration pathways. In addition, SAM is a

universal methyl donor which methylates a number of molecules, some of them are listed in the figure 1.3.

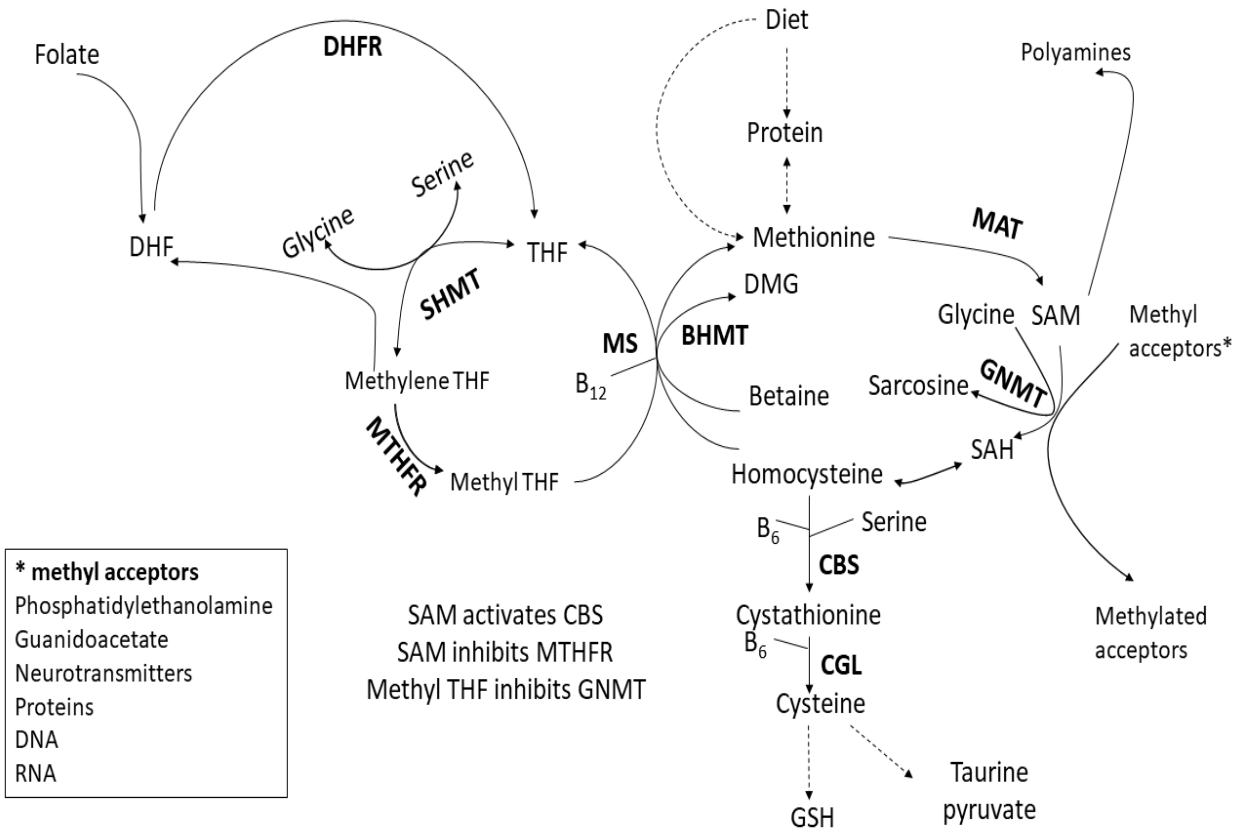

Fig. 1.3. Methionine metabolism pathways. Solid arrows represent a single step while dashed arrows represents multiple steps. Several intermediates, cofactors and enzymes are omitted for clarity.

Abbreviations: SAM, S-adenosyl-methionine; SAH, S-adenosyl- homocysteine; DMG-dimethyl glycine; methyl THF, N-5-methyl-tetrahydrofolate; methylene THF, N-5,10 methylene-tetrahydrofolate; THF, tetrahydrofolate; DHF, dihydrofolate; Enzymes: MAT, methionine adenosyltransferase; GNMT, glycine-N-methyltransferase; BHMT, betaine, homocysteine-methyltransferase; MS, methionine synthase; MTHFR-methylenetetrahydrofolate reductase; SHMT, serine hydroxymethyltransferase; DHFR, dihydrofolate-reductase; CBS,cysathionine-β-synthase; CGL, cystathionine-γ-lyase.

While the dietary methionine is high , SAM is synthesized rapidly and high SAM inhibits MTHFR and activates CBS. The activity of glycine-N methyl transferase (GNMT) and other methylation reactions also increase at high SAM levels. During low methionine intake, N-5-methyltetrahydrofolate concentration goes up and it inhibits GNMT to decrease SAM utilization [88].

Since, homocysteine metabolism is regulated via transsulfuration and transmethylaion pathways, impairment of those pathways can lead to hyperhomocysteinemia. Defective synthesis of N-5-methyltetrahydrofolate, vitamin-B6, B12 deficiencies or defective CBS all can lead to hyperhomocysteinemia [88]

Hydrogen sulphide producing pathways

Hydrogen sulphide (H_2S) was largely considered as a toxic gas for decades. In recent years, H_2S research is gaining attention because H_2S can be synthesized by animals cells [89] and both exogenous and endogenous H_2S seems to have beneficial aspects on delaying aging-related processes as well as in therapeutics (reviewed in [90, 91]). Substantial evidences demonstrated that H_2S can act as a vasorelaxant, antioxidant and also can regulate proinflammatory responses by suppressing NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation (reviewed in [90]). The potential mechanism of H_2S includes sulfhydration [92], thus preventing the exposed -SH group from oxidation. It has also been suggested that H_2S may induce the expression of *klotho* gene which supresses multiple aging phenotypes such as reducing oxidative stress by inducing the expression superoxide dismutase , repressing DAF-2 (insulin/IGF-like) receptors and prevention of angiotensin II-induced kidney damage (reviewed in [90]). The transsulfuration pathway enzymes CBS and CGL, along with another enzyme mercaptopyruvate-

sulfurtransferase (MPST) are the major known contributors to cellular H_2S production [89]. Although CBS and CGL produce H_2S, these are not exactly the same reactions involved in transsulfuration, rather a different set of reactions take place depending on the substrate availability [92] (possible H_2S producing pathways are illustrated in Fig. 1.4). The relative production of H_2S from these three enzymes depends on a number of factors, such as substrate availibility, tissue/organelle specificity of protein expression and also on physiological effects such as the availability of allosteric regulators of these enzymes. For instance, in rodents CBS and CGL proteins are more abundant in liver than in kidney and consistent with this expression pattern H_2S production was higher in liver [93]. Pathological states such as atherosclerosis induced by high fat diet induces the expression of CBS protein while supressing CGL and MPST expression in mice liver (reviewed in [94]). Collectively it can be suggsted that due to the limited availability of sulphur amino acids, dietary methionine restriction might alter the activity of H_2S producing enzymes.

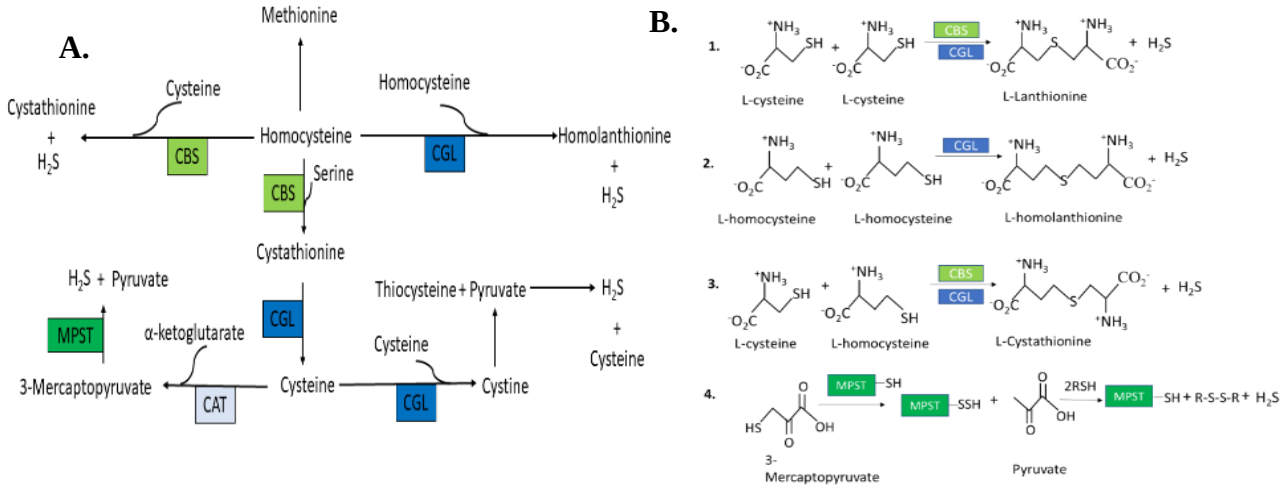

Fig. 1.4. H_2S producing pathways. A) H_2S can be produced from homocysteine and cysteine by cystathionine-β-synthase (CBS) and cystathionine-γ-synthase (CGL). H_2S can also be produced from 3-mercaptopyruvate (3-MP) by mercaptopyruvate-sulfurtransferase (MPST) where 3-MP is a product of cysteine-aminotransferase (CAT). Some reactants or products are omitted for clarity. **B)** Some of the H_2S producing reactions are provided with structures.

Effects of methionine restriction on transsulfuration and H_2S production

Elshorbagy *et al*. studied the effect of methionine restriction on its downstream sulphur containing amino acids using the same methionine restricted diet that extended the lifespan in F-344 rats [57]. They found that this diet decreases serum methionine, cystathionine, total cysteine and taurine levels but contrastingly increases total homocysteine [57]. Although it is debated whether hyperhomocysteinemia is associated with cardiovascular diseases [55], there is evidence that hyperhomocysteinemia is associated with the pathogenesis of Alzheimer's disease [95], schizophrenia [96] and bone decaying diseases [97]. If methionine restriction causes hyperhomocysteinemia in rodents, then the metabolic regulation causing hyperhomocysteinemia is not clearly documented but inferences from the known metabolic regulation allows for predictions to be made. Interruption of homocysteine metabolism through the transmethylation or transsulfuration pathways can elevate the homocysteine. Elevated expression of transmethylation proteins and metabolites suggest that the transmethylation pathway is not impaired by methionine restriction [74, 76]. Therefore, impairment of transsulfuration could be the possible reason in the elevation of homocysteine. Whether or not transsulfuration is impaired by methionine restriction needed to be investigated, some studies suggested that the rate limiting enzyme of the transsulfuration pathway, CBS, might have an association with longevity. Because, some studies reported that the diminished activity of CBS may enhance longevity or delay senescence in different species whereas others demonstrate the opposite. For instance, a decline in CBS induces premature senescence of human endothelial cells *in vitro* [98], which may promote aging. On the contrary, deletion of the human CBS orthologue in yeast, extends its life span [99]. The response of the transsulfuration pathway to methionine restriction become further complicated when Hine *et al*. demonstrated that several forms of diet restriction, including methionine restriction, that

extend lifespan in rodents are correlated with increased production of H_2S, which they speculated was through upregulation of the transsfuration pathway [100]. On the basis of the literature, it can be assumed that if methionine restriction induces hyperhomocysteinemia then elevated H_2S production should not be a result of increased transsulfuration capacity, rather it might be a by-product of other H_2S production pathways. Therefore, to underpin the mechanism of hyperhomocysteinemia and H_2S production further investigations are required.

Oxidative stress and antioxidants

Oxygen and hydroperoxyl radicals including superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and protonated superoxide or hydroperoxyl ($HO_2^{\bullet}$) are collectively called reactive oxygen species (ROS). Apart from these radicals some non radical oxidizing agents like H_2O_2, hypochlorous acid and ozone, which can readily form radicals, are also called ROS [101]. In mammalian cells, ROS originates from many different sources such as mitochondria, endoplasmic reticulum,

peroxisomes, the cytosol, plasma membrane and may also form in the extracellular space [102] (Fig 1.5).

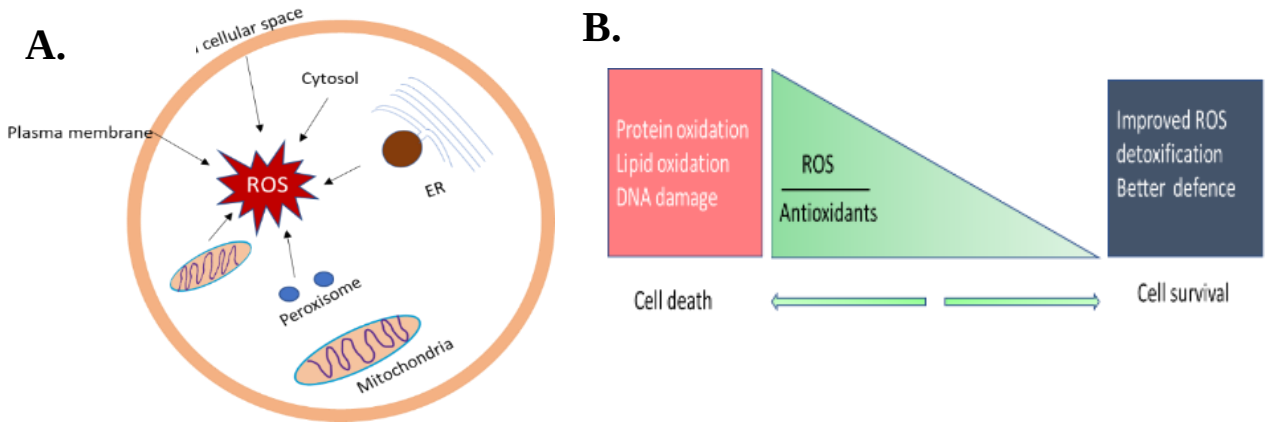

Fig. 1.5. Reactive oxygen species (ROS) production and oxidative stress. A) Mitochondria, endoplasmic reticulum, peroxisomes, cytosol, plasma membrane and extracellular space contribute for ROS production **B)** The increased ratio of ROS and antioxidants trigger oxidative stress whereas decrease ratio demonstrates a protected environment.

The continual formation of low levels of ROS is a part of normal oxygen metabolism [103, 104] which is necessary in cellular signalling [105]. Oxidative stress results due to an imbalance between the production of reactive oxygen species and a biological system's ability to detoxify ROS. The terminology of oxidative stress has been updated recently by Helmut Sies where he recommended that physiological oxidative stress or low levels of oxidative stress termed as 'oxidative eustress' because this is not harmful and that is used in redox signalling, whereas supraphysiological oxidative challenge causes damage to macromolecules causing 'oxidative distress' [106].

Some of the many oxidation damage products of proteins and lipids include protein carbonyl groups and lipid peroxidation respectively. When proteins are oxidized by ROS, carbonyl groups are produced on their side chain [107]. If the damaged proteins are not cleared by cellular systems then they accumulate and can cause certain pathophysiologies such as the death of neuronal cells and apoptosis along the spinal cord in mice [108]. The protein carbonyls are chemically stable, therefore these are convenient marker of oxidative stress [107]. Likewise, lipids get oxidized upon attack by ROS and those oxidized lipids also damage cells [109]. Therefore, these protein carbonyls and lipid peroxidation products can act as oxidative stress markers. To protect themselves from oxidative damage, cells maintain a reducing environment. Tissue thiols are important in maintaining a reducing environment and protecting cell from oxidative damage. Glutathione (GSH) and thioredoxins (Trxs) are considered as the major thiols to preserve this reducing environment [110, 111]. Mitochondrial ROS are mostly produced as superoxide, which is rapidly converted to H_2O_2 by superoxide dismutase [112]. The H_2O_2 from both mitochondria and other organelles is scavenged by catalase, GSH and Trx systems of which the later two are major thiol dependant antioxidants [45, 103]

Effect of methionine restriction on protein and lipid oxidation

Protein, lipid and DNA oxidation increases in tissues with increasing age [113]. A methionine restricted diet was able to reduce these damage markers in liver mitochondria obtained from old rats [114]. Methionine restriction also significantly reduces polyunsaturated double bonds (20:4n-6 and 22:6n-3) whereas it increases the number of a very few double bond containing fatty acids (18:0,18:1n-9 and 18:2n-6) or saturated fatty acids in mitochondria [115]. Decreases in polyunsaturated fatty acids (PUFA) may be beneficial in preventing lipid peroxidation because oxidants primarily attack the carbon-carbon double bonds on PUFA [116] and previous studies

demonstrated that long lived animals have low levels of PUFA in tissues and mitochondria, which might be associated with lower oxidative damage [117]. Although mitochondria are significant source, ROS can also be produced from some other organelles (Fig. 1.5) which also can damage tissue. It remains unclear whether low oxidative damage is limited only to mitochondria or it is also true for the whole tissue level in methionine restricted animals.

Methionine restriction and the glutathione system

Glutathione is one of the major endogenous antioxidants which neutralize free radicals and scavenge reactive oxygen compounds. It is a tri-peptide consisting of glutamate, cysteine and glycine. Since the precursor to cysteine, methionine is limited in methionine restricted diets, it is not surprising that GSH levels may decline in tissues [28, 50]. However, responses are varied in tissues. Total GSH declined in liver, kidney and pancreas of rats on a methionine restricted diet, whereas it did not change in heart and brain tissues [28, 50]. Despite the decrease in other tissues, GSH was elevated in erythrocytes but not in serum [28, 50]. It is important to note that despite a reduction of GSH content in different tissues, mitochondria from these methionine restricted rodents produce less reactive oxygen species [114]. Mitochondria do not have the enzymes to synthesize their own GSH, thereby they rely on cytosolic GSH. It is expected that low cytosolic GSH will affect the mitochondrial GSH pool.

Methionine restriction and thioredoxin system

The thioredoxin system consists of peroxiredoxins, thioredoxin reductase and thioredoxin proteins and participates in many cellular processes. Some of the major functions are to provide reducing equivalents to enzymes such as ribonucleotide reductase [118]. Thioredoxins keep certain transcription factors reduced by donating electrons to cysteine residues and they also induce cell growth and inhibit apoptosis [119, 120]. Beyond this, over expression of thioredoxins in transgenic

mice was found to increase longevity [121]. Telomerase activity, which is suggested to be another determinant of longevity in mammals, was higher in spleen tissues in thioredoxin overexpressing mice compared with wild-type mice [121]. Beyond this, the thioredoxin system may be an alternate source to recycle oxidized glutathione into reduced GSH form, when the GSH recycling system is perturbed [122, 123]. Therefore, there might be a possibility that the thioredoxin system activity will go up in liver tissue in response to low GSH due to methionine restriction.

Mitochondrial electron transport chain

The mitochondrial electron transport chain comprises a series of electron transfer reactions which sequentially move electrons throughout the complexes which are finally received by O_2 (Fig. 1.6). During the transfer of electrons through complex I, III and IV, protons are pumped from the matrix into the intermembrane space, as a result the matrix becomes negatively charged while the intermembrane space becomes positively charged. The proton transfer process generates a proton electrochemical gradient which is called the protonmotive force. This force has two components, chemical potential energy and electrical potential energy which is termed as membrane potential. Protons can return to the matrix via a proton pore-associated with ATP synthase therefore driving conversion of ADP and inorganic phosphate into ATP synthesis, which is termed as oxidative phosphorylation or coupling phosphorylation.

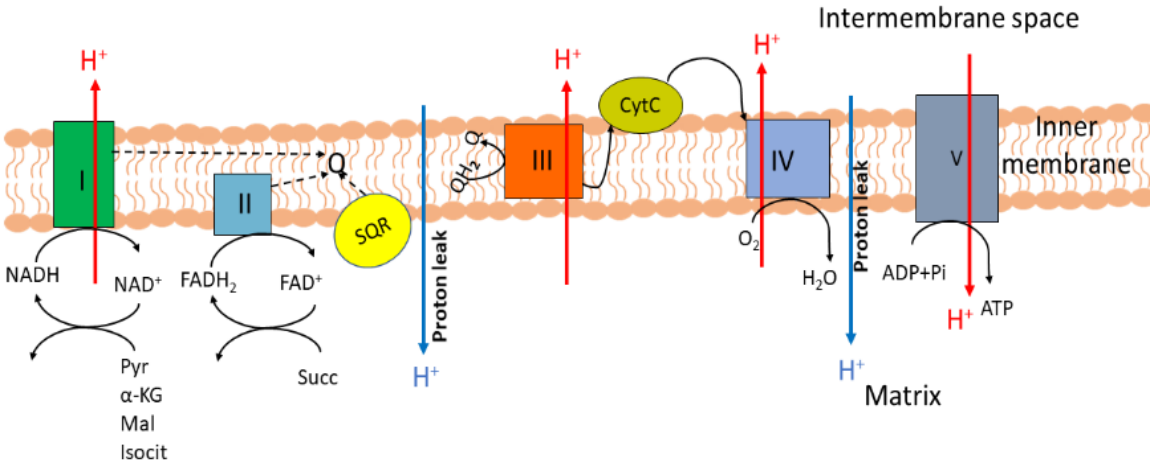

Fig. 1.6. Mitochondrial electron transport chain. Complex I and complex II receive electrons from NADH and $FADH_2$ producing substrates respectively. NADH producing substrates pyruvate (pyr), α-ketoglutarate (α-KG), malate (mal), isocitrate (isocit) and $FADH_2$ producing substrate, succinate (succ). Ubiquinone (Q) has binding sites in complex I, II and sulfur quinone reductase (SQR). Upon receiving electrons from these complexes Q becomes reduced. Reduced Q (QH_2) binds to complex III and from there cytochrome C (Cyt C) passes electrons to molecular O_2 to form H_2O. During the transfer of electrons complex I, II and IV pump out protons from matrix into intermembrane space and these protons are harvested for ATP synthesis. Some protons return to the matrix without ATP synthase causing a proton leak. Here red arrows and red H^+ indicate protons pumping via respiratory complexes whereas blue arrows and blue H^+ indicate proton leak.

Proton leak and ROS production

Protons can return into the matrix independent of ATP synthase, and when not associated with other direct transport processes is collectively called proton leak. Electron leakage can also occur when electrons bypass the respiratory complexes and directly react with molecular O_2. The reduction of dioxygen by a single electron generate superoxide ($O_2^{\bullet-}$). A complex relationship exists between proton leak and electron leak because proton leak decreases protonmotive force which can decreases ROS production [124]. If the protonmotive force elevates at a faster rate, respiration rate cannot cope with the increase, and as a result respiration slow downs which sequesters electrons on the enzyme complexes instead of transferring the electrons sequentially through the respiratory complexes to molecular oxygen. This in turn increases the possibility of reducing O_2 prematurely and thus increases ROS. Conversely, while mild proton leak decreases the protonmotive force, it no longer faces resistance in transferring electrons through the respiratory complexes and electrons are transferred sequentially to O_2. Thus, mild proton leak could help in the ROS regulation in isolated mitochondria [125]

Role of H_2S in mitochondrial bioenergetics

The localization of three H_2S producing enzymes, cystathionine-β-synthase, cystathionine-γ-lyase and mercaptopyruvate sulfurtransferase are varied in tissues and also in organelles [126]. Prior to the identification of MPST in mitochondria [127], it was assumed that mitochondria are net consumer of cytosolic H_2S obtained from CBS and CGL rather than potentially being a source of H_2S [128]. Although CBS and CGL are primarily considered cytosolic in certain situations they were reported in mitochondria. For instance, mitochondria obtained from smooth muscle cells localizes CGL at the outer membrane of mitochondria while the cells are treated with stressors, i.e. A23187 (Ca^{+2} ionophore) [129]. In another study, although CGL was not found in liver mitochondria, CBS has been reported in isolated liver mitochondria and its expression decreased but did not disappear when treated with partial trypsin digestion [130]. Of note, *in vitro* trypsin treatment can generate mitoplast, a mitochondrion without an outer membrane [130]. In the same study, MPST expression in liver mitochondria did not change with trypsin treatment [130]. This suggests that MPST is more likely to be localized in the matrix, but there might be other enzymes which can also produce H_2S in liver mitochondria.

It is well established that H_2S inhibits the activity of cytochrome-c oxidase (complex IV) which is the terminal electron acceptor of the mitochondrial respiratory chain [131, 132]. However, studies have demonstrated that H_2S displays a double-edged pattern with the mitochondrial electron transport chain, being capable of both stimulatory and inhibitory action depending on the concentration [129, 133]. For instance, addition of an exogenous source of H_2S (0.1 μM-1 μM NaHS) stimulates respiration in liver mitochondria but respiration was inhibited by higher concentrations (3-30 μM) of NaHS [133]. Low doses of 3-mercaptopyruvate (3-MP) (10 μM) also

stimulates respiration whereas high doses (30-1000 μM) inhibits respiration [133] which suggests that 3-MP serves as a H_2S producing source via MPST and therefore follows the similar pattern as observed with a H_2S liberating salt. When ruptured mouse brain mitochondria are incubated with a chemical reductant dithiothreitol or biological reductants like dihydrolipoic acid or recombinant thioredoxin in the presence of 3-MP, H_2S is formed [134]. However, incubation of 3-MP with GSH, cysteine or NADPH does not produce H_2S in mouse brain mitochondria [134] suggesting dihydrolipoic acid and thioredoxin would be the possible biological reductant in releasing H_2S *in vivo*.

Overview of research chapters

In this thesis, we addressed thiol metabolism in rats. To do so, we have designed three primary research projects each of which contributes to a research chapter. The investigations carried out in three projects are summarized here under each research chapter.

Chapter two

This chapter investigates how methionine restriction induces hyperhomocysteinemia and enhances H_2S production capacity simultaneously. To understand the mechanisms, we tested the hypotheses that reduced cystathionine-β-beta synthase activity in liver induces hyperhomocysteinemia and whether the limitation of sulphur amino acid intake enhances compensatory mechanisms other than transsulfuration to contribute toward H_2S production in methionine restricted rats. To investigate these hypotheses, Fischer-344 rats were fed with AIN-76A formulated semi-purified control and diets for 8 weeks.

Chapter three

In this chapter enzymatic GSH synthesis capacity, antioxidant function and oxidative stress were explored in F-344 rats. It was hypothesized that methionine restriction decreases liver GSH due to impaired enzymatic synthetic capacity and we also tested whether methionine restriction decreases oxidative damage in liver tissue and the mitochondrial compartment by activating other antioxidants.

Chapter four

Mitochondrial H_2S producing enzyme(s) and their role in respiration were investigated in this chapter. To investigate the mechanisms of H_2S production, liver mitochondria were isolated from Sprague-Dawley rats. It was hypothesized that liver mitochondria have more than one H_2S producers and it was tested what was the major H_2S producer in rat liver mitochondria. It was found that MPST has a greater capacity to produce H_2S than CBS and CGL, therefore it was pharmacologically tested whether thioredoxin or dihydrolipoic acid are the possible biological reductant which release H_2S from MPST as well as stimulate mitochondrial respiration.

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Chapter Two: Methionine restriction leads to hyperhomocysteinemia and alters hepatic H_2S production capacity in Fischer-344 rats

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Nahid Tamanna[a], Shyamchand Mayengbam[b], James D. House [b], Jason R. Treberg[a,b,c]

[a] Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada.

[b] Department of Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada.

[c] Centre on Aging, University of Manitoba, Winnipeg, MB, Canada.

Author contribution: NT and JRT designed the experiments, JDH and SM helped in designing biochemical assays, NT did the experiments and analyzed the data, JRT and NT prepared the manuscript, JDH and SM edited the manuscript

Abstract

Dietary methionine restriction (MR) increases lifespan in several animal models. Despite low dietary intake of sulphur amino acids, rodents on MR develop hyperhomocysteinemia. On the contrary, MR has been reported to increase H_2S production in mice. Enzymes involved in homocysteine metabolism also take part in H_2S production and hence, in this study, the impact of MR on hyperhomocysteinemia and H_2S production capacity were investigated using Fischer-344 rats assigned either a control or a MR diet for 8 weeks. The MR animals showed elevated plasma homocysteine accompanied with a reduction in liver cysteine content and methylation potential. It was further found that MR decreased cystathionine-β-synthase (CBS) activity in the liver, however, MR increased hepatic cystathionine-γ-lyase (CGL) activity which is the second enzyme in the transsulfuration pathway and also participates in regulating H_2S production. The relative contribution of CGL in H_2S production increased concomitantly with the increased CGL activity. Additionally, hepatic mercaptopyruvate-sulfur-transferase (MPST) activity also increased in response to MR. Taken together, our results suggest that reduced CBS activity and S-adenosylmethionine availability contributes to hyperhomocysteinemia in MR animals. Elevated CGL and MPST activities may provide a compensatory mechanism for maintaining hepatic H_2S production capacity in response to the decreased CBS activity.

Keywords: methionine restriction, longevity, methylation potential, transsulfuration, sulfur amino acids, H_2S

Introduction

Manipulation of dietary regimen has been widely applied to evaluate the influences of diet on longevity and the processes of aging. While restriction of total calories receives a great deal of attention [1-3], restriction of specific macronutrients has also been extensively investigated. In rats, restriction of carbohydrate or lipid intake may have limited effects on lifespan relative to isocaloric controls, whereas protein restriction can increase lifespan equivalent to some caloric restriction studies [4]. However, it appears that protein restriction may operate via a different mechanism than caloric restriction because the increase in lifespan seen with protein restriction occurs with *ad libitum* feeding [3]. Targeting specific amino acids has demonstrated that when methionine is the sole source of sulfur amino acids in the diet, then restriction of dietary methionine can enhance lifespan in yeast, fruit-fly, and rodents [5-8]. Cysteine supplementation of rodents on a methionine restriction (MR) diet reverses the phenotype of MR [9, 10], suggesting that the effects exerted by MR are either due to absolute sulphur amino acid limitation in the diet or a metabolic insufficiency to convert enough methionine to downstream metabolites like cysteine which is required for the synthesis of important metabolites like glutathione [11].

Despite the low dietary intake of sulfur amino acids, MR rodents have been reported to develop hyperhomocysteinemia [12, 13] which is a downstream metabolite of methionine metabolism. Although hyperhomocysteinemia is a known risk factor of cardiovascular and neurological diseases [14, 15], there is an ongoing debate in favour of the concept that homocysteine might not be directly related to cardiovascular disease rather it might be an indicator of unhealthy condition which can lead to cardiovascular disease [16]. Therefore, hyperhomocysteinemia in MR animals is in apparent discord with the lifespan increasing effect of MR. Homocysteine is a metabolic

intermediate between methionine and cysteine, therefore elevation of homocysteine levels with high methionine intake is expected [17]; however, hyperhomocysteinemia with the limited dietary intake of sulphur amino acids in a MR diet seems to conflict with conventional expectations [18].

Further complicating understanding of how MR may alter metabolism, recently it was hypothesized that increased flux in the pathway that converts homocysteine to cysteine was associated with the elevated H_2S production in several animal models of increased longevity by dietary restriction, including MR in rodents [19]. Elevated H_2S is thought to be beneficial because this molecule may delay aging processes and reduce age-associated diseases by mutltiple pathways such as reducing oxidative stress, activating the SIRT1(Sirtuin 1) pathway, and also by inducing the expression of the aging associated "klotho" gene which supresses multiple age associated phenotypes. For instance, it is considered that the oxidative stress increase with aging and "klotho" gene alters aging phenotypes such as increasing the expression of the antioxidant enzyme superoxide dismutase to enhance resistance towards oxidative stress [20]. In addition, "klotho" is known to repressing DAF-2 (insulin/IGF-like) receptors to modulate lifespan and also preventsion of angiotensin II-induced kidney damage [20]. While an elevated capacity to transform homocysteine to cysteine might enhance H_2S production, increased metabolic transformation of homocysteine to cysteine is inconsistent with the observation of hyperhomocysteinemia with MR in rats.

To place the interrelationships between the metabolic responses to a MR diet and the conversion of methionine to its downstream metabolites in context with H_2S production, it is important to appreciate the regulation of homocysteine metabolism (Fig. 2.1). In rats, both the liver and kidney play a major role in metabolising homocysteine, and both organs possess the enzymes necessary

for homocysteine synthesis from methionine [21, 22]. Homocysteine is synthesized from methionine via S-adenosyl-methionine (SAM) and S-adenosyl-homocysteine (SAH) as intermediates. Initially, SAM is formed from methionine by the action of methionine-adenosyl-transferase and SAM then is converted to SAH during methylation reactions. The reversible enzyme S-adenosylhomocysteine-hydrolase interconverts SAH and homocysteine. Once formed, homocysteine can have one of the two fates, either remethylation to methionine or conversion to cysteine via the transsulfuration pathway. The switching between transmethylation and transsulfuration is triggered by low and high methionine content respectively [23]. Furthermore, the control over flux between the transmethylation of homocysteine and the transsulfuration to cysteine is elicited by the availability of SAM [23]. High levels of SAM can act as an inhibitor of methionine-tetrahydrofolate-reductase in the remethylation process and as a positive modulator of cystathionine-β-synthase (CBS), which condenses homocysteine and serine to cystathionine and subsequently forms cysteine via transsulfuration [24]. Homocysteine is first converted to cystathionine by CBS. Cystathionine is further converted by cystathionine-γ-lyase (CGL) to produce cysteine.

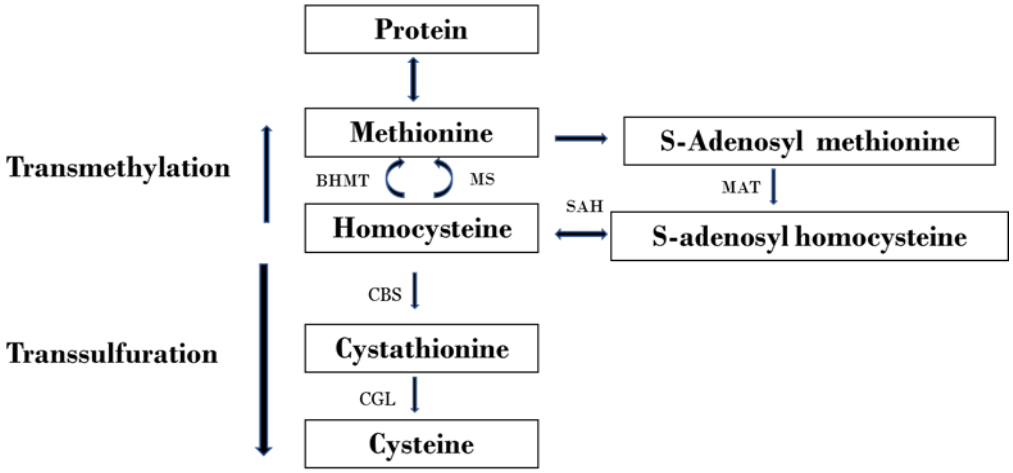

Fig. 2.1. Schematic diagram of transmethylation and transsulfuration pathways. MAT-methionine adenosyl transferase, MS-methionine synthase, BHMT- betaine homocysteine methyl transferase, SAH- S-adenosylhomocysteine hydrolase, CBS-cystathionine-β-synthase, CGL- cystathionine-γ-lyase.

If MR leads to hyperhomocysteinemia in rodents then the metabolic regulation leading to the increased homocysteine levels is not clear. Expression of hepatic betaine-homocysteine-methyltransferase and methionine-tetrahydrofolate-reductase proteins were elevated and CBS protein content was reduced by MR [10], supporting a shift towards remethylation being favoured over transsulfuration. Although the supplementation of cysteine in MR diet decreased the expression of betaine-homocysteine-methyltransferase and methionine-tetrahydrofolate-reductase, it could not restore CBS protein levels [10] suggesting that CBS is regulated by some other factor(s) not obtained via cysteine supplementation. Cystathionine-β-synthase is allosterically regulated by SAM [25] and there is evidence that SAM also enhances the stability of CBS [26]. Unlike methionine, cysteine lacks a methyl group, thereby adding cysteine in the MR diet may fail to change the SAM levels and thus possibly could not restore CBS function reported by previous studies [10].

Along with converting homocysteine to cysteine, CBS and CGL are also major sources for production of H_2S [27]. It is important to note that H_2S formation is not simply a by-product of the transsulfuration reaction, rather the same transsulfuration enzymes can produce H_2S via a different series of reactions with overlapping substrates and products [28]. Increased transsulfuration may not necessarily increase H_2S production, as cysteine derived via the transsulfuration pathway may end up in synthesizing glutathione or taurine [28]. The relative contrbitution of CBS and CGL vary depending on the concentration and availibity of substrates as well as the influence of allosteric activator(s). For example, at high subsrate concentration (20 mM cysteine and 20 mM homocysteine), CBS and CGL produce H_2S at similar rates, however, at physiologically relevant concentrations of these substrates (100 μM of cysteine and 10 μM of homocysteine), CBS only accounts for approximately 3% of total H_2S production in rodent liver [29]. Besides, CBS derived H_2S production decreases while CGL derived H_2S production increases under conditions mimicking *in vivo* simulated hyperhomocystemic conditions [30] emphasizing that CGL may be more important to H_2S production during MR. Beyond the enzymes of the transulfuration pathway, mercaptopyruvate-sulfur-transferase can also produce H_2S from 3-mercaptopyruvate, which is formed from cysteine and α-ketogluterate by cysteine aminotransferase [27]. These three major H_2S producing enzymes may respond differently towards a physiological condition; for example, mercaptopyruvate-sulfur-transferase is sensitive to oxidative stress [31] while CGL is not [32]. Alternatively, high-fat-diet increased the expression of CBS protein in rat liver while it decreased the translation of CGL and mercaptopyruvate-sulfur-transferase protein [32].

The observed elevation in homocysteine levels with MR [12], which would be consistent with decreased transsulfuration flux, appears to be in conflict with the suggested enhanced

transsulfuration capacity reported to increase H_2S production concomitant with MR in rodents [19]. The current study addresses this apparent conflict by focusing on two major questions: First, how does MR alter homocysteine metabolism? Second, in what way does MR lead to altered enzymatic H_2S production capacity in rodents? Given that, MR appears to favour the transmethylation pathway [10, 33, 34], in the current study we focused on the transsulfuration pathway to explore shifts associated with homocysteine metabolism and H_2S production capacity. First, we have confirmed hyperhomocysteinemia and then we have estimated SAM, SAH, homocysteine and cysteine levels to investigate key intermediates of the transsulfuration pathway and the cellular methylation potential. We also measured the activity of CBS, CGL and the transsulfuration mediated H_2S production capacity. Finding changes in response to MR, we also evaluate mercaptopyruvate-sulfur-transferase activity to see if this pathway of H_2S production may also respond to MR. We found that, despite evidence for reduced transsulfuration flux, MR increases the H_2S production capacity from CGL and mercaptopyruvate-sulfur-transferase activity, emphasizing that increased H_2S production capacity may be a core component to this lifespan enhancing dietary treatment.

Materials and methods

Animals and experimental design

All studies were approved by the University of Manitoba Animal Care Committee (protocol-F2013-036). Five-week-old Fischer-344 (F-344) male rats (n=45) were purchased from Charles River Laboratories (Quebec, Canada) and housed in an air-conditioned environment (22±1°C, 12h light-dark cycle) at the Department of Biological Sciences Animal Holding Facility. Rats were initially kept in shoebox cages and after one week they were individually housed in wire bottom

cages to measure food consumption throughout the experiment. For two weeks, rats were gradually acclimatized with commercial rodent chow diet and control diet pellets (AIN-76 formulated semi purified diet from Research Diet, New Brunswick, New Jersey, USA). At the age of seven weeks, rats were randomly assigned to a MR or control diet [5]. The diet consisted of the following (in percentage of diet by mass): L-arginine 1.12, L-lysine 1.44, L-histidine 0.33, L-leucine 1.11, L-isoleucine 0.82, L-valine 0.82, L-threonine 0.82, L-tryptophan 0.18, L-phenylalanine-1.16, L-glycine-2.33, corn starch 43.61, sucrose 20.00, solca floc 5.00, choline bitartrate 0.20, vitamin mix 1.00, mineral mix 3.50, corn oil 8.00 and dextrin 5.00. The control diet had 0.86% methionine as the sole source of sulfur amino acids whereas in the MR diet this was reduced to 0.17% as in past studies demonstrating the longevity impacts of MR in this strain of rat [5, 6]. To compensate for the reduced amount of methionine, glutamic acid content was increased (from 2.70% to 3.39% in controls and MR respectively). Food and water were provided *ad libitum*. During the experiment, food consumption was measured twice a week by calculating the food left in the cage and the amount of spillage in the cage tray. Rats were weighed twice weekly throughout the study period. Rats were sacrificed after 4 weeks or 8 weeks using isoflurane as an anaesthetic. Once anaesthetized, arterial blood was collected using a heparinized syringe and centrifuged at 5500 × g for 5 minutes to separate plasma and red blood cells. Following blood collection, liver and kidney were collected and flash frozen in liquid nitrogen. Frozen liver and kidney samples were pulverized using a mortar and pestle, chilled with dry-ice to facilitate subsampling without freeze-thaw cycles. All samples were stored at -80°C until analyzed.

Biochemical assays

Cysteine and homocysteine

Plasma total cysteine and homocysteine were measured by UHPLC with fluorescence detection

[35, 36]. Briefly 150 µL of plasma was incubated with 10% (w/v) of 20 µL tris-carboxyl-ethyl-phosphine to give a final volume 170 µL. After 30 minutes of incubation at room temperature proteins were precipitated by addition of 125 µL 0.6 M of perchloric acid. Extracts were then centrifuged at 10,000 × g for 10 minutes to remove precipitated protein. An aliquot of the supernatant was mixed with a double volume of potassium borate buffer (pH-10.5) and an equal volume of ammonium 7-fluoro-benzo-2-oxa-3-diazole-4-sulfonate (1mg/mL) followed by an incubation at 60°C for 1 hr to produce fluorescent derivatives of the reduced thiols. Derivatized samples were then cooled to 4°C, filtered with a 0.2 µm syringe filter and either directly analyzed or stored in -20°C until analysis. For liver, kidney and red blood cell analysis, samples were initially homogenized with 10 volumes of 50 mM of imidazole buffer. Following homogenization, samples were processed as the same way as the plasma. All analyses were conducted with a Dionex ultimate 3000 UHPLC system and injections were resolved using an Acclaim C-18 reversed phase column (3 µm particle size, diameter 4.6 mm, 100 mm length) and eluted isocratically with a mobile phase containing 95% of 0.1M potassium phosphate buffer (pH-6.5) and 5% methanol.

SAM-SAH assay

SAM and SAH were analysed from freeze-clamped tissues by UHPLC using UV detection [37]. Briefly, liver and kidney tissues (500 mg) were mixed 1:2 (w/v) with ice cold 0.1M sodium acetate (pH 6.0) and homogenized on ice using an IKA T25 Ultraturrax homogenizer. Following homogenization, 750 µL of 40% of trichloroacetic acid (w/v) was added. The samples were then centrifuged for 5 minutes at 5,900 × g at 4°C. The supernatant was collected and washed with 1 mL of petroleum ether to extract the of trichloroacetic acid from the sample and this process was

repeated three times. Samples were stored in -20°C until analysis. Following filtering with 0.2 µm syringe filter, SAM and SAH were eluted using gradient mobile phase containing solvent A (25 mM sodium monophosphate with 10 mM 1-heptanesulfonicacid (pH-3.2) and Solvent B (Methanol containing 10 mM 1-heptanesulfonic acid) using a C-18 (3 µm particle size, diameter 4.6 mm, 100 mm length column). SAM and SAH were detected directly based on absorbance at 254 nm.

CBS activity

CBS activity was assessed in liver using a radio-isotopic method [38]. Liver was homogenized with 10 volumes of 50 mM imidazole buffer (pH-7.4) and stored on ice. In a 2 mL centrifuge tube a reaction mixture containing, 0.15 mM L-cystathionine, 41.67 mM DL-homocysteine, 0.32 mM SAM and 2.08 mM DL-propargylglycine were added to make a final volume of 500 µL, followed by 150 µL of homogenized liver sample. Then the mixture was incubated for 3 minutes at 37°C. Following the preincubation, reactions were started by adding serine (mixture of unlabelled serine and ^{14}C-serine (ubiquitously labelled) at a final concentration of 25 mM and a specific activity of 0.035 µCi/µmol in the reaction mixture). Then the mixture was vortexed and incubated at 37°C for 1 hour. The reaction was stopped by adding 300 µL of 15% of trichloroacetic acid and the reaction mixture was transferred to 2 mL of prepacked analytical grade 50W-X8 resin, 200–400 mesh, 63–150 particle size column (Bio-Rad). The column was initially washed twice with 4 mL of water followed by removal of unreacted serine by adding 6 volumes of 4 mL of 1N HCl. Following removal of serine, the column was washed with 4mL of water for four more times. Finally, radio-labelled cystathionine was eluted with 5 mL of 3N ammonium hydroxide. A 1 mL subsample was

taken from the eluent and mixed with 1 mL of scintillation fluid and vortexed. Radioactivity of the subsample was determined using a Perkin Elmer scintillation counter (Model-Tri-Carb 3110 TR).

CGL assay

The activity of cystathionine-γ-lyase was assessed spectrophotometrically via oxidation of NADH by lactate dehydrogenase in presence of cystathionine [39]. In brief, phosphate buffer (pH 7.5, 0.04 M), L-cystathionine (4 mM), NADH (0.32 mM) and lactate dehydrogenase (1.5 U) were added in a cuvette to give a final volume of 1 mL. The liver sample was homogenized with 10 volumes of 0.05 M potassium phosphate buffer (pH 6.8) and then the assay was started by adding the homogenized liver sample. The decline in absorbance at 340 nm was monitored at 37°C for 20 minutes and it was linear over the duration of the assay. Reaction blank cuvettes were prepared in the same way except cystathionine was omitted from the reaction mixture. Activity was determined assuming a molar extinction coefficient of 6220 M^{-1} cm^{-1} for NADH and blank values were subtracted from rates in the presence of cystathionine.

MPST activity

Liver was homogenized with 10 volumes of 0.1 M potassium phosphate buffer (pH 7.4, at 25°C). In a 1.5 mL micro centrifuge tube, 0.12 M sodium phosphate buffer (pH-8), 1.0 M sodium sulfite, 0.1 M dithiothreitol, 60 µL sample and 60 µL of H_2O were added to make a final volume of 400 µL. The reaction mixture was incubated for 3 min at 37°C. Following this step, 40 µL of 0.1 M sodium-mercaptopyruvate was added in the reaction mixture and was incubated at 37°C for 15 min and for the substrate-free control rate everything except sodium-mercaptopyruvate was

included during incubation and the control rates were subtracted from the sodium-mercaptopyruvate added sample during calculation. The reaction was carried out at 37°C for 15 min. The reaction was stopped by adding 200 μL of 1.2 M perchloric acid in both samples and blank tubes. Following termination of the assay with perchloric acid, sodium mercaptopyruvate was added in the blank sample. All tubes were kept on ice for 5 mins to precipitate protein, followed by being centrifuged for 10 min at 10,000 × g. The supernatant was collected and assayed for pyruvate. Pyruvate content was measured via the following assay condition: 0.12 M disodium phosphate, N-ethylmaleimide, 0.1 M NADH (approx. 0.3 mM), perchloric acid filtrate 0.2 ml in a total volume of 2.9 mL. After equilibration at 37°C, 5~10 μL of lactate dehydrogenase (7-8 IU) was added and the decrease in absorbance was measured spectrophotometrically at 340 nm. Pyruvate content was measured using a molar extinction coefficient of 6220 $M^{-1}cm^{-1}$ for NADH [40].

H_2S production capacity

Liver H_2S production capacity was measured by adding the substrates of CBS and CGL as described in [27]. To differentiate the contribution of CBS or CGL to H_2S production, propargylglycine was used as an inhibitor of CGL. In brief, liver was homogenized in 10 volumes of 50 mM potassium phosphate (pH 6.9) buffer and centrifuged at 15000 × g for 30 min at 4°C. Following centrifugation, 300 μL of supernatant was added to a 25 mL Erlenmeyer flask which was then flooded with N_2 gas for 20 sec and immediately capped with a septum stopper containing a hanging centre-well. The centre-well contained a piece of filter paper soaked in an alkaline zinc acetate solution (15:1; 1% (w/v) zinc acetate:12 % (w/v) NaOH). Erlenmeyer flasks were preincubated in a shaking water bath for 3 min at 37°C followed by the addition of reaction mixture

containing (final concentrations) 10 mM cysteine, 10 mM homocysteine, 2 mM pyridoxal phosphate and 0.05 mM of SAM (all made in 100 mM, pH 7.4 potassium phosphate buffer solution) which were injected through the septum stopper in the Erlenmeyer flask. In reaction blanks, substrates were omitted but the same volume of 100 mM potassium phosphate buffer was added. After 30 min of incubation at 37°C in a shaking water bath the reaction was stopped by injecting 500 μL of 30% (w/v) trichloroacetic acid. Flasks were left at room temperature for 1 hr to collect the remaining H_2S. Filter paper strips were then placed in a test tube containing 3.5 mL of H_2O. Color formation reagent, consisting of 0.4 mL 20 mM N,N–dimethyl-p-phenylene diamine sulphate (dissolved in 7.2 M HCl) and 0.4 mL of 30 mM $FeCl_3$ (dissolved in 1.2 M HCl) were added, tubes were mixed and the reaction was carried out for 10 min in the dark. Following incubation with color formation reagent, the absorbance at 670 nm was measured. Sodium hydrosulfide was used as a standard for H_2S detection and concentrations between 2.5 to 100 μM were used as the standards [27].

Protein assay

Protein concentration of homogenates were analyzed by using Pierce BCA protein assay kit (Rockford, Illinois, USA) using bovine serum album as a standard.

Statistical Analysis

Data were analysed using JMP13 statistical software. A Shapiro-Wilks test was performed to check the normality of the data and log transformed when needed. A generalized linear model (GLM) was used to analyse the interaction between diet and week which was followed by Tukey's post-hoc (parametric) or Steel-Dwass test (non-parametric) when the data failed the assumption of normality. $p < 0.05$ was considered as significantly different. Data are presented as mean ± 1

standard error of the mean (SEM).

Results

Food consumption and growth rate

After the initial transition to the MR diet, rats lost a small amount of mass (~2.5%), followed by a period where body mass stabilised and then increased. The MR animals were smaller than control animals throughout the experiment. Both the control and MR animals gained mass during the experiment, but the growth was very low in MR compared to controls (Fig. 2.2A). Being smaller, MR animals consumed less food than controls on an absolute basis (Fig. 2.2B), which is comparable to previous studies [6, 41]. However, consistent with past studies, MR rats consume more food on a mass-specific basis (Fig. 2.2C).

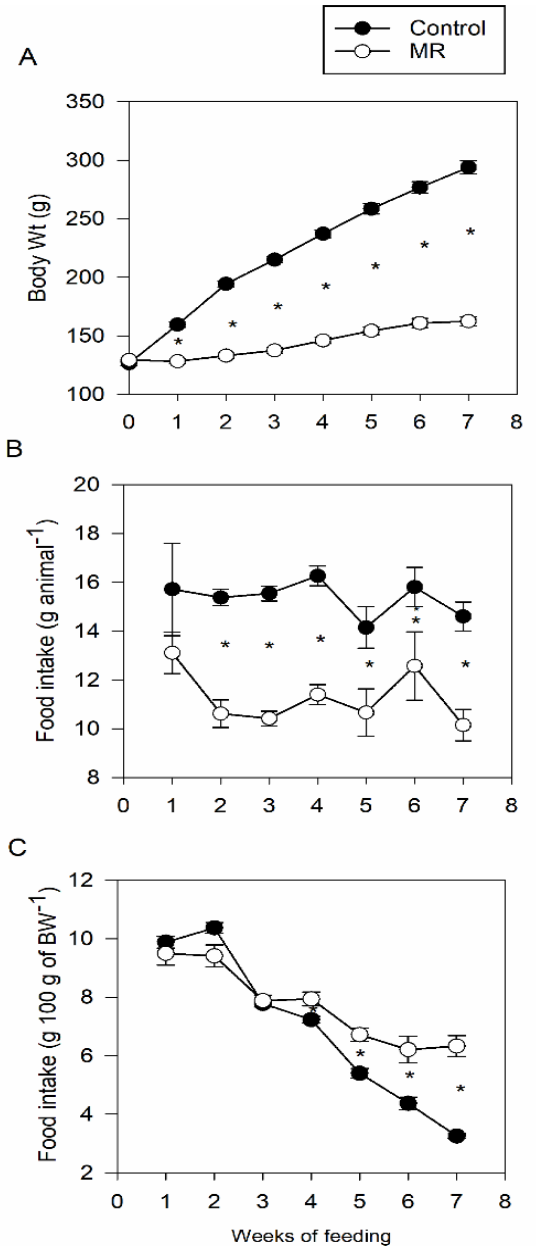

Fig. 2.2 Food consumption and growth of F-344 rats. A) MR animals grew less compared to controls **B)** On average body weight (BW) MR animals consume less food than controls **C)** MR animals consume more food on a mass-specific basis. Initially 28 rats were fed with control and MR diets, after 4 weeks some rats were sacrificed, and the remaining were on diet for the 8 weeks. Data are presented as mean±SEM (*, $p < 0.05$; One-way ANOVA).

Response of homocysteine and cysteine levels to methionine restriction

Homocysteine and cysteine in blood

Plasma homocysteine increased in rats on the MR diet after 8 weeks, although following 4 weeks of MR there was no evidence for hyperhomocysteinemia relative to control animals (*p = 0.61)* (Table 2.1). It is important to note that both control and MR rats had high plasma homocysteine compared to chow fed Fischer-344 rats weighing approximately 200 g (4.11±1.84 μmol L^{-1}, n=5). In the red blood cells, the MR group also displayed elevated homocysteine levels after 8 weeks (Table 2.1). For both plasma and red blood cells, the cysteine levels did not differ between treatments (Table 2.1). However, compared to the 4 week sample the cysteine increased in red blood cells after 8 weeks in both control and MR animals (Table 2.1).

Homocysteine and cysteine in liver and kidney

Liver homocysteine levels did not change between treatments over the entire experiment (Table 2.1). In the kidney, MR led to increased kidney homocysteine at both 4 and 8 weeks (Table 2.1). Liver cysteine levels decreased after 8 weeks of dietary MR (Table 2.1), whereas kidney cysteine levels remained relatively constant at both time periods and between MR and control animals (Table 2.1). The kidney cysteine and homocysteine contents were much higher than in liver (Table 2.1).

Table 2.1: Homocysteine and cysteine levels in Fischer-344 rat tissues

Tissue	Metabolite	4 Weeks		8 Weeks	
		Control	MR	Control	MR
Plasma	homocysteine	35.2±6.9	41.8±5.5	36.3±5.6	87.2±4.5^{*a}
	cysteine	90.8±14	96.4±19	208±37	122±11
RBC	homocysteine	38.8±5.2	39.8±4.1	43.5±3.1	176±34 ^{*a}
	cysteine	77.4± 8.8	69.5±4.7	164±21 ^{a}	209±45 ^{a}
Liver	homocysteine	5.77±1.6	9.26±1.7	2.91±0.24	2.29±0.34
	cysteine	117 ±30	118±17	183±38	89.1±11*
Kidney	homocysteine	22.3±1.7	34.3±1.5*	55.5±2.6	71.5±1.9*
	cysteine	3384±23.7	3253±174	2827±177^{a}	2611±172^{a}

Values are in mean±SEM, unit-μmol L^{-1}(plasma)/ μmol kg^{-1} (liver/kidney), (n=6-8), data were analysed by GLM followed by Tukey's post-hoc test or Steel-Dwass test
$p < 0.05$=* denotes significant difference between controls and MR at same time period.
$p < 0.05$=^{a} denotes significant difference between same treatment at different time periods

Methylation potential in liver and kidney

Dietary MR led to reduced SAM contents in liver at both 4 and 8 weeks (Fig. 2.3A), whereas the SAH content did not change in the liver (Fig. 2.3B). The resulting ratio of SAM to SAH (methylation potential) was lower in the MR rat liver at both the 4 and 8 week periods (Fig. 2.3C). In contrast, SAM and SAH levels did not change between treatments in kidney tissue (Fig. 2.3D and Fig. 2.3E). In addition, SAM levels were higher and SAH levels were lower in kidney compared to liver (Fig. 2.3A-D). Although the methylation potential decreased in kidney tissue after 8 weeks because of high SAH levels (Fig. 2.3F), the kidney methylation potential remained high compared to the liver. The kidney SAM content and cysteine level (Table 2.1) were not

different between control and MR rats even at 8 weeks when hyperhomocysteinemia was apparent with MR (Table 2.1). Since these transsulfuration-related metabolites were unchanged by MR in the kidney, despite induction of hyperhomocysteinemia, we focused on homocysteine metabolism and H_2S production capacity in the liver where MR led to a significant decrease in methylation potential along with declining cysteine levels, both of which are consistent with reduced transsulfuration flux.

Transsulfuration enzyme activities in liver

Cystathionine-β-synthase and CGL sequentially convert homocysteine into cysteine. At both time period (4 and 8 weeks), MR rats had significantly lower CBS activity than controls (Fig. 2.4 A). The activity of CGL was not different between control and MR rats after 4 weeks (*p = 0.92)*, however CGL activity significantly increased in the MR rat liver between the 4 and 8 week sample points (Fig. 2.4B).

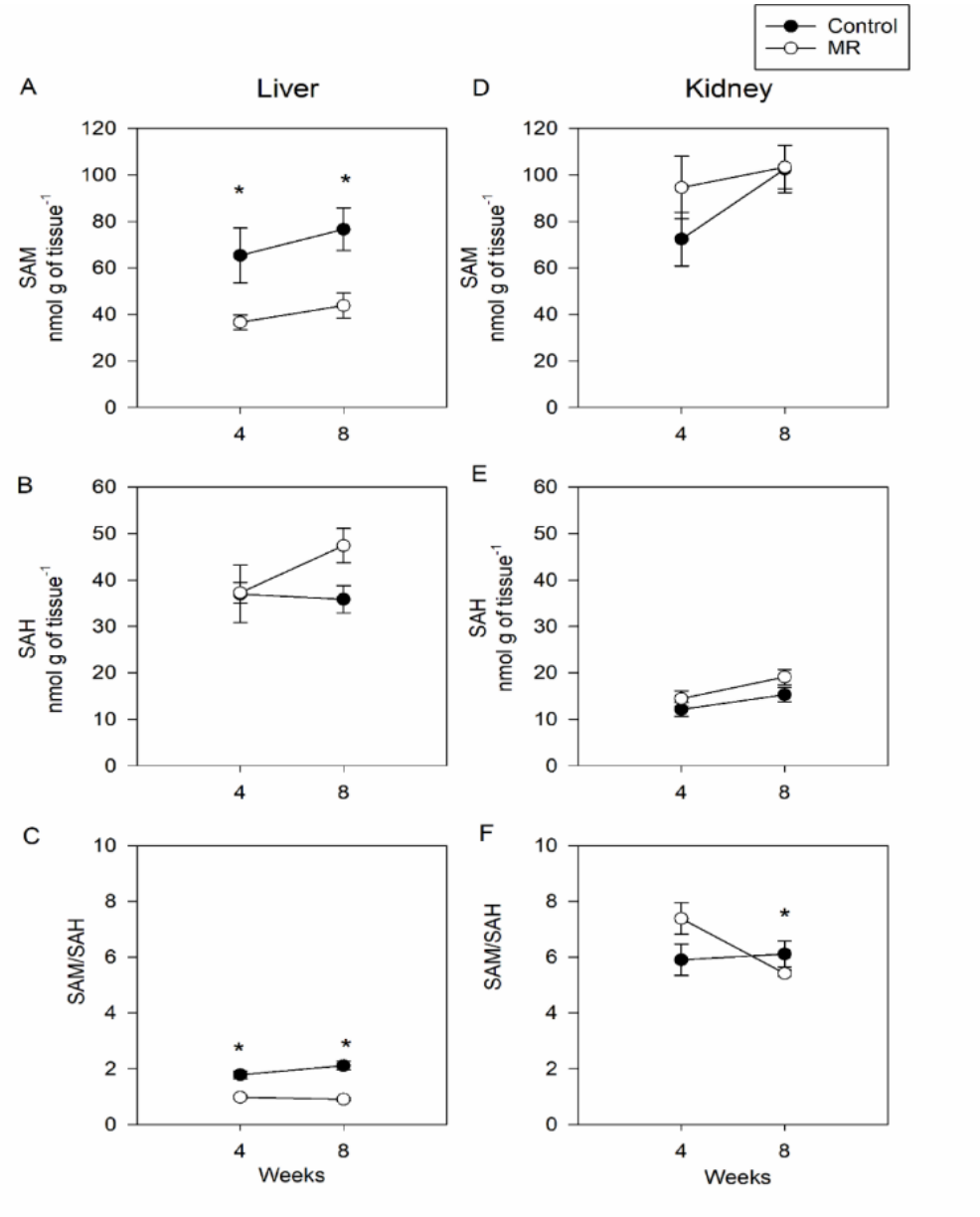

Fig. 2.3 Levels of SAM and SAH in liver and kidney. A) Liver SAM, **B)** kidney SAM, **C)** liver SAH, **D)** kidney SAH, **E)** ratio of liver SAM and SAH, **F)** ratio of kidney SAM and SAH. Data are presented as mean ± SEM (n=8), (* $p < 0.05$ between different diet groups; GLM followed by Tukey's post-hoc or Steel-Dwass test)

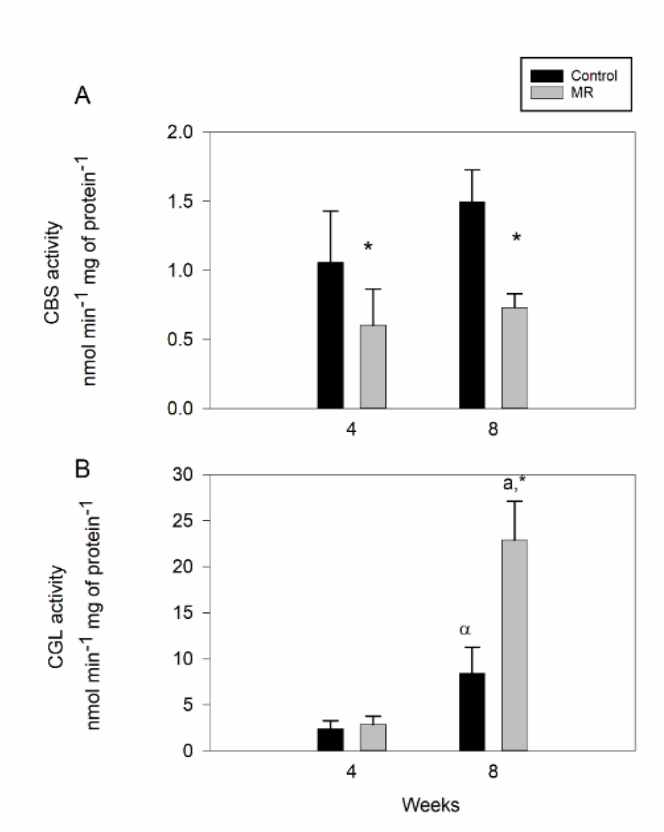

Fig. 2.4 CBS and CGL activity in liver. A) CBS activity in liver **B)** CGL activity in liver. Data are presented as mean ± SEM (n=8), (*, $p<0.05$ between the two diet groups; ^{a}, $p<0.05$ same dietary group at different time points; $^{\alpha}$, $p= 0.0552$ between same diet at different time periods; GLM followed by Tukey's post-hoc or Steel-Dwass test)

H_2S production from the transsulfuration pathway

Having identified that a decrease in liver CBS activity coincides with apparent impaired transsulfuration flux, as indicated by hyperhomocysteinemia, the marked elevation of the downstream CGL activity in response to MR seems contradictory to a potential decrease in transsulfuration flux. However, since CGL can also produce H_2S from homocysteine and cysteine, it is possible that CGL is upregulated to enhance H_2S production in the liver. I We tested this hypothesis by measuring H_2S production capacity, pharmacologically separating the contributions from CBS and CGL [27]. Total H_2S production capacity from cysteine plus homocysteine was not different between treatments (Fig. 2.5A). Cystathionine-γ-lyase derived H_2S production, determined as the propargylglycine sensitive component of total H_2S formation, was prominent in both treatments by 4 weeks and after 8 weeks was higher in MR than controls. Despite observed differences in CBS activity, the propargylglycine insensitive H_2S production capacity did not vary between treatments (Fig. 2.5C).

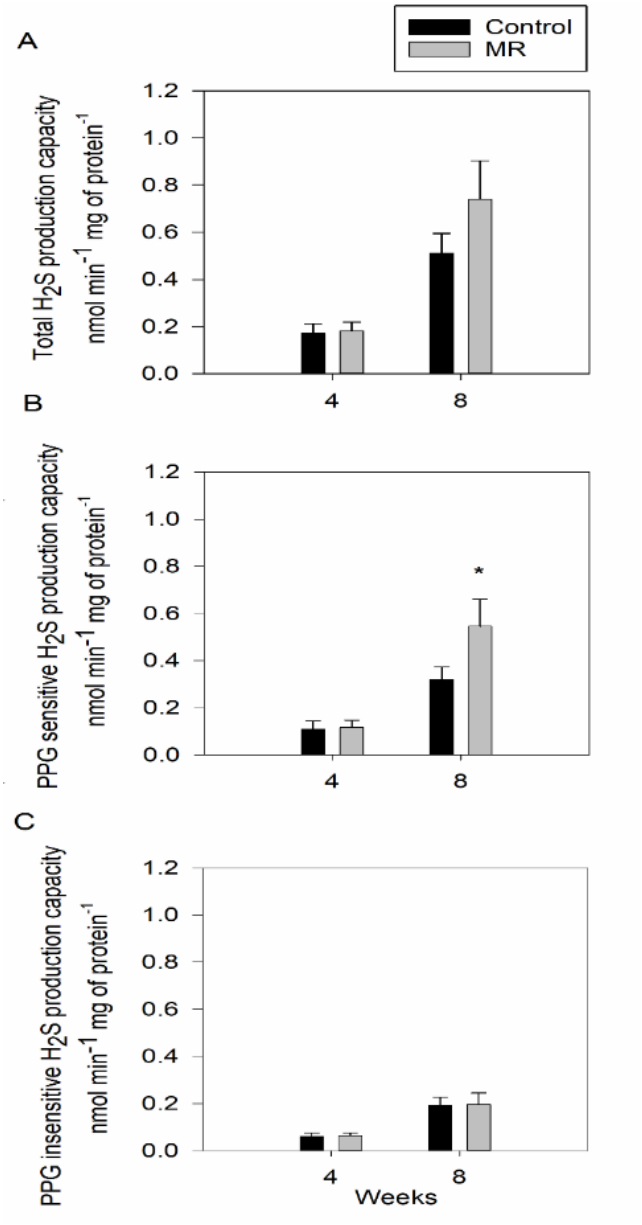

Fig. 2.5 H_2S production capacity by liver. A) Total H_2S production by tissue protein unit, **B)** H_2S production by CGL enzyme on tissue protein unit, **C)** H_2S production by CGL on tissue protein unit. Data are presented as mean ± SEM (n=8), (* $p < 0.05$; GLM followed by Tukey's post-hoc or Steel-Dwass test)

Mercapto-pyruvate-sulfur-transferase activity

Mercapto-pyruvate sulfur-transferase is an alternative source of H_2S production. This enzyme produces H_2S from 3-mercaptopyruvate (3-MP). Several studies reported that the endogenous level of 3-MP is very low and it is often below the quantitation level or not found at all [42-44]. Therefore, we measured the activity of MPST in the liver to test for possible compensatory changes in response to MR. The activity of MPST was elevated in MR animals compared to controls at both 4 and 8 weeks (Fig. 2.6).

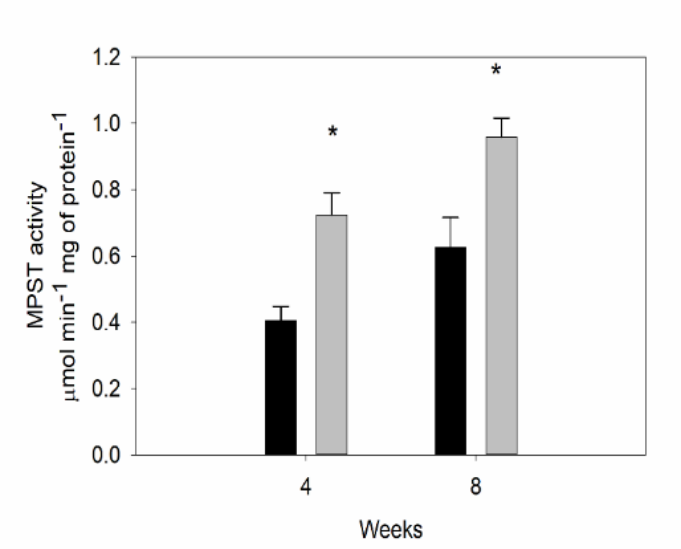

Fig. 2.6 MPST activity in liver. Data are presented as mean ± SEM (n=8), * denotes significance between the two diet groups, (* $p < 0.05$; GLM followed by Tukey's post-hoc or Steel-Dwass test)

Discussion

Methionine restricted model confirmation and rationale of the study design

Currently there are few MR studies where metabolites involved in methionine metabolism have been investigated in F-344 rats [10], C57BL/6 mice [34, 45] or Ames dwarf/wild-type mice [33]. Although there was some variation in the amount of dietary methionine among studies in the MR diet (0.12%, 0.16% and 0.17%, respectively), the metabolites relevant to methionine metabolism were found to either increase or were unaltered in liver in all three studies except the ratio of SAM-SAH and the levels of cystathionine [33, 34, 45]. To specify, 0.12% methionine in the diet reduced SAM and SAH levels, the ratio of SAM-SAH, and also cystathionine in C57BL/6 mice [45], whereas 0.17% methionine decreased SAH levels, but not the SAM-SAH ratio as SAM levels were increased by MR [33]. While MR mostly increased transmethyation, transsulfuration and remethylation metabolites in the mouse model, in rats MR shows different responses. For instance, in F-344 rats levels of all of the methionine metabolites including cysteine and cystathionine were decreased, with the notable exceptions of other than choline, serine and homocysteine [10]. However, elevated expression of hepatic betaine-homocysteine-methyltransferase and methionine-tetrahydrofolate-reductase proteins suggest that the remethylation pathway is presumably increased by MR [10]. Perrone *et al*. suggested that reduced vitamin B6 and 5-methyl-tetrahydrofolate in MR animals plays a key role in the elevation of homocysteine [10]. However, they could not report SAM levels either in controls or MR which limits conclusions on homocysteine elevation [10]. Moreover, it is unclear how animals on MR maintain or elevate H_2S production [19] if hyperhomocysteinemia is a result of reduced transsulfuration capacity.

Hence, the current study investigated how the lifespan increasing MR diet alters homocysteine metabolism and H_2S production capacity in F-344 rats. Prior to investigating metabolic responses, it was crucial to confirm MR animals display the typical characteristics of previous MR studies including those studies showing increased lifespan. The current experiment demonstrated that the MR rats consumed more food on a mass-specific basis but grew less, which is consistent with previous MR studies on rodents [5]. Based on these altered growth and feeding dynamics, along with confirmation of hyperhomocysteinemia after 8 weeks [12], we concluded that our system recapitulated the typical MR phenotype.

Of note, dietary restriction strategies, either by total calorie intake or specific nutrients, have been widely applied for decades in studies of longevity and aging. For rodents, within weeks after the introduction of dietary restriction, biomarkers of metabolic health start to improve and this is often maintained throughout the lifespan of the study organisms. As such, although the current study only spans 8 weeks, my findings may still have relevance to understanding the metabolic outcomes related to the lifespan increasing MR diet in rodents.

Metabolic regulators of homocysteine metabolism

To date, three studies reported that MR elevated serum homocysteine levels in rodents [12, 13, 46], an observation that has met with some criticism [18]. We found that after 8 weeks of MR, rats develop hyperhomocysteinemia compared to control animals (Table 1). Although the magnitude of homocysteine elevation between treatments was similar between our 8 week and a 12 week study [12], the absolute increase was higher in my study. We found that chow-fed F-344 rats had much lower homocysteine levels than both control and MR animals (data not shown). Of note,

chow-fed F-344 rats had similar plasma homocysteine levels to values reported for other chow-fed rats [47]. It is important to note that the AIN-76 A diet contains less vitamin B12 and choline than the AIN-93 diet [48]. Although the semi-purified diet is formulated based on the composition of casein, casein provides an additional source of vitamins in the diet when provided as a whole protein. Taken together, we suggest that low B12, choline and lack of other nutrients from casein in the semi-purified might make the controls hyperhomocysteinemic relative to chow-fed rats found in other studies [12,47] and also in this study. However, despite the semi-purified diet appearing to induce hyperhomocysteinemia in the control animals, the MR diet still led to even higher plasma homocysteine levels after 8 weeks. In addition, we reported for the first time that MR markedly elevated red blood cell homocysteine levels at 8 weeks, the pattern is similar to plasma homocysteine which was not different at 4 weeks (Table 2.1).

Following demonstration that MR leads to elevated homocysteine levels in the circulation, we further investigated the associated metabolites in liver and kidney, because these are the major organs involved with metabolising sulfur amino acids in rodents [21, 49]. The liver is a major site of homocysteine transformation by remethylation or transsulfuration, and can be a net exporter of homocysteine [50]. For example, with cultured hepatocytes, when homocysteine formation exceeds the metabolic capacity for removal, either due to enhanced formation or via pharmacological inhibition of further metabolism, excess of homocysteine is exported from the cells to the extracellular medium [51]. In rodents, unlike humans, the kidney plays a major role in removing extracellular homocysteine and thus contributes to maintaining plasma homocysteine levels [52, 53]. Thus, altered homocysteine clearance by either the liver or kidney could explain the hyperhomocysteinemia observed in the current study.

We found that kidney homocysteine increased with MR (Table 2.1) which supports the conventional notion that kidney tissue is a net uptake system for amino acids [54]. In addition, we reported that renal SAM levels were unchanged (Fig. 2.3D) while methylation potential was decreased by MR (Fig. 2.3 F). In rodents, renal homocysteine is predominantly metabolised via transsulfuration [21] and therefore, reduced methylation potential by MR could lead to an enhanced remethylation in the kidneys, similar to what may take place in the liver of MR mice [33, 34, 45]. However, the level of cysteine was unchanged by MR suggesting that reduced homocysteine clearance from declining kidney transsulfuration flux is unlikely to explain the elevated plasma homocysteine levels. In contrast to the kidney, the liver showed reduced SAM levels, decreased methylation potential (Fig. 2.2), and lower cysteine concentration in response to MR (Table 2.1). All of these traits would be consistent with preferential retention of homocysteine via remethylation and a reduced transsulfuration flux. Therefore, MR rats might retain homocysteine via methionine resynthesis, thus limiting the downstream production of cysteine due to a low transsulfuration flux.

Taken together, the above implicates altered transsulfuration in the liver as a source of the hyperhomocysteinemia induced by MR. For this reason, we next focused on liver tissue to assess enzyme activities associated with homocysteine conversion to cysteine.

Enzymatic regulators of liver homocysteine metabolism

Homocysteine is an intermediate metabolite between the remethylation and transsulfuration pathways. Although homocysteine can be remethylated via methionine synthase or betaine homocysteine-methyl-transferase, both *in vitro* and *in vivo* studies have shown that homocysteine is primarily metabolised by transsulfuration which is largely regulated by CBS [47, 55]. We found

that the activity of CBS (Fig. 2.4A) and the levels of SAM, an allosteric activator of CBS, were lower in the MR rat liver (Fig. 2.3C), which suggests that the activity of CBS is downregulated in response to MR. The concomitant lower SAM levels should further decrease the flux from homocysteine to cysteine in the liver of MR rats. Consequently, enhanced homocysteine export from the liver to the plasma may contribute to both the hyperhomocysteinemia and reduced levels of cysteine in the liver.

Although the first committed step in the transsulfuration pathway was perturbed due to MR, we found that the activity of CGL, the second enzyme of the transsulfuration pathway, increased at 8 weeks. Along with its canonical role in the transsulfuration pathway, CGL also catalyzes reactions that produce H_2S and the contributions of those reactions are thought to be responsive to the grade of hyperhomocysteinemia [30].

Enzymatic H_2S production

Several studies have reported that H_2S could attenuate the damage caused by hyperhomocysteinemia [20]. Likewise, enhanced H_2S production has been postulated to be a unifying aspect of dietary regimes that increases lifespan across animal models [19]. We also found that CGL contributes more to H_2S production than CBS when H_2S generating substrates for both CBS and CGL (homocysteine and cysteine) are present at the same time (Fig. 2.5B). Importantly, the MR group increased the relative reliance on CGL-dependent H_2S production in parallel with the development of the hyperhomocysteinemic condition. A recent study reported that 4 months of MR enhanced H_2S production capacity in mice [19]. They further suggested that increased H_2S production capacity is accompanied by increased transsulfuration rate [19]. Here, we assert that if transsulfuration in the liver was increased then hyperhomocysteinemia would not be exhibited

by the MR animals. Instead, our study indicates that transsulfuration may be reduced in the liver by MR combined with enhanced CGL, where the latter could accentuate H_2S production.

Emphasizing that enhanced H_2S production may be the key to the overall metabolic adjustments that occur with MR in rodents, we found that the activity of MPST increased with MR. When the transsulfuration pathway is impaired, then MPST derived H_2S might be an important source to conserve H_2S production. However, it is important to note that MR greatly reduces liver cysteine content. Cysteine is the source of sulfur for H_2S produced by MPST and a major constituent of the H_2S produced by CGL [27]. Taken together, elevated CGL and MPST activity might provide a compensatory mechanism to H_2S production in response to an impaired transsulfuration pathway. Based on my results we propose following mechanism for hepatic H_2S production in MR for F-344 rats (Fig. 2.7).

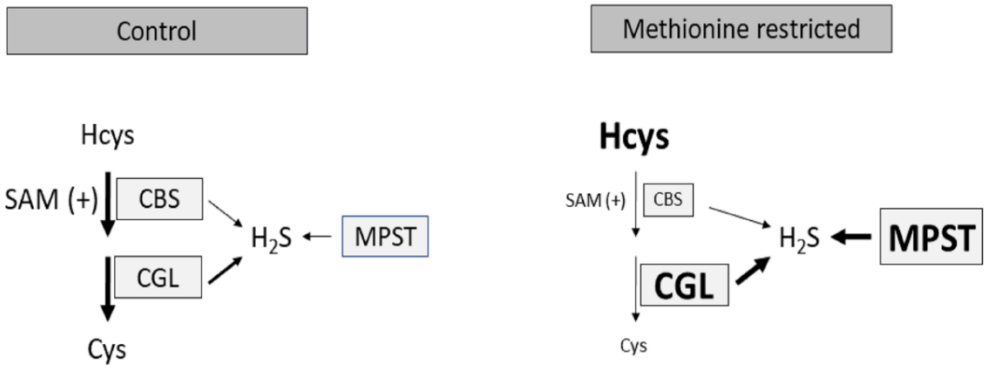

Fig. 2.7 Summary of transsulfuration and H_2S production in a schematic diagram. Reduced SAM levels in MR liver does not enhance CBS activity compared to controls, which ultimately impairs the transsulfuration pathway resulting lower cysteine content on a MR diet. Reduced CBS activity contributes less in H_2S production whereas enhance CGL and MPST might act as a compensatory mechanism in producing H_2S in MR rat liver. Larger font size and thick arrows denote the relative elevation between pathways and metabolites of MR and control diets.

Conclusions

Methionine restriction induces two apparently opposing effects: hyperhomocysteinemia, which typically would be interpreted as a negative outcome for lifespan, and an increased capacity for H_2S production which is associated with increased lifespan. In the current study, we investigated how MR synchronizes these two paradoxical phenotypes. We observed that MR induces hyperhomocysteinemia by repressing the transsulfuration pathway in the liver, and it likely regulates H_2S production capacity by a compensatory activation of CGL and MPST. One of the limitations of our study is that we did not measure the transsulfuration flux or endogenous H_2S

levels from the fresh liver tissue and direct measurement of these two factors are warranted in future work to provide better insight regarding *in vivo* H_2S metabolism. Further studies assessing the beneficial H_2S production in naturally long-lived species and their comparison with laboratory used aging models would provide better insight on aging research.

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Chapter Three: Glutathione regulation and alternate antioxidant response(s) to a short-term methionine-restricted diet

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Nahid Tamanna[a], Kathryn Kroeker [a], Kristen Braun[a], Sheena Banh[a], Jason R. Treberg[a,b*]

[a] Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada.

[b] Centre on Aging, University of Manitoba, Winnipeg, MB, Canada.

Author contribution: NT and JRT designed the experiments, NT did most of the experiments, KK conducted the respirometry, KB did the protein carbonyls and SB did the mitochondrial GSH assay. NT analyzed the data. NT and JRT prepared the manuscript.

Abstract

Dietary methionine restriction (MR), where methionine is the sole source of sulphur containing amino acids, increases lifespan in diverse species. Methionine restricted rodents experience a decrease in glutathione (GSH), a major antioxidant, in several tissues, which is paradoxical to longevity interventions because tissues with low GSH might experience more oxidative damage. Liver tissue plays a key role in GSH synthesis and here we examined how MR influences GSH metabolism in the liver. We also hypothesised that low GSH might be ameliorated by compensatory pathway(s) in the liver. To investigate GSH synthesis and antioxidant responses, Fischer-344 rats were provided either a MR diet or a control diet for 8 weeks. Based on γ-glutamylcysteine synthetase activity, GSH synthetic capacity was unaffected by low dietary methionine availability. Tissue-level protein and lipid oxidation markers do not provide any evidence of elevated oxidative damage, despite low GSH availability. Whole tissue and mitochondrial responses to MR differed. Specifically, the activity of glutathione reductase and thioredoxin reductase increased in whole liver tissue which might offset the effects of limited GSH availability whereas mitochondrial GSH levels were unperturbed by MR. Moreover, enhanced proton leak in liver mitochondria by MR presumably diminishes ROS production.

Keywords: Methionine restriction, glutathione, thioredoxin, protein carbonyls, oxidative stress, proton leak

Introduction

Aging is often characterized by the progressive or irreversible accumulation of deleterious processes and cellular modifications in a species [1]. Currently there are several hypotheses on the proximate cause of aging and senescence; the 'free radical theory of aging' being one of the more prominent. The free radical theory of aging initially postulated that aging is a process of progressive accumulation of products from reactions between biomolecules and free radicals, the production of which is ubiquitous throughout aerobic cells [2]. The concept of aging being linked to damage by radicals has been refined over the years to include other oxidants, such as non-radical reactive oxygen species (ROS) and nitrogen species which can also cause damage to cells. More recent modifications of this theory hypothesizes that the imbalance between pro-oxidants and antioxidants triggers oxidative stress, and as a result, aging occurs [3, 4]. Therefore, it is predicted that the functional lifespan of a species may be increased by lowering oxidative stress in the cellular environment, or at least within specific subcellular compartments. While the idea that oxidative stress itself causes age related damage is still a matter of debate (reviewed in [3, 5]), several lines of evidence support the idea that ROS balance in mitochondria is important in the aging process [6-9].

For decades it has been known that dietary manipulation through calorie restriction can extend lifespan in different species from diverse taxa [10]. Although many hypotheses have been proposed to explain the anti-aging effects of calorie restriction (reviewed in [11]), reducing oxidative stress by calorie restriction is a commonly accepted explanation (reviewed in [11, 12]). Calorie restriction attenuates age-associated oxidative damage [12] as well prevents the decline of

the small molecule antioxidant glutathione (GSH) as well as antioxidant enzymes glutathione reductase, glutathione peroxidase and thioredoxin reductase with increasing age in rodents [13].

Methionine restriction (MR) is another dietary regimen shown to increase lifespan in diverse species [14-20]. Of note, several key distinctions in rodent models occur between calorie and methionine restriction. For example, calorie restricted animals consume less food by experimental design and grow less whereas methionine restricted animals are typically fed *ad libitum* and consume more food on a mass-specific basis but grow less than either control *ad libitum* or pair-fed animals [4]. While calorie restriction prevents age-associated declines in GSH, MR decreases GSH levels in several tissues including liver [14, 17, 21-25]. Glutathione is the most abundant intracellular non-protein thiol and contributes to cellular defence and is a major component of the intracellular reducing environment and has roles in the regulation of protein and gene expression [26]. A major connection of GSH to the aging process comes from GSH serving as a major protectant against oxidative stress experienced by cells [26]. Hence, in MR rodents where lifespan is increased by the dietary intervention, the decreased hepatic GSH appears to be in conflict with the lifespan increasing effect of MR. However, decreased GSH in most tissues is not the only characteristic exerted by MR that is counterintuitive to increased longevity. For example, contrary to any pace-of-life notion that slower metabolic rates or decreased food intake may lead to longer lifespans, MR in rodents results in an elevated metabolic rate combined with mass-specific hyperphagia [27]. The elevated metabolic rate and smaller size of MR rodents is not explained by increased locomotory activity but is associated with proliferation of brown adipose tissue [27], Moreover, homocysteine, which is regarded as an extracellular pro-oxidant, becomes elevated in the blood with MR [28, 29], and studies suggest that homocysteine might have direct or indirect influence on the progression of cardiovascular disease [30]. Having a decrease of GSH, a major

antioxidant, and a simultaneous increase of pro-oxidants suggest that MR animals should be more susceptible to oxidative stress. However, MR causes lower oxidative damage to mitochondrial protein and DNA in several tissues [31-34] suggesting the activation of alternate mechanism(s) to regulate oxidative damage and ROS production compensate for low tissue GSH at least at the mitochondrial level.

Given that low cellular GSH availability would be anticipated to have negative outcomes, it is important to consider how MR influences GSH metabolism and the consequences this may have. One organ that shows a pronounced decrease in GSH with MR in rodents is the liver. This is particularly striking because, while many cell types can synthesize GSH, the liver is a major contributor to whole organism synthesis of GSH and a significant exporter of GSH for distribution throughout the body via the circulation [35]. Hepatic GSH content is known to be influenced by the content and nature of the diet [36]. For instance, either fasting or a low protein diet decreases hepatic GSH content in rats which can be restored by supplementing sulphur amino acids in the diet [37-39].

Glutathione, which is a tripeptide comprised of glutamate, cysteine and glycine, is synthesized in the cytosol in a two-step process [40]: in the first step γ-glutamylcysteine is formed from the condensation of glutamate and cysteine. This step is the rate controlling component of GSH synthesis and is catalyzed by γ-glutamylcysteine-synthetase. In the second step, glycine is added by glutathione synthetase forming GSH. Among the three precursor amino acids, glutamate and glycine are dispensable [21] since they are obtained from a number of metabolic cycles, especially in the liver, whereas cysteine is obtained either from the diet, catabolism of endogenous cysteine containing molecules, such as protein, or from methionine by the transsulfuration pathway [41]. Evidence suggests that rate of hepatic GSH synthesis is mainly dependant on the availability of

cysteine rather than glutamate or glycine [41]. For MR diet studies the sulfur containing amino acid content of the diet is normally only supplied as methionine, meaning that all other sulfur containing amino acids and their derivatives must intially come from the conversion of dietary methionine. Previously, it has been shown that MR decreases hepatic cysteine levels in rats which appears to be due to decreased transsulfuration flux and retention of sulfur amino acids as methionine via remethylation pathways [18, 24, 42, 43]; however, it is not clear if this lower cysteine availability limits the GSH synthetic capacity of liver in MR rodents..

Following synthesis, GSH participates in several reactions including protein and nucleic acid synthesis as well as detoxifying peroxides [44]. Glutathione along with two glutathione-dependant enzymes detoxify H_2O_2, which is a primary mitochondrial ROS product, in both the cytosol and mitochondria (Fig. 3.1). Glutathione reductase recycles oxidised GSH to reduced GSH and thus assists glutathione peroxidase in the detoxification process (Fig. 3.1). However, the glutathione system is not alone in the capacity to scavenge peroxides, with the thioredoxin antioxidant system being of particular relevance to the scavenging of H_2O_2 (Fig. 3.1). The thioredoxin system is composed of thioredoxins, peroxiredoxins and thioredoxin reductases. Similar to the GSH system, the thioredoxin system is found in both the cytosol and mitochondria and relies predominantly on the NADPH pool of cofactors to supply reducing equivalents to maintain H_2O_2 consumption activity [45-47]. It is not known how the thioredoxin system responds to MR. However, since MR decreases hepatic availability of GSH, it is anticipated that the thioredoxin-dependant pathway could act to compensate for impaired GSH-dependent H_2O_2 consumption or act as an alternate means for augmenting the reduction of GSH. Of note, mitochondria do not synthesize their own GSH, rather they rely on the cytosolic GSH pool via a transport system to maintain matrix levels

of this important antioxidant [48, 49]. Mitochondrial GSH plays an important role in oxidative stress and declines in the intramitochondrial GSH concentration are linked to the development of liver disease [48, 49]. Since MR decreases overall cellular GSH level, it is anticipated that the mitochondrial GSH content may also decline.

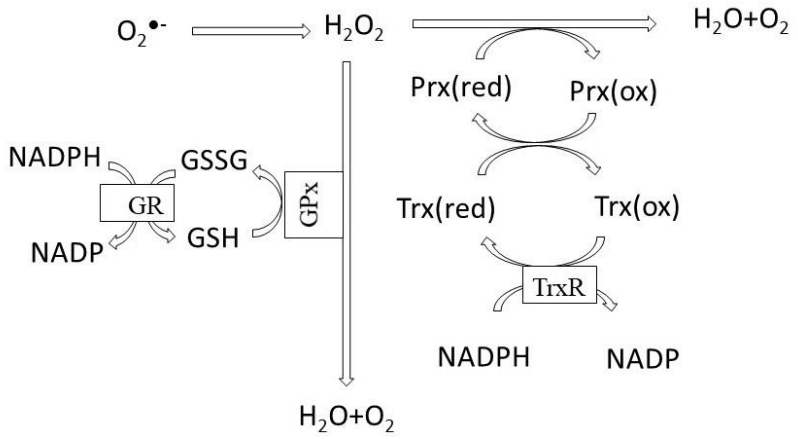

Fig. 3.1 Glutathione and thioredoxin-dependent pathways of peroxide consumption. The glutathione and thioredoxin pathways scavenge H_2O_2 in both cytosol and mitochondria. The scavenging system of glutathione consists of GSH, glutathione-reductase (GR) and glutathione-peroxidase (GPx), whereas thioredoxin consists of thioredoxin (Trx), peroxiredoxin (Prx) and thioredoxin-reductase (TrxR)

An alternative explanation of how MR may be beneficial is that this diet may decrease the overall amount of oxidative molecular species cells experience, therefore alleviating the impact of decreased GSH content. Mitochondria are often considered a substantial source of ROS in mammalian cells [50, 51]; however, this contention has been challenged [52]. Importantly, previous studies found that MR decreases mitochondrial ROS production by liver mitochondria

which is concomitant with lower mitochondrial protein carbonylation and DNA damage, implicating improved mitochondrial function as a potential benefit of MR [31-34]. All reports of MR decreasing mitochondrial ROS production that we are aware of have relied on assays detecting H_2O_2 emission [31-34]. However, despite repeated findings of lower mitochondrial H_2O_2 emission, from multiple tissues [31-34], MR does not consistently show altered mitochondrial respiration rates in liver [31-34], leaving it unclear how MR may alter mitochondrial ROS formation. A decrease in H_2O_2 efflux from isolated mitochondria could be explained by two hypotheses indirectly related to the actual rate of ROS formation; increased mitochondrial antioxidant activity or by increased proton leak. The mitochondrial matrix has antioxidant enzymes which consume H_2O_2, which can interfere with detecting H_2O_2 emission because only that H_2O_2 which is not scavenged in the matrix is estimated by conventional H_2O_2 trapping systems of detection. Therefore, extramitochondrial trapping systems reflect apparent H_2O_2 production rather than the actual rate [53, 54]. On that account, it is not clear whether MR leads to mitochondria in the liver producing less ROS or whether the lower H_2O_2 emission reflects enhanced mitochondrial antioxidant activities.

Lower mitochondrial ROS formation with MR in rodents could, alternatively, reflect increased basal mitochondrial proton leak. During substrate oxidation by mitochondria, complexes I, III and IV generate a protonmotive force across the inner membrane. Protons return to the matrix via complex V (ATP synthase) and couple the to drive ATP synthesis by oxidative phosphorylation. During proton leak, protons return to matrix independent of complex V and this disrupts the coupling between substrate oxidation, which generates the ATP synthesis at complex V [55]. The relationship between proton and electron leak on ROS production have been reviewed in detail by M.D Brand [56]. In brief, at high protonmotive force, electron flow through the electron transport

system is restricted leading to the accumulation of electrons in the electron carrier pools (Q, NAD^+, cytochrome c) and within enzyme complexes as electrons flow through respiratory complexes to oxygen. As a result, the steady state availability of electrons that can 'leak' to molecular oxygen, which leads to the formation of ROS, also increases. With mild uncoupling, under the same capacity for substrate oxidation the increased proton leak causes the proton motive force to decline and oxygen consumption increases along with a decreasing barrier to electron flow. At the same time, mild uncoupling can shift the electron carriers and redox centres in enzyme complexes to a more oxidized state, decreasing residence of electrons on the sites where electrons can prematurely leak onto oxygen, forming ROS [56].

The current study addressed several unknown aspects associated with how MR influences liver metabolism, specifically with regards to GSH synthesis and the related impacts on systems that may be involved with counteracting the consequences of low GSH in liver. Fischer-344 rats were fed on the same methionine restricted diet that is known to enhance lifespan [14] and known patterns for blood GSH were used to confirm the expected metabolic response to methionine restriction [22, 57]. We, then focused on the GSH synthetic capacity and measured key enzyme and metabolites to determine if low liver GSH reflects low cysteine availability, decreased enzymatic capacity for synthesis or a combination thereof. Next, we investigated if MR influences oxidative stress, and if it does, then what could be the possible mechanism. To this end, we measured protein carbonyl and malondialdehyde levels as oxidative stress markers at the whole tissue level in the liver as well as antioxidant enzyme activities relevant to the glutathione and thioredoxin dependent H_2O_2 consumption pathways. We also focused on mitochondrial responses to MR in the liver, assessing mitochondrial GSH content, the activities of antioxidant enzymes and proton leak status as possible explanations for the previously observed decline of mitochondrial

ROS production by liver mitochondria [32, 58]. The results from this study provide a better insight about how a lifespan extension dietary regimen which is limited in sulphur amino acids regulates a major antioxidant system and also attenuates ROS production in liver.

Materials and method

Reagents

All chemicals were purchased from Sigma-Aldrich (Canada).

Animals and experimental design

In this study, samples were obtained from same rats which were used for the second chapter. However, for the completeness, given this is a stand-alone chapter, all of the feeding and sample collection procedures have been added here. All studies were approved by the University of Manitoba Animal Care Committee (protocol- F2013-036). Five-week-old male Fischer-344 (F-344) rats were purchased from Charles River laboratories (Quebec, Canada) and housed at the Biological Sciences Animal Holding Facility. Rats were initially kept in shoebox cages and after one week were housed individually in wire bottom cages to measure food consumption throughout the experiment. For two weeks, rats were gradually acclimatized with commercial rodent chow diet and control diet pellets (AIN-76 formulated semi purified diet from Research Diet, New Brunswick, New Jersey, USA). At the age of seven weeks, rats were randomly assigned to a MR or control diet [14]. Both diets contained the following (in percentage of diet by mass): L-arginine 1.12, L-lysine 1.44, L-histidine 0.33, L-leucine 1.11, L-isoleucine 0.82, L-valine 0.82, L-threonine 0.82, L-tryptophan 0.18, L-phenylalanine-1.16, L-glycine-2.33, corn starch 43.61, sucrose 20.00, solca floc 5.00, choline bitartrate 0.20, vitamin mix 1.00, mineral mix 3.50, corn oil 8.00 and

dextrin 5.00. The control diet had 0.86% methionine as the sole source of sulfur amino acids whereas in MR diet this was reduced to 0.17% as in past studies demonstrating the longevity impacts of MR in this strain of rat [14, 15]. To compensate for the reduced amount of methionine, glutamic acid content was increased from 2.70 % to 3.39 % in controls and MR, respectively. Food and water were provided *ad libitum*. Rats were sacrificed after 4 weeks or 8 weeks using isoflurane as an anaesthetic; once anaesthetized, arterial blood was collected using a heparinized syringe and centrifuged at 5500 × g for 5 min to separate plasma and red blood cells. Following blood collection, a sample of liver tissue was flash frozen in liquid nitrogen and the remaining liver was placed in a beaker containing mitochondrial isolation buffer (see details in the mitochondrial isolation section). Frozen liver was subsequently pulverized using a mortar and pestle, chilled with dry-ice to facilitate subsampling without freeze-thaw cycles. All samples were stored at -80°C until analyzed.

Mitochondrial isolation

Liver mitochondria was isolated by a differential centrifugation method. All of the centrifugations were carried out at 4°C and any step in between centrifugation was processed on ice. The liver was cut into small pieces and then was transferred to a glass tube containing 50 mL of isolation buffer (pH-7.2, 255 mM mannitol, 70 mM sucrose, 10 mM Hepes, 1 mM EGTA). Homogenization was performed sequentially with loose, medium and tight-fitting pestles using a 55 mL mortar (Wheaton 55 mL, Potter-ELV Ctd). In addition, 0.5% of bovine serum albumin was added in the isolation buffer prior to homogenization to minimize the effect of lipolysis during the initial preparation of the sample. Following homogenization, the suspension was centrifuged at 1000 x g

for 10 mins to remove nuclei and cell debris. The supernatant was then collected in a beaker by filtering through several layers of cheesecloth (mesh size-grade 10, VWR) and centrifuged at 10,000 x g for 10 mins. Any over laying fat was removed, and pellets were re-suspended with 15 mL of isolation buffer taking care to not re-suspend red blood cells or other fast sedimenting non-mitochondrial material. The suspension was centrifuged at 1000 x g for 10 mins and supernatant was collected. The supernatant was centrifuged at 10,000 x g for 10 mins and the subsequent mitochondrial pellet was re-suspended in a small volume of isolation buffer. Mitochondrial protein content was measured by the Pierce BCA kit (Thermo fisher scientific, Rockford, Illinois, USA). Fresh isolated liver mitochondria were used for respiration and proton leak experiments whereas frozen mitochondria were used to estimate total GSH levels and enzyme activities.

Biochemical assays

Total γ-glutamylcysteine and GSH

Plasma γGC and GSH were measured by UHPLC with fluorescence detection [59, 60]. 150 µL of plasma was incubated with 10% (w/v) of 20 µL tris-carboxyl-ethyl-phosphine to reduce any oxidized thiols thereby giving the total γGC and GSH (a combined total of the reduced and oxidized forms) in the sample. After 30 min of incubation at room temperature, protein was precipitated by addition of 125 µL 0.6 M perchloric acid. Extracts were then centrifuged at 10,000 x g for 10 min to remove any precipitated protein. An aliquot (50 µL) of the supernatant was mixed with a double volume of potassium borate buffer (pH 10.5) and an equal volume of ammonium 7-fluoro-benzo-2-oxa-,3-diazole-4-sulfonate (1 mg/mL) followed by an incubation at 60°C for 1 hr to produce fluorescent derivatives of the reduced thiols. Derivatized samples were cooled on an ice bath, filtered with a 0.2 µm syringe filter and injected into a HPLC. For liver and

thiol analysis, frozen samples were initially homogenized with 10 volumes of 50 mM of imidazole buffer. Following homogenization, samples were processed in the same way as plasma. All analyses were conducted with a Dionex Ultimate 3000 UHPLC system and injections were resolved using an Acclaim C-18 reversed phase column (3μm particle size, diameter 4.6mm,100 mm length) and eluted isocratically with a mobile phase containing 95% of 0.1M potassium phosphate buffer (pH 6.5) and 5% methanol.

γ-glutamylcysteine synthetase activity

To assess the activity of γ-glutamylcysteine synthetase in liver, frozen tissue was homogenized with five volumes of homogenization buffer (50 mM imidazole buffer, 0.1 mM EDTA, pH-7.4). A 0.6 mL aliquot of homogenized sample was filtered through a bio-spin column (Zeba spin-desalting column, 7K MWCO, 2 mL) to remove small molecular weight compounds. Following filtration, the activity of γ-glutamylcysteine synthetase was assessed in liver homogenates following previously described methods [61-63]. Briefly, 10 mM glutamate, 5 mM cysteine, 10 mM ATP, 0.1 M Tris-HCl (pH 8.2), 0.15 M KCl, 2mM EDTA and 20 mM $MgCl_2$ were added to a 1.5 ml microcentrifuge tube and pre-incubated at 37°C for 3 min. Following this step, 450 μL of homogenized sample was added to the reaction medium. Samples were withdrawn at 0, 10 and 20 min intervals and the reaction was stopped by adding 330.75 μL of 0.6M perchloric acid. The samples were then cooled on ice and centrifuged at 10,000 × g for 10 mins to remove any precipitated protein. The samples were then processed by following established methods [59, 60], using the same procedures as described for total γ-glutamylcysteine and GSH.

Glutathione reductase

The activity of glutathione reductase was analyzed by measuring the rate of oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) at 37°C in the presence of oxidized glutathione [64]. Frozen liver tissue was homogenized with 15 volumes of homogenization buffer and same homogenate was used for GPx and TrxR assays. Briefly, 100 mM potassium phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.2% (w/v) triton X100, and 0.1 mM NADPH were added in a cuvette and the rate of NADPH oxidation monitored at 340 nm either in the presence or in the absence of substrate (2 mM oxidized GSH). The activity of glutathione reductase was determined using the difference between rates with substrate and substrate free controls assuming a millimolar extinction coefficient of 6.2 for NADPH.

Glutathione peroxidase

The activity of glutathione peroxidase was measured at 37°C following the method described by Flohe and Gunzler [65]. 100 mM potassium phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.2% (w/v) triton X100, 3 mM sodium azide, 0.24 IU/mL of glutathione reductase, 0.2 mM NADPH, 2 mM GSH and sample homogenized with 15 volume of imidazole buffer (50 mM imidazole, 0.1mM EDTA, pH-7.4) were added into a cuvette. In the chemical control, everything except the sample was added. Following 5 min of incubation, 1.5 mM of (30% w/v) H_2O_2 was added in the reaction mixture and then the rate of absorbance change was monitored at 340 nm. The background absorbance change due to spontaneous reactions was determined in the absence of homogenate and subtracted from the rate of absorbance change in of the sample cuvette which had homogenate present. Rates of enzyme activity were determined assuming a millimolar extinction coefficient of 6.2 for NADPH.

Thioredoxin reductase

The activity of thioredoxin reductase was measured at 37 °C following the method of Luthman and Holmgren [64] with minor modifications [66]. Similar to the glutathione reductase and glutathione peroxidase assays, liver samples were homogenized with 15 volumes of homogenization buffer (50 mm imidazole, 0.1 mM EDTA, pH-7.4). 100 mM potassium phosphate buffer (pH 7.0), 10 mM EDTA, 0.2% triton X100, 0.2 mM NADPH, 5 mM 5,5'-Dithiobis-(2-Nitrobenzoic Acid), 0.2 mg/mL of bovine serum albumin and 1% ethanol were used as substrates. In another substrate containing cuvette, 1 µM of auranofin was added which is an inhibitor of thioredoxin reductase. The rate of absorbance change was monitored at 412 nm while 5,5'-Dithiobis-(2-Nitrobenzoic Acid) is converted to thionitrobenzoate. The difference of absorbance between the auranofin free and auranofin added reaction mixture was obtained and the enzyme activity was expressed as micromoles of NADPH oxidized per minute. The millimolar extinction coefficient for 5,5'-Dithiobis-(2-Nitrobenzoic Acid) is 13.6, since 1 mole of NADPH yields 2 mole of thionitrobenzoate , the rate of absorbance change was divided by 13.6 × 2 [64].

Protein carbonyl group

The concentration of protein carbonyls was assessed based on the method described by Levine *et al.* [65]. Liver was homogenized with 10 volumes of 0.1 M EDTA containing 50 mM imidazole buffer (pH 7.4). Then, 200 µL of sample was transferred into two separate tubes (control and sample tube). Four volumes of 10 mM 2.4 dinitrophenylhydrazine dissolved in 6.0 M guanidine hydrochloride (pH 2.5) and 4 volumes of 6.0 M guanidine hydrochloride, (pH 2.5) were added to

the sample and control tubes respectively. Dinitrophenylhydrazine is light sensitive, hence, the reaction was carried out in the dark for 1 hour with intermittent vortexing. After 1 hour, 500 µL of 20 % (w/v) trichloroacetic acid was added to stop the reaction and the tubes were placed on ice for five mins. The tubes were then centrifuged at 10,000 g for 10 mins and the pellet was collected and further re-suspended with 1 mL of 10% of trichloroacetic acid. The re-suspended pellet was again centrifuged at 10,000 g for 10 mins at 4°C. This time the pellet was washed by resuspension with 1 mL of ethanol: ethyl acetate (1:1) mixture. This procedure was repeated twice and then the pellet was dissolved with 6N guanidine hydrochloride by vortexing and incubation at 37ºC for 25 mins and the absorbance taken at 370 nm. Protein content of the sample was measured from the control tubes at 280 nm where a portion of sample was 10 times diluted with 6 N guanidine hydrochloride. Bovine serum albumin was used a standard for protein content. Using 22 mM^{-1} cm^{-1} as the milimolar extinction coefficient of dinitrophenylhydrazine, protein carbonyl content was measured at 370 nm and was expressed in nmol per mg of protein.

Malondialdehyde assay

Malondialdehyde was assessed based on the method described by Mateos *et al*. [67]. Liver tissue was homogenized with 5 volumes of 0.1 M EDTA containing 50 mM imidazole buffer (pH 7.4). The homogenate was centrifuged at 10,000 g for 30 min at 4°C and the supernatant was collected. Then, 500 µL of supernatant was incubated with 100 µL of 6 N NaOH for 30 min at 60°C to hydrolyse the protein. The reaction was stopped by adding 250 µL of 35% (v/v) perchloric acid. After cooling on ice, processed samples were centrifuged for 5 min at 5,600 g. Following centrifugation, the supernatant was incubated with 50 µL of 5 mM of dinitrophenylhydrazine for 30 minutes at room temperature. Samples were then filtered and analyzed on a 3000 dionex

UHPLC for analysis. 1,1,3,3 tetraethoxypropane was used as standards for malondialdehyde estimation. 1 mM of tetraethoxypropane stock was prepared with deionised water from 4.004 M tetraethoxypropane. Malondialdehyde was formed upon hydrolysis of 1mM tetraethoxypropane stock solution in 50 ml of 1% sulphuric acid and incubation of 2 h at room temperature. The resulting malondialdehyde standard of 20.00 nmol per mL was further diluted with 1% sulphuric acid to yield different concentrations of (2-10 nmol per ml). After malondialdehyde standards were prepared, they were processed the same as the liver supernatant. The samples were detected at 310 nm using 0.2% acetic acid and acetonitrile (62:32) as an isocratic mobile phase and eluted through Acclaim C-18 reversed phase column (3 μm particle size, diameter 4.6 mm,100 mm length).

Oxygen consumption rate and proton leak

The rate of mitochondrial oxygen consumption was measured at 37°C with a computer-controlled Clark-type O_2 electrode (Oxygraph, Innsbruck, Austria) in 2 mL of the respiration medium (120 mM KCl, 20 mM Hepes, 5 mM KH_2PO4, 2.5 mM $MgCl_2$, 1 mM EGTA and 0.3% BSA, pH 7.2 at 37 °C). The state 2 oxygen consumption rate was measured either in the presence of 5 mM glutamate and malate or in the presence of 5 mM succinate and 2 μM rotenone. State 3 oxygen consumption was obtained by adding 500 μM of ADP and 1 μg mL^{-1} of oligomycin was added to inhibit ATP synthase to measure oxygen consumption under state 4o (an oligomycin defined state 4 rate). To check the consistency of mitochondrial preparations, the respiratory control ratio was measured as the ratio of state 3 respiration to state 4o respiration. Additionally, a cytochrome c test was performed every day to evaluate the outer mitochondrial membrane integrity. During the isolation process, the mitochondrial outer membrane can be ruptured and as a result cytochrome c is lost from the intermembrane space. Loss of cytochrome c can therefore limit the oxygen

consumption rate by constraining electron flow between complexes III and IV. If addition of exogenous cytochrome c elevates the mitochondrial oxygen consumption rate that indicates damage of the mitochondrial outer membrane. To ensure the mitochondrial integrity was adequate only the samples passing the criteria of less than 15% activation of succinate fuelled respiration, in the presence of rotenone and ADP, by exogenous cytochrome c (10 µM) were used for further experiments.

Proton leak rates need to be compared at a common proton motive force and to do so we generated proton leak curves. The bulk of the proton motive force is made up of the membrane potential. We made relative estimates membrane potential using safranin-O dye based on the relative fluorescence quenching, described elsewhere [68]. This approach normalizes the relative fluorescence quenching under a specific condition of substrate oxidation to the total fluorescence measured in the presence of fully de-energized mitochondria. Liver mitochondria (0.3 mg/mL) were incubated with 2 µM rotenone, 1 µg mL^{-1} oligomycin, to inhibit ATP synthase and 5 µM safranin-O dye in respiration medium at 37°C. Once oxygen consumption and fluorescence signals stabilized, 5 mM succinate was added as a respiratory substrate followed by sequential additions of 0.5 mM malonate to inhibit substrate oxidation to generate proton leak curves.

Statistical analysis

Data were analysed using the JMP13 statistical software package. A Shapiro-Wilks test was performed to check the normality of the data and log transformed when needed. A generalized linear model (GLM) was used to analyse the interaction between diet and week which was followed by Tukey's post-hoc (parametric) or Steel-Dwass test (non-parametric) when the data

failed the assumption of normality. A $p<0.05$ was considered as significant. Data are presented as mean ± 1 standard error of the mean (SEM).

Of note, for the proton leak analysis we needed to compare the control and MR mitochondria at a common estimate of membrane potential, measured as $\Delta F/F_{total}$ in our case. Because the mitochondria from MR rats did not reach as high of membrane potential as the mitochondria from control animals we took the mean maximum fluorescence quenching with MR mitochondria (a $\Delta F/F_{total}$ value of 0.56) as our point of comparison. For mitochondria from the control animals, we estimated the rate of respiration for each individual by interpolating between the two $\Delta F/F_{total}$ values that were lower and higher than 0.56. For simplicity, we assumed a linear fit between these two values of $\Delta F/F_{total}$ in control mitochondria flanking 0.56. This approach would always provide some degree of overestimate for the values from control animals because of the nature of proton leak, which becomes non-ohmic at high potentials [55]. These estimates of respiration from control mitochondria were then compared using a Student's t-test ($p < 0.05$ being considered significant) to the rate of respiration in mitochondria from MR animals in the absence of malonate.

The data for all analysis are presented for the 4 and 8 weeks, or if no difference was evident between 4 and 8 weeks then the data were pooled. Exceptions to this are the mitochondrial respiration and proton leak data, where data are only available for the 4 week animals, as well as the protein carbonyl and malondialdehyde levels for whole liver tissue where data are available only for animals that were on the diets for 8 weeks.

Results

GSH and GSH metabolites in blood tissue

We initially examined GSH in the blood because it is now well established [14, 23] that MR in rodents leads to a paradoxical increase in GSH in the red blood cells , with most other tissue compartments showing a decrease in GSH. After just 4 weeks the red blood cell GSH concentration was higher in MR and this elevation in GSH was maintained for 8 weeks on MR (Table 3.1). Although 4 weeks of MR did not change plasma GSH, after 8 weeks MR decreased plasma GSH (Table 3.1). We also measured γ-glutamylcysteine, a key intermediate in GSH synthesis, in the plasma and erythrocytes with MR leading to no difference in the plasma at 4 weeks but causing a significant drop from control levels in the plasma by 8 weeks. The red blood cell level of γ-glutamylcysteine did not respond to MR (Table 3.1).

Table 3.1: Levels of GSH and GSH intermediate in blood tissue.

Tissue		4 weeks		8 weeks	
plasma	metabolite	control	MR	control	MR
	γGC	7.80±2.0	8.20±1.7	25.6±2.2[a]	13.8±1.3[*a]
	GSH	25.2±6.0	21.5±5.0	85.7±4.9[a]	43.3±3.9 [*a]
red blood cells	γGC	24.2±2.8	34.0±4.0	169±10 [a]	153±15 [a]
	GSH	2545±552	5116±325[*]	1424±206	3294±324 [*]

Values (μmol L^{-1}) are presented as mean±SEM (n=6-8), γ-glutamylcysteine- γGC
*Denotes significant difference between controls and MR at same time period ($p < 0.05$). [a] Denotes significant difference ($p < 0.05$) between same treatment at different time periods

GSH synthesis in liver

Having confirmed that we reproduce the expected rodent blood response to MR for GSH levels, we looked at the levels of GSH in liver tissue and found that MR decreased liver GSH content (Fig. 3.2A), which is also consistent with previous methionine restricted studies [14, 69]. We and others have reported that liver cysteine is decreased by MR following 8 weeks of diet and remained low throughout the feeding period (~30 months) [14, 22, 29]. Since, γ-glutamylcysteine is the intermediate metabolite between cysteine and GSH, we measured the levels of γ-glutamylcysteine in liver. After 8 weeks γ-glutamylcysteine levels declined in response to MR, but similar to plasma (Table 3.1), no change was evident after 4 weeks (Fig. 3.2B).

Production of GSH proceeds by two enzymatic steps, however, γ-glutamylcysteine synthetase is the regulatory enzyme in GSH synthesis [40]. Although the GSH content in liver declines in response to MR, the activity of γ-glutamylcysteine synthetase did not respond to MR (Fig. 3.2C).

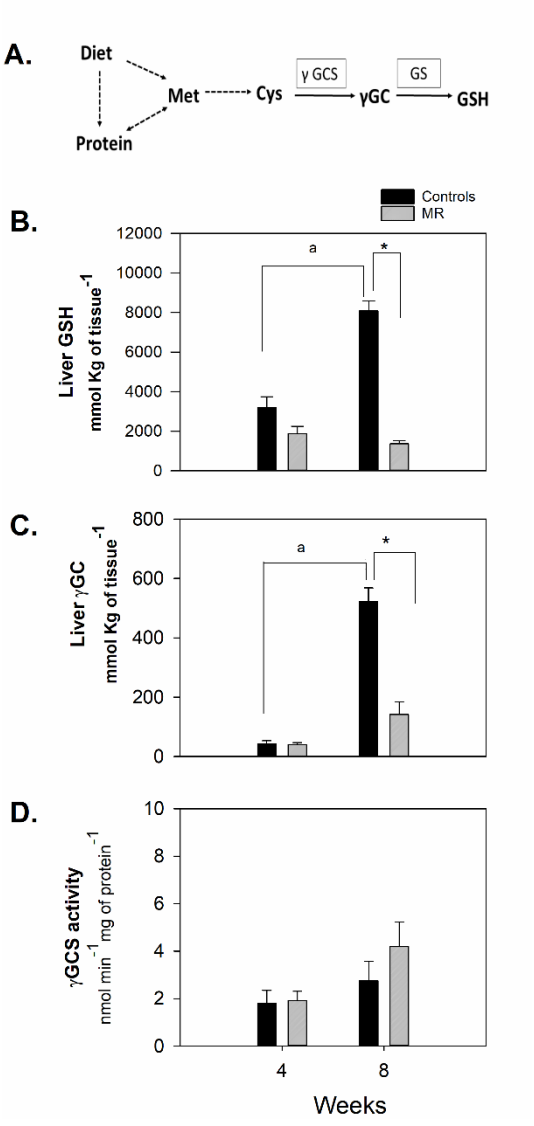

Fig. 3.2 The influence of methionine restriction (MR) on GSH metabolism in the liver. **A)** Simplified schematic of GSH synthesis **B)** liver GSH content **C)** γ-glutamylcysteine content, and **D)** activity of γ-glutamylcysteine synthetase. Solid and dashed arrows in A indicate a single step and multiple steps respectively; boxes in A denote enzymes, γGCS-γ: glutamyl cysteine and GS: glutathione synthetase. Values are in mean±SEM (n=6-8), (*, $p<0.05$ between the two diet groups ; [a] , $p<0.05$ same dietary group at different time points; GLM followed by Tukey's post-hoc or Steel-Dwass test)

Whole tissue level oxidative stress markers and antioxidant enzyme activities in liver

Oxidative stress markers

Protein carbonyls and malondialdehyde were evaluated as markers of protein and lipid damage respectively. Methionine restriction significantly decreased protein carbonyl content in liver (Fig. 3.3 A) whereas malondialdehyde levels did not change between two dietary groups after 8 weeks (Fig. 3.3B).

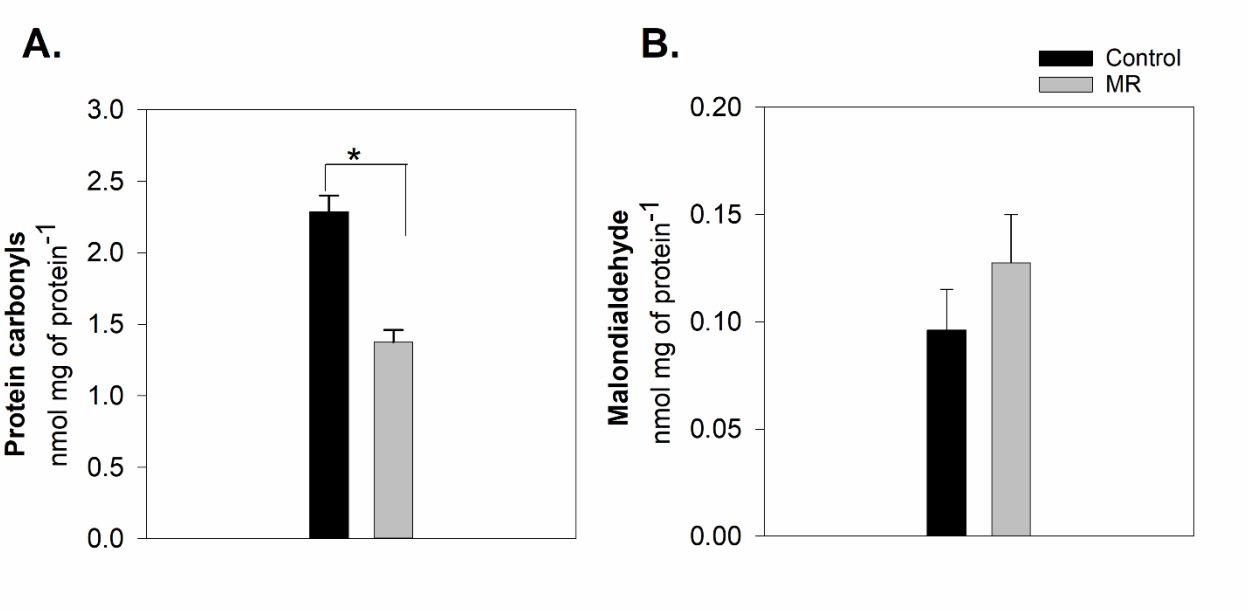

Fig. 3.3 Markers of oxidative modification in rat liver. A) Levels of protein carbonyl and **B)** malondialdehyde. Values are mean±SEM (n=6-8), (*, $p<0.05$, One-way ANOVA). Data are for 8 week liver samples only.

Thiol-dependant H_2O_2 consumption pathways

Since oxidative damage markers were either unchanged or even decreased by MR, despite greatly diminished GSH content, we examined if enhanced antioxidant enzyme capacity may compensate for low levels of the antioxidant GSH. In the liver, at the whole tissue level, the activity of glutathione reductase increased in both 4 and 8 weeks (Fig. 3.4A), while the activity of glutathione peroxidase was unchanged (Fig. 3.4B). Moreover, after 8 weeks of methionine restriction the activity thioredoxin reductase in liver also increased (Fig. 3.4C).

Factors that could influence ROS metabolism in mitochondria

Since others have shown that liver mitochondria from MR rodents show lower H_2O_2 emissions [31-34], we tested how two mechanisms that can alter mitochondrial H_2O_2 emissions respond to MR, with the first being altered enzymatic capacity to consume H_2O_2 with the other being changes in proton leak.

Glutathione and enzymes of thiol-dependant H_2O_2 consumption pathways in liver mitochondria

Lower tissue-level GSH content in the liver could lead to a decline in the mitochondrial GSH pool. However, despite having low GSH levels in whole tissue, mitochondria from methionine restricted rats maintain GSH at a level comparable to control animals (Fig. 3.5A). Similar to mitochondrial GSH levels, none of the antioxidant enzyme activities were altered by methionine restriction in liver mitochondria (Fig. 3.6 B, C, D).

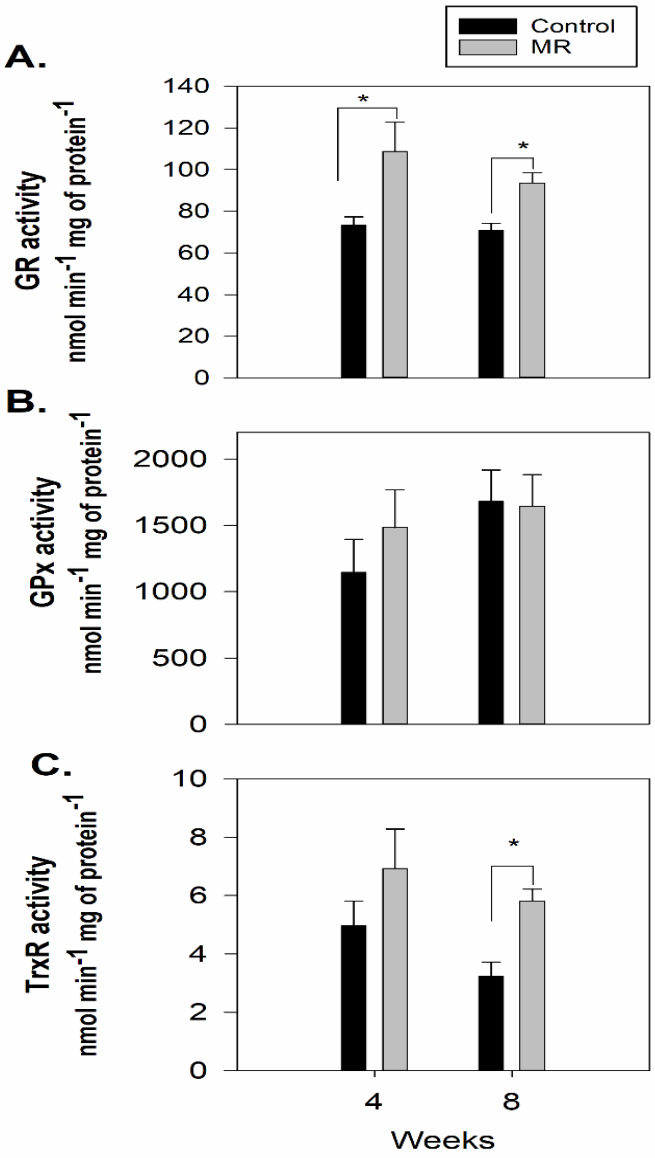

Fig. 3.4 Activities of thiol-linked enzymes associated with peroxide consumption in rat liver. **A)** Glutathione reductase (GR) activity, **B)** glutathione peroxidase (GPx) activity, **C)** thioredoxin reductase (TrxR) activity. Values are mean±SEM, (n=6-8), (*, $p < 0.05$ between the two diet groups ; GLM followed by Tukey's post-hoc or Steel-Dwass test)

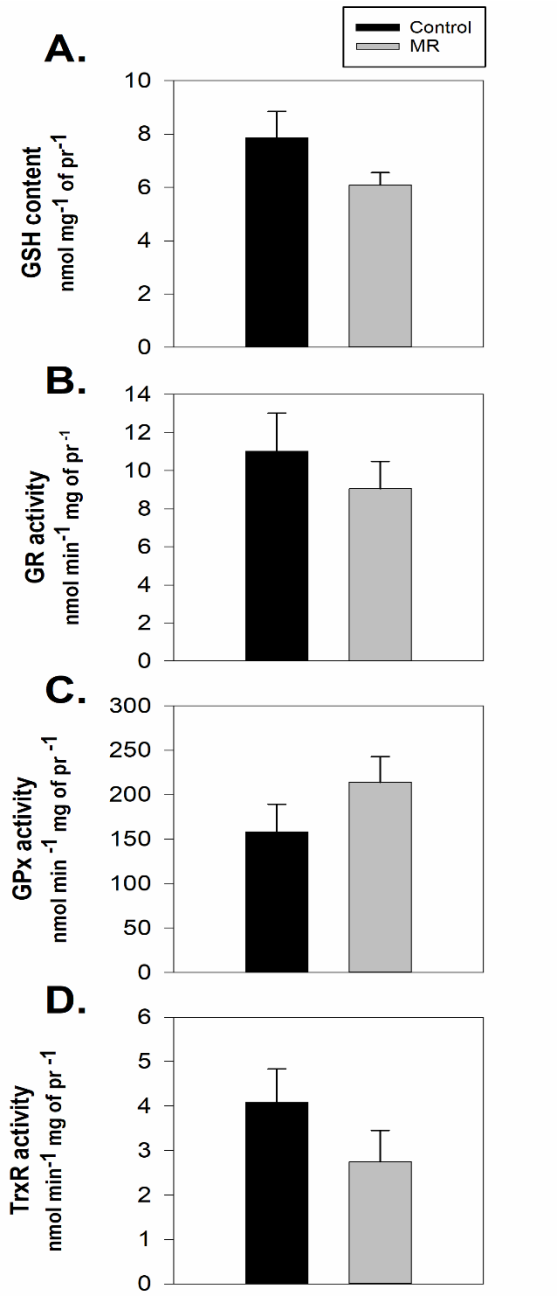

Fig. 3.5 GSH and antioxidant enzymes activities in isolated liver mitochondria. **A)** Glutathione content, **B)** glutathione reductase (GR) activity, **C)** glutathione peroxidase (GPx) activity, **D)** thioredoxin reductase activity (TrxR). Samples from weeks 4 and 8 were pooled. Values (μmol L^{-1}) are presented as mean±SEM (n=6-7).

Respiration and proton leak in liver mitochondria

Liver mitochondria from control and MR rats have similar respiration rates using either complex I-linked substrates (glutamate-malate) or a complex II specific respiratory condition (succinate plus rotenone) (Fig. 3.6 A-B), however, MR led to a lower respiratory control ratio for mitochondria respiring on glutamate + malate (Fig. 3.6A) which suggests a shift in coupling efficiency. When steady state respiration was compared while accounting for the effect of membrane potential on proton influx, higher respiration rates indicate that MR increases basal proton leak in liver mitochondria (Fig. 3.6 C).

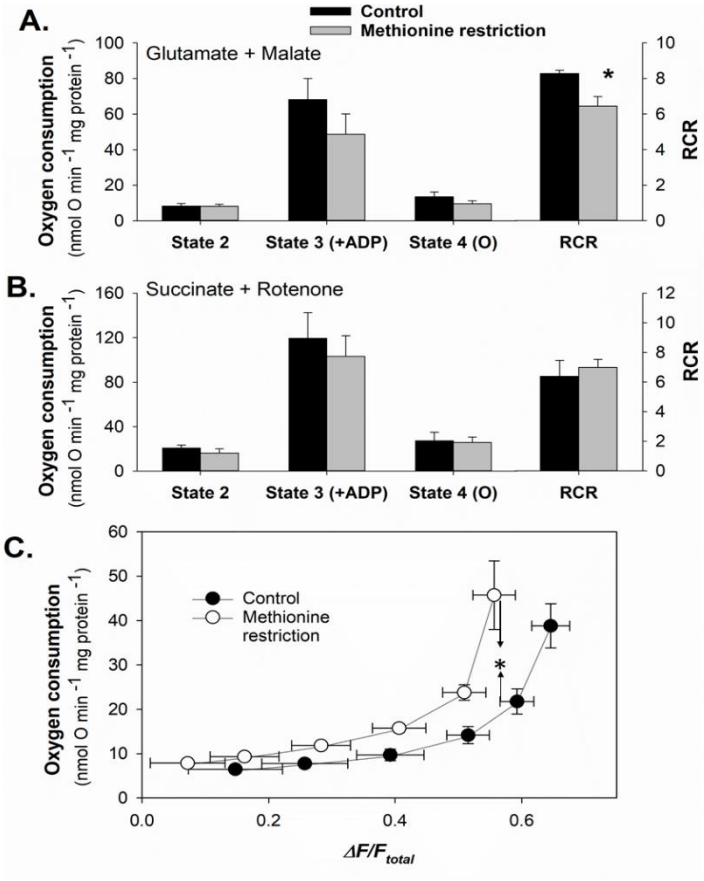

Fig. 3.6 Comparison of substrate oxidation and proton leak in isolated liver mitochondria from MR and control rats. A) Glutamate-Malate dependant state 2, 3 and 4o respiration rates. **B)** Succinate dependant state 2, 3 and 4o oxygen consumption rates. **C)** Proton leak curves with mitochondria respiring on succinate. The relative fluorescence quenching with safranin O ($\Delta F/F_{total}$) is a relative measure of mitochondrial membrane potential. Data obtained from 4 week liver samples (n=4), and are presented as mean±SEM, (*, $p < 0.05$ between the two diet groups, One-way ANOVA)

Discussion

Methionine restriction is known to decrease hepatic GSH content in rodents as early as 4 weeks on this diet with GSH remaining low throughout the dietary restriction (2-32 months) [14, 17, 21-25]. Moreover, MR is known to increase red blood cell GSH level [15, 22]. Our results show the same responses demonstrated by previous MR studies, indicating that animals from current study recapitulate the MR phenotype associated with increased lifespan in rats.

Given that liver is the major organ responsible for synthesizing and exporting GSH in rodents [40], we focused on aspects of GSH synthesis in this tissue in response to MR. The synthesis of GSH depends on multiple factors, with precursor availability being one of those factors of particular relevance. Limited availability of substrates such as cysteine and glutamate can decrease the GSH pool without affecting enzyme activities [70]. Previous studies reported that MR decreases hepatic cysteine and glycine levels, but not glutamate [18, 29], suggesting the availability of limiting amino acids can affect GSH synthesis. In the current study, we found that the activity of γ-glutamylcysteine synthetase, the rate limiting enzyme of GSH synthesis, did not change in response to MR. Depleted GSH with unchanged γ-glutamylcysteine synthetase activity is similar to other diet limited circumstances; for instance, while rats were starved for 48 hours, hepatic GSH declined without altering GSH synthesizing enzyme activities [70]. Of note, the enzymes involved in GSH synthesis also take part in opthalmic acid synthesis which is a structural analogue of GSH and has a 2-aminobutyrate groups instead of cysteine [71, 72]. It has been suggested that ophthalmic acid is a by-product of glutathione synthetase when cysteine and GSH levels are low [71]. Several studies have found that elevated ophthalmic acid is an indicator of low GSH,

however, there have been some reports where this pattern was not found (reviewed in [73]); nevertheless ophthalmic acid appears as a functional analogue of GSH where thiol groups are not required, i.e. transporting of exogenous glucuronide conjugates [71]. Methionine restriction decreases hepatic GSH level but increases opthalmic acid in both C57BL/6J mice and F-344 rats [18, 21]. Taken together, this is consistent with low liver GSH due to MR and GSH synthetic capacity is not a result of impaired γ-glutamylcysteine synthetase activity, rather it appears to be from low cysteine availability favouring ophthalmic acid formation over GSH synthesis.

Cysteine availability can affect GSH synthesis and on the defined diets used in rodent MR studies, where methionine is the only exogenous source of sulfur amino acids, transsulfuration is a necessary pathway to replenish cellular cysteine. The responses of upstream metabolites relevant to the transsulfuration pathway reported by different MR studies were varied among species. For instance, C57BL/6J mice or Ames dwarf/wild type mice both were reported to have elevated levels of hepatic cysteine compared to their methionine sufficient controls [21, 24]. On the contrary, hepatic cysteine content decreases in MR with F-344 rats which has been reported by me [29] and others [18, 22, 57]. Different species, genotypes or methionine levels used in the control/MR diet might explain the inconsistency found in metabolites level as well in phenotypes. Therefore, the responses noticed in one species might not necessarily explain the underlying mechanisms in other species on MR diets, and hence assumptions across the species level may be unreliable even for relatively closely related species like rats and mice.

A substantial decline in the tissue level of a major antioxidant like GSH might expose cellular systems toward a more oxidative environement, as argued by Ghosh *et al*. [21]. Therefore, we have

assesed protein carbonyl content and the lipid oxidation product as candidate markers of oxidative stress. Despite a decline in GSH content, protein oxidation was significantly lower in methionine restricted liver compared to controls (Fig. 3A) and malondialdehyde was not different between methionine restricted and control animals. This suggests that liver cells of methionine restricted animals experience no more oxidative damage than control animals and this observation is in agreement with the low mitochondrial macromolecular damage reported by previous studies [31-34]. Lack of oxidative damage in conjunction with low tissue GSH level could be explained by either an upregulation of antioxidant pathways, production of less ROS or a combination of both. For these reasons, we focused on an alternative thiol-dependant antioxidant pathway in liver tissue and possible alterations to ROS release from mitochondria.

The increased activity of glutathione reductase in liver at the whole tissue level (Fig. 3.4A) might be a benificial mechanism for methionine restricted animals to enhance GSH use under conditions of lower GSH availability. In addition, thioredoxin reductase activity in the liver increased by 8 weeks of methionine restriction. Of note, both glutathione and thioredoxin demonstrate overlapping function in consuming H_2O_2. Altough it is generally considered that the glutathione and thioredoxin systems work independently, recent evidence shows that the glutathione and thioredoxin systems may not be completely independent, rather they may interact to some extent and components from these two pathway might compensate for each other (reviewed in [74]). For instance, the glutathione system can reduce human recombinant thioredoxin *in vitro* when thioredoxin reductase was inhibited [75] and the thioredoxin system functions as an alternate system to reduce oxidized GSH in *Saccharomyces cerevisiae* and can rescue GSH deficiency in γ-glutamylcysteine synthetase knock out mouse embryonic cells [76, 77]. Therefore, we suggest that

increased thioredoxin reductase activity might be a compensatory mechanism in response to low GSH to deal with oxidative stress in methionine restricted animals.

Following assessing the antioxidant enzyme activities at the whole tissue level, we focused on mitochondrial ROS regulation because mitochondria are a significant source of ROS in most animal cells and previous studies demonstrated that MR lowers ROS production, measured as H_2O_2 emission from isolated liver mitochondria [31-34]. However, neither GSH content nor any mitochondrial antioxidant enzyme activities were changed by MR (Fig. 3.5). Mitochondria do not have the enzymes needed to synthesize GSH [49, 78, 79]; therefore, they rely on cytosolic GSH for matrix and intermembrane space GSH and depletion of this mitochondrial GSH is associated with oxidative stress [49, 78, 79]. Therefore, unaltered GSH in mitochondria suggests that the influx of GSH from cytosol to mitochondria is not impaired by MR. This preferential maintenance of mitochondrial GSH in the face of declining extra-mitochondrial GSH may specifically protect the mitochondria from possible oxidative damage. Importantly, we saw no effect of MR on antioxidant enzyme activities in isolated mitochondria, which means lower ROS release by mitochondria from MR rat liver [31-34] is not likely due to increased consumption of H_2O_2 before it escapes the mitochondrion. It is known that antioxidants consume reactive oxygen species (ROS) whereas proton leak in mitochondria can regulate ROS production. Since, MR enhances proton leak in liver mitochondria it can be suggested that the mild uncoupling induced by MR will reduce the protonmotive force which in turn will direct electron carriers and redox centres in enzyme complexes to a more oxidized state and thus ultimately will reduce ROS production. Futile cycling of proton pumps and proton leak can cost approximately 20 to 25% of a rat's basal metabolic rate [78] and increased proton leak can also account for heat increment due to decreased efficiency of

mitochondrial respiration [55]. Therefore, the increased metabolic rate with concomitant increase of core body temperature reported by previous MR studies [27] could also be related to the increased basal proton leak induced by MR along with the proliferation of brown adipose tissue and uncoupling protein 1 [80].

Conclusions

In the liver of MR rats, the lack of oxidative stress despite low GSH could be due to a mix of increased antioxidant activity (glutathione reductase and thioredoxin reductase) in whole tissue and altered ROS formation via increased proton leak in the mitochondria. The stability of liver mitochondrial GSH content in MR rats further suggests specific importance of maintaining intramitochondrial ROS regulation perhaps at the cost of GSH in other cellular compartments. Therefore, with methionine restriction the liver appears to alter cellular metabolism to compensate or at least accommodate for low GSH, which fits the contention that this type of nutritional intervention may act though protecting the cellular redox environment, despite the lower GSH availability at the whole tissue level.

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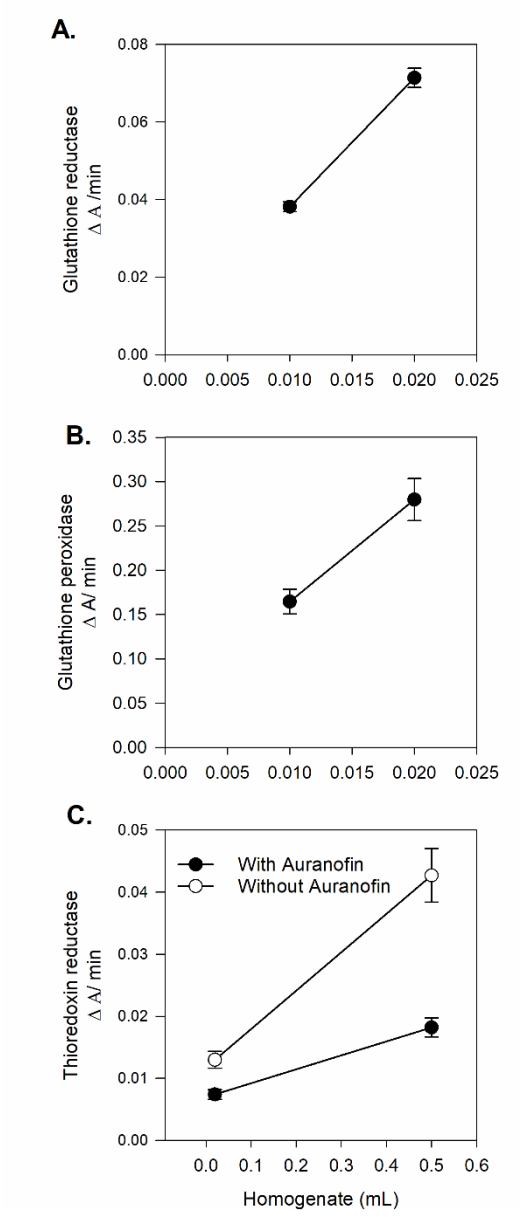

Supplementary Fig 3.1. Enzyme assay optimization**.** Y axis from A, B and C respectively shows the absorbance changes following the addition of different volumes of homogenate (X axis) were linear for a given enzymatic assay.

Chapter Four: Inhibitors of thioredoxin reductase and lipoamide dehydrogenase impair intramitochondrial hydrogen sulphide formation measured as mercaptopyruvate sulfurtransferase dependant mitochondrial respiration

Nahid Tamanna[a] and Jason R. Treberg[a,b]

[a] Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada.

[b] Centre on Aging, University of Manitoba, Winnipeg, MB, Canada.

Author contributions: NT and JRT designed the experiments, NT did the experiments and analyzed the data, NT and JRT wrote the manuscript

Abstract

Hydrogen sulphide (H_2S), which was viewed as a poisonous gas for decades, is now emerging as an important signalling molecule. The use of both exogenous H_2S donors and endogenous H_2S producers are receiving simultaneous attention in therapeutic and aging research. Endogenous H_2S is produced from three enzymes; cystathionine-β-synthase, cystathionine-γ-lyase and mercaptopyruvate sulfurtransferase (MPST). Recent studies demonstrated that H_2S enhances mitochondrial bioenergetics while MPST is considered as the sole mitochondrial H_2S producer. However, other H_2S producers have also been reported in mitochondria suggesting MPST might not be only enzyme which enhances respiration rate. Isolated MPST requires reductants in releasing H_2S *in vitro*; however, the need of these reductants in MPST mediated release of H_2S with intact and functional mitochondria has not yet been shown. The current study investigates the predominant liver mitochondrial H_2S producer(s), where MPST is shown to have far greater potential to produce H_2S. After demonstrating that MPST requires a chemical reductant *in vitro*, it was assumed that biological reductants of MPST might be needed during respiration. Our results demonstrated that MPST has the maximum capacity to produce H_2S in rat liver mitochondria and requires thioredoxin and dihydrolipoic acid as biological reductants to enhance mitochondrial bioenergetics.

Keywords: H_2S, mitochondria, bioenergetics, mercaptopyruvate sulfurtransferase, respiration

Introduction

Hydrogen sulphide (H_2S) is well recognized as an environmental hazard, however, this perspective has changed as studies have demonstrated that, at appropriate intracellular and extracellular levels, H_2S has beneficial aspects on numerous pathophysiological conditions including cardiovascular and nervous diseases, in delaying aging processes and as well potentially acting as an anticancer agent (reviewed in [1, 2]). Numerous cellular sources of endogenous H_2S production are known, with three enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CGL) and mercaptopyruvate sulfurtransferase (MPST) being the major sources in animal cells. The first two enzymes, CBS and CGL, also take part in transsulfuration where homocysteine is converted to cysteine while MPST participates in the cysteine desulfhydration pathway [3, 4]. All three enzymes are found in a number of tissues including liver and kidney (reviewed in [5]). The subcellular localization of these enzymes can vary, for instance; CBS and MPST were reported in both cytosolic and mitochondrial fractions of rat liver mitochondria whereas CGL was reported to predominantly occur in the cytosol [6]. In mouse brain tissues this pattern was different, MPST and CBS were reported in the mitochondrial and cytosolic fraction respectively whereas CGL was absent in both cytosol and mitochondria [7].

Whether imported from the cytosol or synthesized within the mitochondria, H_2S can be broken down into a non-toxic form via the mitochondrial sulphur oxidation pathway which couples electron removal from H_2S to mitochondrial oxidative phosphorylation [8]. Sulfide quinone reductase (SQR) is the first enzyme in the sulphur oxidation pathway and reduces ubiquinone (Q) upon receiving electrons from H_2S. The subsequently formed reduced Q donates electrons to complex III of the respiratory chain thereby allowing H_2S consumption by mitochondria to

contribute to respiration [8-10]. Therefore, measurement of mitochondrial oxygen consumption can be a potential proxy measure of H_2S availability within isolated mitochondria. For example, when isolated liver mitochondria were incubated with succinate and rotenone, addition of 3-mercaptopyruvate (3-MP) as a H_2S producing substrate causes an increment of both phosphorylating and uncoupler mediated respiration rates [11].

Recent studies demonstrate that either direct administration of exogenous H_2S liberating sodium salts (NaHS) or the substrates of endogenous H_2S synthesis alter mitochondrial respiration rate [6, 11, 12]. Specifically, addition of 3-MP, cysteine or cysteine plus α-ketoglutarate has been reported to increase respiration at low concentrations and decrease respiration rates at high concentrations [6, 11, 12]. While both CBS and CGL can directly use cysteine to produce H_2S, MPST requires an additional enzyme, cysteine aminotransferase (CAT) which converts cysteine and α-ketoglutarate to 3-MP and glutamate. The 3-MP is subsequently used by MPST to generate H_2S and pyruvate [3]. Therefore, all three enzymes rely on cysteine to some extent to produce endogenous H_2S which in turn can theoretically enhance the respiration rate. However, cysteine (10-1000 μM) dependant increases in mitochondrial respiration rate in the presence of 10 mM succinate is thought to be a result of MPST derived H_2S production [6]. Since rotenone was absent in the assay medium in this study, and because MPST can also produce pyruvate from cysteine via 3-MP produced by the CAT reaction, there is a possibility that electron donation from pyruvate, rather than just H_2S, enhances the respiration rate in rat liver mitochondria [6]. If H_2S, obtained from cysteine enhances respiration rate there is an alternative possibility that cysteine dependant increases in respiration can also be mediated by CBS and CGL depending on the availability of these enzymes in rat liver mitochondria.

There is a complex pattern between H_2S and mitochondrial respiration. The addition of 3-MP, or direct addition of H_2S liberating sodium salts can either increase or inhibit mitochondrial respiration depending on the concentration of H_2S generating substrates added. The interaction between the mechanisms involved warrants some further explanation. Of note, at low levels H_2S donates electrons to SQR, however at high levels H_2S inhibits complex IV thus inhibiting respiration [6]. Therefore, H_2S production can be monitored via O_2 consumption experiments. Therefore, low doses of 3-MP (10-100 μM) have been shown to stimulate respiration whereas high doses (300-1000 μM) inhibit respiration in isolated rat liver mitochondria [11]. A similar pattern is seen with H_2S liberating sodium salts indicating that H_2S released from 3-MP is likely acting as an electron donor to the electron transport chain via SQR and thus increasing electron flow to oxygen reduction. Therefore, as long as H_2S concentration is low the H_2S is readily oxidized by SQR which is coupled to respiration. However, if concentrations get too high then H_2S interferes with O_2 binding at complex IV and thus inhibits the respiration rate [6].

Mercaptopyruvate sulfurtransferase is expected to require accessory reductants to release H_2S because recombinant MPST only produces appreciable H_2S from 3-MP in the presence of reducing agents like dihydrolipoic acid (DHLA), thioredoxin or dithiothreitol [7]. Mikami *et al*. (2011) further demonstrated that while sonicated mouse brain mitochondria are incubated with either 3-MP or the combination of cysteine plus α-ketoglutarate, in presence of varied amount of thioredoxin or dihydrolipoic acid, H_2S is produced in a concentration dependant manner [7]. It is suggested that following transfer of the thiol from 3-MP, persulfide is formed on the active site of MPST which is released as H_2S in the presence of additional reductants [7]. Based on this context,

we hypothesized that if MPST enhances respiration rate by producing H_2S from 3-MP, there may be a physiological requirement for intramitochondrial reductants to maximally stimulate the respiration. Interruption in the supply of potential reductants should cause MPST meditated respiration to decline. Therefore, in the current study we questioned whether functional mitochondria need any reductant to produce H_2S which in turn enhances mitochondrial respiration rate.

While H_2S production capacity can vary by tissue [5], as well as with differences occurring across organelles [6, 7], one of the best studied systems to date is the liver [6, 11]. For example, liver mitochondria have been well studied for monitoring H_2S release via changes in respiration [11], therefore we focused on liver mitochondria to understand H_2S metabolism. In the current study, we initially investigated whether liver mitochondria have more than one substantial producer of H_2S and if so, which should be the major mitochondrial H_2S producer. For this, we used both physiological and supraphysiological levels of homocysteine, cysteine and 3-MP where the first two are the direct substrates of CBS and CGL, and the third one, 3-MP is the substrate of MPST. Having found that MPST has vastly greater potential to make H_2S and that MPST requires the chemical reductant DTT to release H_2S *in vitro*, we investigated whether MPST requires any physiological reductant *in situ* within a functional mitochondrion and, if so, to test what reductants may be involved. For that, we followed a strategy where pharmacological inhibition of electron supply to MPST from physiological reductants, i.e., thioredoxin or DHLA were induced and if, MPST require those reductants on H_2S generation the effect will be evident by a declined oxygen consumption rate. This study design will identify whether thioredoxin or DHLA act as physiological candidate(s) for MPST within intact mitochondria.

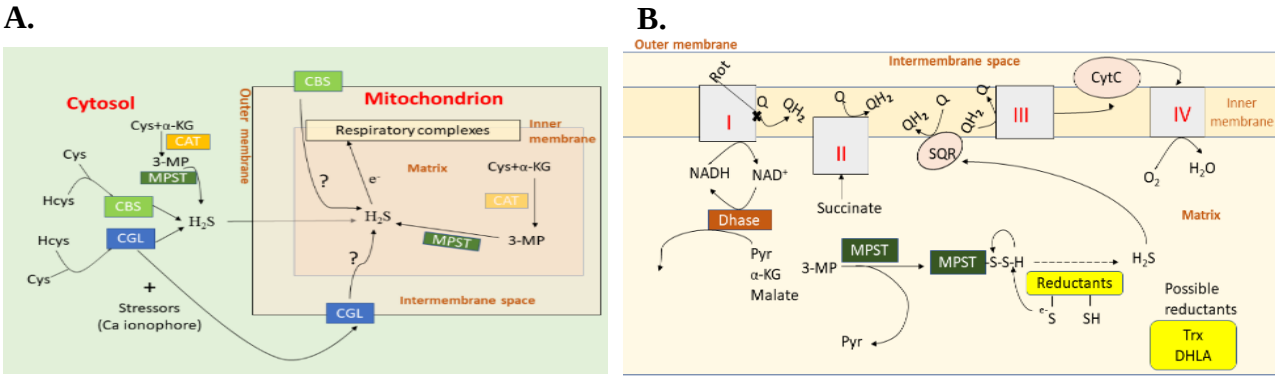

Fig. 4.1. H_2S production from enzymes. A) Cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CGL) produce H_2S from homocysteine (Hcys) and cysteine (Cys) in the cytosol which can diffuse through the mitochondrial membrane and can be oxidized. CBS is also localized to the outer membrane of mitochondria whereas CGL moves toward mitochondria during stress conditions. MPST produces H_2S from 3-mercaptopyruvate (3-MP), which is the product of cysteine aminotransferase (CAT). **B)** Schematic of mitochondrial H_2S metabolism. The mitochondrial electron complex is comprised of four respiratory complexes (I-IV), of which complex I derives electrons from pyruvate (Pyr), α-ketoglutarate (α-KG) or from any NADH generating substrates, whereas succinate is the electron donor for complex II. Both complex I and II donate electrons to the Q pool (ubiquinone) which subsequently binds to complex III and transfers electrons there. From there cytochrome c (Cyt C) accepts the electrons and transfers the electrons to complex IV which is ultimately taken by O_2 and H_2O is formed. From meracaptopyruvate sulfurtransferase (MPST), both pyruvate and H_2S are formed. If rotenone is added in the assay medium, any electron flow from complex I will be inhibited and thus pyruvate will not donate electrons to the electron transport chain. Following its synthesis, H_2S is oxidized by sulfide quinone reductase (SQR) and thus electrons from H_2S are transferred to the Q pool. Likewise, electrons derived from complex I and II, reduced Q from SQR electron donation, binds to complex III and thus enhances respiration. MPST might need reductants to release H_2S inside the mitochondria, it is not known whether thioredoxin (Trx) and dihydrolipoic acid (DHLA) are the possible candidates.

Materials and methods

Reagents

Sodium mercaptopyruvate was purchased from Santa Cruz biotechnology (Dallas, Texas, USA). All other reagents were purchased from Sigma-Aldrich (Canada).

Animals and mitochondrial isolation

All procedures were approved by the Animal Care Committee at the University of Manitoba, Canada. Male Sprague-Dawley rats (180-250 g) were housed in a controlled environment (22±1°C, 12h light-dark cycle) at the Biological Sciences Animal Holding Facility. Rats had free access to laboratory chow diet and water. Rats were killed by CO_2 asphyxiation and dissected liver was collected in liver isolation buffer (details are discussed below).

Liver mitochondria were obtained by differential centrifugation following a previously described method with some modifications [3]. The liver was minced and homogenized with 55 mL of isolation buffer (75 mM sucrose, 255 mM mannitol, 10 mM hepes, 1 mM EGTA, pH 7.2 at 25°C). In addition, 0.5% of bovine serum albumin was added to the isolation buffer prior to homogenization to minimize the effect of lipolysis during the initial preparation of the sample. Homogenization was performed sequentially using loose, medium and tight-fitting pestles in a 55 mL mortar (Wheaton 55 mL, Potter-ELV Ctd). Following homogenization, the suspension was centrifuged at 1000 × g for 10 mins to remove nuclei and cell debris and the supernatant centrifuged at 10,000 × g for 10 mins. Any overlaying lipid was removed, and pellets were re-suspended with 15 mL of isolation buffer taking care to not resuspend underlying red blood cells and other non-mitochondrial material that sedimented below the mitochondrial layer. The

suspension was centrifuged at 1000 x g for 10 mins and supernatant was collected. The supernatant was centrifuged at 10,000 x g for 10 mins and subsequently mitochondria were obtained by suspending the pellet. Mitochondrial protein content was measured by Pierce BCA kit (Rockford, Illinois, USA) using bovine serum albumin as a standard. Fresh isolated liver mitochondria were used for respiration and membrane potential measurements whereas frozen mitochondria were used to estimate H_2S production capacity.

H_2S production capacity

For CBS and CGL derived H_2S production capacity, homocysteine or cysteine were tested as H_2S generating substrates either individually or in combination [3, 13]. In brief, ~3-5 mg/mL of freeze-thawed liver mitochondria were added to a 25 ml Erlenmeyer flask which was then flooded with N_2 gas for 20 secs and immediately capped with a septum stopper containing a hanging centre-well. The centre-well contained a piece of filter paper soaked in an alkaline zinc acetate solution ((15:1; 1% (w/v) zinc acetate: 12 % (w/v) NaOH)). Erlenmeyer flasks were pre-incubated in a shaking water bath for 3 mins at 37°C followed by the addition of reaction mixture containing (final concentrations) 0.1, 0.2 or 10 mM of homocysteine or cysteine individually or in combination, 2 mM pyridoxal phosphate and 0.05 mM of S-adenosyl methionine (all made in 100 mM, pH 7.4 potassium phosphate buffer solution) which were injected through the septum stopper in the Erlenmeyer flask. In reaction blanks, substrates were omitted but the same volume of 100 mM potassium phosphate buffer was added. After 30 min of incubation at 37°C in a shaking water bath the reaction was stopped by injecting 500 µL of 30% (w/v) trichloroacetic acid. Flasks were left mixing at room temperature for 1 hr to collect any remaining H_2S. Filter paper strips were then placed in a test tube containing 3.5 mL of H_2O. Color formation reagent, consisting of 0.4 mL 20

mM N,N-dimethyl-p-phenylene diamine sulphate (dissolved in 7.2 M HCl) and 0.4 mL of 30 mM $FeCl_3$ (dissolved in 1.2 M HCl) were added, tubes were mixed and the reaction was carried out for 10 min in the dark. Following incubation with color formation reagent, the absorbance at 670 nm was measured. Sodium hydrosulfide was used as a standard for H_2S detection and concentrations between 2.5 to 100 μM were used as the standards [3]. To asses H_2S production capacity by MPST, 3-5 mg/mL of mitochondria were added in all Erlenmeyer flasks and in some flasks 10 mM dithiothreitol was added. Then the flasks were covered in the same way as described for the CBS and CGL assays and were placed in a shaking water bath at 37°C. After 3 mins 10, 30, 100 or 500 μM of 3-MP were added in different flasks. In all cases the effect of an exogenous reducing agent, dithiothreitol, on H_2S formation was tested. For a reagent blank, dithiothreitol was added without any 3-MP. Following 30 mins of incubation, the reactions were stopped by 30% (w/v) of trichloroacetic acid and followed the same procedure to estimate H_2S production as described for CBS and CGL.

Mitochondrial Oxygen Consumption

The rate of mitochondrial oxygen consumption was measured at 37°C with a computer-controlled Oroboros O2K (Oxygraph, Innsbruck, Austria) in 2 mL of respiration medium (120 mM KCl, 20 mM Hepes, 5 mM KH_2PO4, 2.5 mM $MgCl_2$, 1 mM EGTA and 0.3% BSA, pH 7.2 at 37°C). Mitochondrial oxygen consumption rates were measured at different respiratory states: 2, 3 and 4o. In state 2, substrates are oxidized without ATP synthesis whereas state 3 occurs in the presence of ADP and therefore ATP is synthesized while in state 4o oligomycin, an inhibitor of ATP synthase (complex V), blocks the contribution of ATP synthesis to respiration. State 2 and 4o can also be viewed as non-phosphorylating states of respiration while state 3 can be addressed as a

maximally ADP-stimulated phosphorylating respiratory state. Under non-phosphorylating states, respiration is largely under the control of the substrate oxidation capacity maintaining the protonmotive force in the face of any proton leak or other passive dissipation of the electrochemical gradient. Under phosphorylating respiratory conditions, the control over respiratory flux is shared between the substrate oxidation capacity and the phosphorylation system. The respiratory control ratio (state 3/state 2), was used everyday to check for consistency in the mitochondrial integrity. Additionally, a cytochrome c test was performed daily to evaluate the outer mitochondrial membrane integrity. During the isolation process, the mitochondrial outer membrane can be ruptured and as a result cytochrome c can be lost from the intermembrane space. Loss of cytochrome c can therefore limit the oxygen consumption rate by constraining electron flow between complex III and IV. If the addition of exogenous cytochrome c elevates the mitochondrial oxygen consumption rate that indicates damage of the mitochondrial outer membrane. To ensure the mitochondrial integrity was adequate only the samples passing the criteria of less than 15% activation of succinate fuelled respiration, in the presence of rotenone and ADP, by exogenous cytochrome c (10 μM) were used for further experiments.

Following the addition of mitochondria, in all experiments 10 mM succinate and 2 μM of rotenone were added. Then state 2 oxygen consumption rate was measured either in presence or absence of 3-MP, cysteine or homocysteine. State 3 and state 4o respiration rates were estimated by the sequential addition of 500 μM of ADP and, following stabilization of rates, 1μg mL^{-1} of oligomycin, respectively.

Thioredoxin and dihydrolipoic acid dependant oxygen consumption

To investigate the dependency of 3-MP enhanced respiration on reduced thioredoxin or dihydrolipoic acid, auranofin and 5-methoxy indole 2-carboxylic acid were added in the respiration medium which are the inhibitors of thioredoxin reductase and lipoic acid reduction process, respectively.

Membrane potential

The protonmotive force, Δp, is mostly comprised of membrane potential in mitochondria [14], therefore membrane potential was used as a proxy measure of the proton motive force. Relative mitochondrial membrane potential was measured simultaneously with oxygen consumption using an Oroboros O2K in presence of florescence intensity, using wavelengths of 525 and 620 nm for excitation and emission respectively, based on the method described by Treberg *et al*. [15] with minor modifications. Tetramethylrhodamine methyl ester fluorescence was used as an indicator of membrane potential. Briefly, in absence of any mitochondrial polarization, tetramethylrhodamine methyl ester emits high fluorescence. When mitochondria are energized by the presence of respiratory substrates, the mitochondria become polarized leading to a membrane potential dependent up take of tetramethylrhodamine methyl ester into the matrix. Under these assay conditions tetramethylrhodamine methyl ester accumulation within the mitochondrion leads to a shift in fluorescence and an overall quenching, or decrease, in fluorescence strength. The relative changes in fluorescence quenching was normalized to total fluorescence in unenergized mitochondria according to the method described Treberg *et al*. [15]; however, instead of carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone as the uncoupler [15], in the current experiment,

carbonyl cyanide *m*-chlorophenyl hydrazone was used. An experimental run progressed in the following sequence: following the stabilization of analytical signal, 2.5 μM of tetramethylrhodamine methyl ester (dissolved in dimethyl sulfoxide) was added in the respiration buffer. Once fluorescence and respiration rates stabilized, 10 mM of succinate, 2 μM of rotenone and 100 μM of 3-MP were added to one of the two chambers while in the other chamber everything except 3-MP was added. Addition of these substrates energizes mitochondria and thus decreases the tetramethylrhodamine methyl ester fluorescence signal. Following stabilization of the respirometry and fluorescence signals, 500 μM of ADP was added to both chambers which causes depolarization as protons re-enter the matrix with a simultaneous phosphorylation of ADP. A portion of tetramethylrhodamine methyl ester exits the matrix because of the depolarization and thus emits more fluorescence. Once the phosphorylating state 3 rate is stabilized, oligomycin was added, which inhibits ADP phosphorylation; thus tetramethylrhodamine methyl ester again re-accumulates in the matrix and a weaker fluorescence signal is found. Finally, we added carbonyl cyanide *m*-chlorophenyl hydrazone to completely depolarize the mitochondria and which shows the maximum fluorescence.

Statistical analyses

SigmaPlot 13 software was used for statistical analysis. Data were analysed either by paired t-test or One-way ANOVA, repeated measures when appropriate, followed by Bonferroni correction when needed for multiple pair-wise comparisons, with $p<0.05$ being considered as significant. Data are presented as mean ± 1 standard error of the mean (SEM).

Results

H_2S production capacity by liver mitochondria

It has been reported that mitochondria contain a higher concentration of cysteine than the cytosol [16]. Therefore, we used both low and high concentrations of cysteine to study mitochondrial H_2S production capacity. Isolated liver mitochondria had the capacity to produce H_2S in the presence of high concentrations of homocysteine (10 mM) and cysteine (10 mM) and the H_2S production capacity increased when 10 mM homocysteine and 10 mM of cysteine were combined (Fig. 4.2A). We could not quantify H_2S production capacity in presence of more physiologically relevant cysteine or homocysteine concentrations of either 100 μM or 200 μM individually or in combination (Fig. 4.2A). Of note, 3-MP derived H_2S production depends on dithiothreitol, in the absence of dithiothreitol negligible amount of H_2S production was measured. Contrary to homocysteine and cysteine, 3-MP at micromolar concentrations could produce H_2S and the H_2S production capacity increased concomitantly with elevating concentrations of 3-MP (Fig. 4.3B). This pattern is similar to a previous study where sonicated mouse brain mitochondria produced substantial amounts of H_2S but only in the presence of dithiothreitol [7].

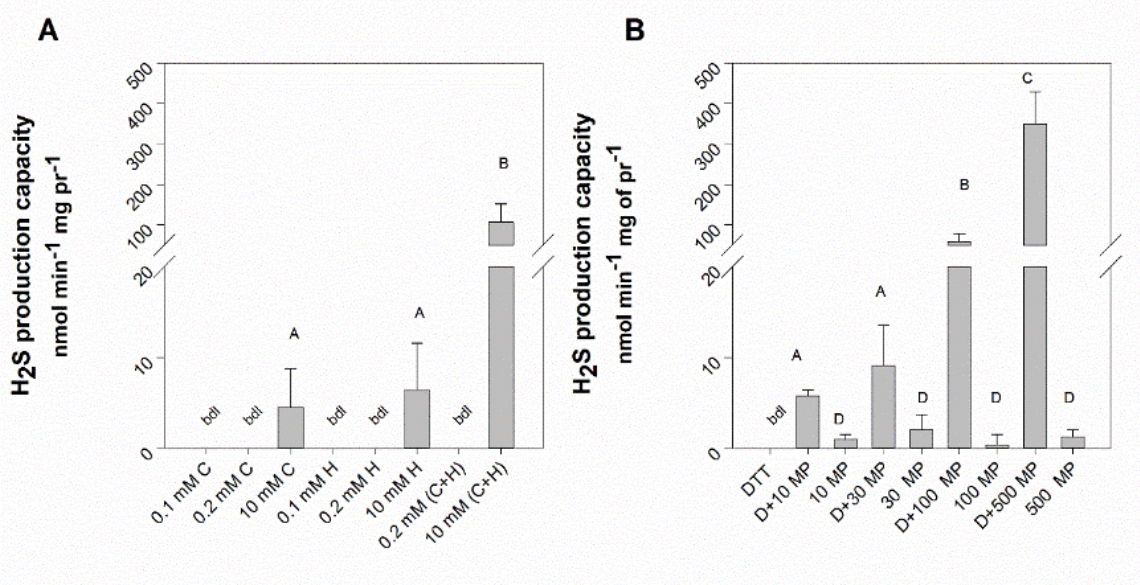

Fig. 4.2. H_2S production capacity by isolated liver mitochondria. **A**) H_2S production capacity by liver mitochondria was estimated in the presence of cysteine (C) and homocysteine (H), **B**) 3-MP derived H_2S production capacity was estimated either in presence or absence of dithiothreitol labelled as DTT and D. Data are presented as mean ± 1 SEM, n=3, (different letters denote $p < 0.05$, One-way repeated measurement followed by Bonferroni correction) bdl-below detection limit (~1 nmol min^{-1} mg of $protein^{-1}$)

Oxygen consumption rate in the presence of cysteine and homocysteine

We investigated both cysteine and homocysteine dependant respiration in presence of 10 mM succinate considering these two enzymes of transsulfuration could be possible candidates of H_2S production. We used rotenone to block electron flow from complex I via pyruvate oxidation or any other NADH forming reactions. Using 100 or 200 µM cysteine or with 200 µM of homocysteine, neither cysteine nor homocysteine altered the mitochondrial oxygen consumption rate in any respiratory state examined (Fig. 4. 3 A, B, C). In addition, a combination of 200 µM of cysteine and 200 µM of homocysteine did not alter mitochondrial oxygen consumption rate at least for mitochondrial preparations from two separate rats (data not shown). We noticed that addition of cysteine causes an initial and transient increase in oxygen consumption rate which stabilizes to a lower steady-state rate with time (traces are shown in supplementary figure 4.1). Because of the transient nature of this response we only used the later stable rates of respiration in analysis for data presented (Fig. 4.3).

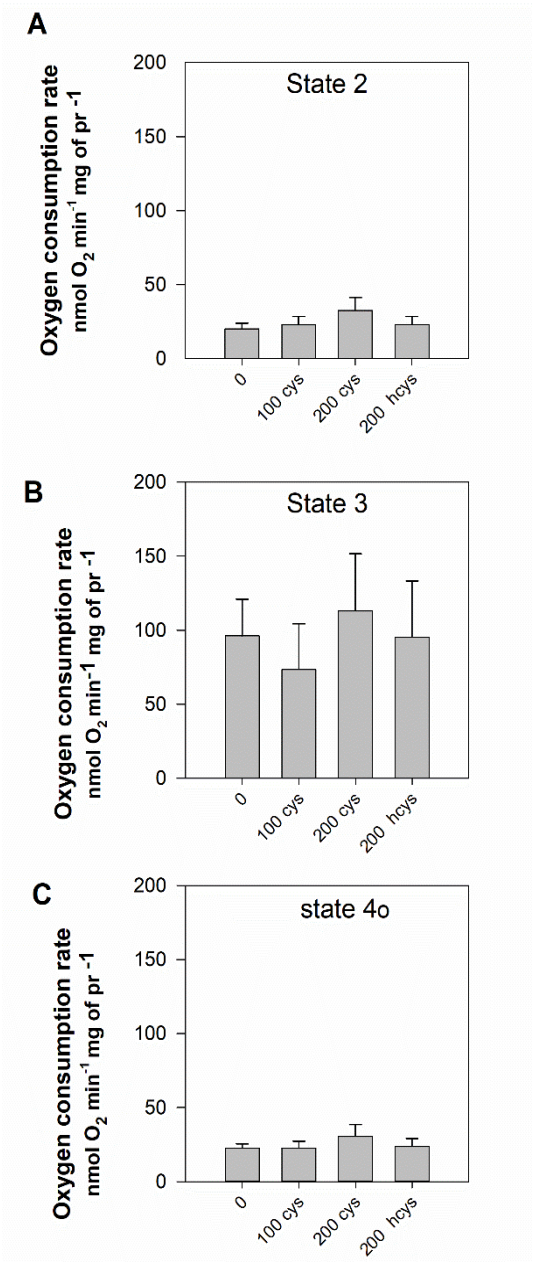

Fig. 4.3. Cysteine and homocysteine dependant respiration of rat liver mitochondria. A) State 2 respiration experiments were carried out in presence of 10 mM succinate +2 μM rotenone, **B)** State 3 respiration experiments were carried in presence of, 500 μM of ADP, **C)** State 4o was estimated in presence of oligomycin (1μg/mL)). 100 μM ,200 μM cysteine (cys) and 200 μM homocysteine (hcys) were compared with 0 group where no hcys or cys were added, only succinate and rotenone were present. Data are presented as mean ± SEM, n=3

The effect of 3-mercaptopyruvate on mitochondrial oxygen consumption rate

When added to isolated respiring liver mitochondria, both 100 and 500 µM of 3-MP increased state 2 respiration, whereas 30 µM had a trend towards enhanced respiration (Fig. 4.4 A). In state 3, neither 10 nor 30 µM 3-MP enhanced respiration while 100 µM of 3-MP increased respiration, but 500 µM had an inhibitory effect on respiration (Fig. 4.4 B). Following addition of oligomycin, an inhibitor of ATP synthase, the effect of 3-MP was similar to that seen in state 2, where 100 and 500 µM of 3-MP elevated respiration while 10 and 30 µM of 3-MP showed a trend toward increased respiration (Fig. 4.4C)

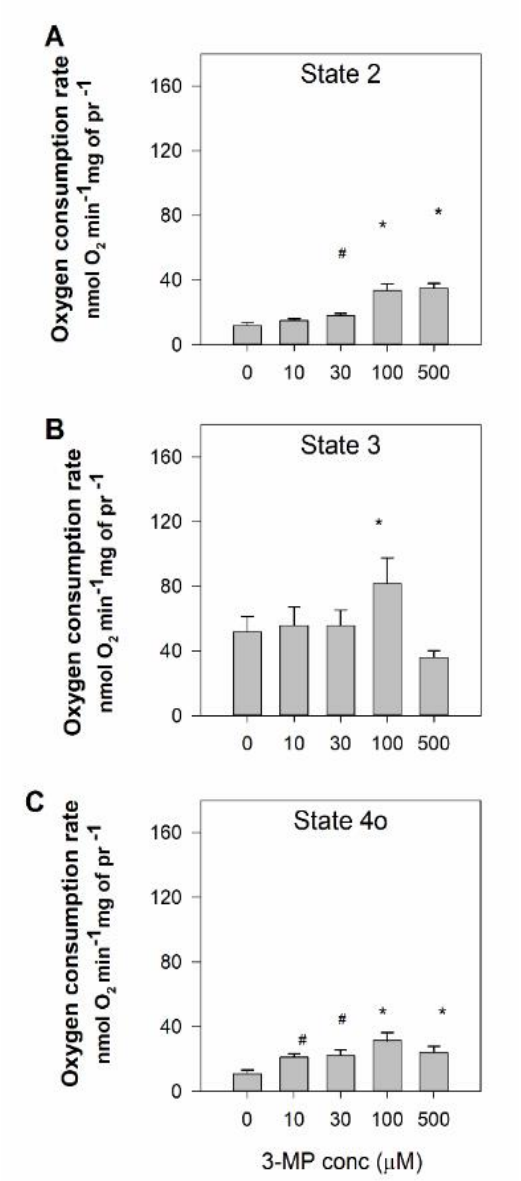

Fig. 4.4. 3-MP derived respiration of rat liver mitochondria. A) State 2 respiration were obtained in presence of 10 mM succinate +2 μM rotenone, **B)** State 3 were carried in presence of 500 μM of ADP, **C)** State 4o was estimated in presence of oligomycin (1μg/mL). 10, 30, 100 and 500 μM of 3-MP are compared with 0 μM group, where only 10 mM of succinate and rotenone were present. Data are presented as mean ± SEM, n=5, (* and # denotes $p<0.05$ and $p<0.1$ respectively than 0 group where no 3-MP was added, One-way repeated measurements, followed by Bonferroni test)

The effect of 3-mercaptopyruvate on mitochondrial membrane potential

A previous study reported that 3-MP enhances the rate of respiration under phosphorylating conditions (state 3) but not during non-phosphorylating respiration (state 2) [11], while we found that 3-MP enhances the rate of respiration under both respiratory states. Because of this discrepancy, we tested whether the increase in respiration is due to an increment of substrate oxidation capacity or possibly a shift that leads to uncoupling. Addition of 3-MP increased respiration rates in both state 2 and state 3 but the relative mitochondrial membrane potential, measured by tetramethylrhodamine methyl ester fluorescence quenching, was not significantly different (Fig. 4.5) suggesting an uncoupling event. If there was no uncoupling, membrane potential should also concomitantly increase with the increased respiration rate as the substrate oxidation capacity increases [17, 18]. Conversely, an increase in respiratory flux, particularly under non-phosphorylating conditions, with no change or a decrease in membrane potential would indicate a greater requirement for proton extrusion at a given steady-state capacity to drive the return of protons to the negative matrix space. Since in the steady-state proton extrusion equals proton return across the inner mitochondrial membrane, this result of increased respiration at a common membrane potential suggests a high rate of proton return to the matrix via proton leak pathways.

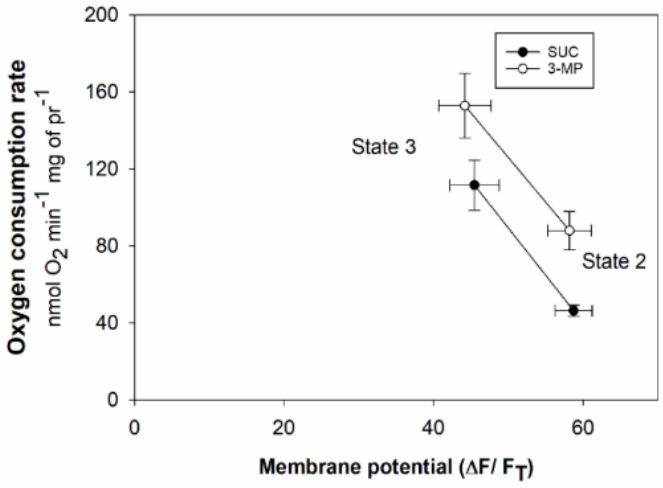

Fig. 4.5. Effects of 3-mercaptopyruvate (3-MP) on oxygen consumption rate and membrane potential. In both state 2 and state 3 in presence of 3-MP oxygen consumption rates were higher than succinate alone. Despite an increase of oxygen consumption in the presence of 3-MP, membrane potential was not significantly different between 3-MP and succinate mediated respiration suggesting uncoupling. In all experiments, 10 mM succinate and 2 μM rotenone were present. Data are presented as mean± SEM, n=4

Role of endogenous reductants in MPST mediated respiration

This study demonstrates that MPST has the greatest maximal capacity to produce predominant H_2S compared to CBS and CGL. We confirmed that 100 μM of 3-MP enhances mitochondrial respiration, in the presence of rotenone which will remove the effects of NADH generating substrates. Since, *in vitro* MPST requires a chemical reductant DTT (Fig. 4.2B), here we investigated whether the pharmacological manipulation of 3-MP dependant respiration could deduce the possible biological reductant(s) that might be important to MPST within intact mitochondria.

Therefore, we have used auranofin and 5-methoxy indole 2-carboxylic acid which are established inhibitors of thioredoxin reductase and dihydrolipoamide dehydrogenase respectively [19, 20].

Effect of auranofin on MPST mediated respiration

Auranofin inhibits the reduction of thioredoxin from oxidised thioredoxin, thereby if reduced thioredoxin is necessary to maximize H_2S release from MPST, in the presence of auranofin MPST mediated H_2S production will be impaired and as a result the 3-MP induced respiration rate will drop. We noticed that auranofin does not interfere either of state 2, 3 or 4_O respiration while only succinate and rotenone are present (Fig. 4.6A, B, C). While 3-MP is present in the respiration medium auranofin inhibits state 3 respiration (Fig. 4.6B) as well a trend of inhibition towards state 4o respiration was noticed (Fig. 4.6C).

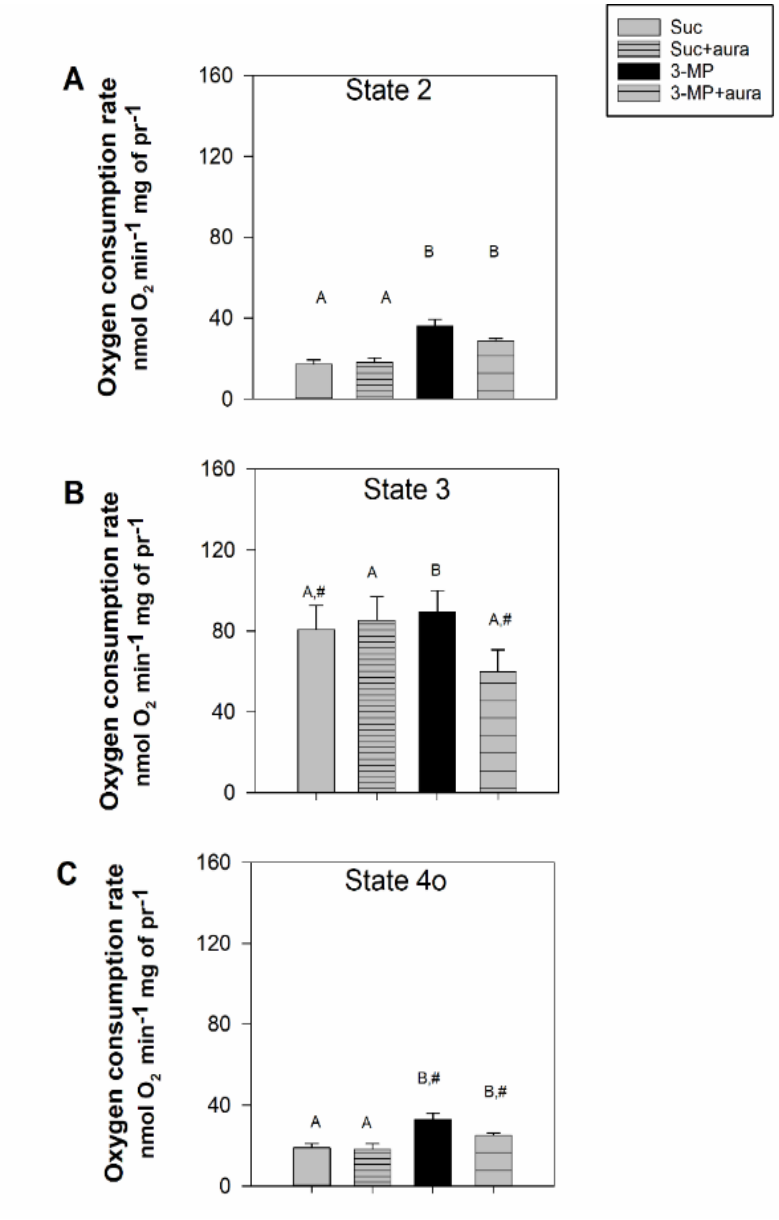

Fig. 4.6. 3-mercaptopyruvate (3-MP) dependant respiration in presence of auranofin. A) State 2 respiration were obtained in presence of 10 mM succinate +2 µM rotenone, in addition, 100 µM of 3-MP was added to 3-MP and 3-MP+Aura group **B)** State 2 was estimated in presence of 500 µM of ADP **C)** State 4o was analyzed in presence of oligomycin (1µg/mL). 2 µM of Auranofin have been added in suc+aura and MP+aura group, auranofin was dissolved in dimethyl sulfoxide, therefore same volume of DMSO was added in control group. Data are represented as mean ±SEM, n=8, (different letter denote $p<0.05$, and groups having # denotes $p<0.1$ between them, paired t-test followed by a Bonferroni test when needed)

Effect of MICA on MPST mediated respiration

Having established the 3-MP dependent increase in liver mitochondrial respiration is inhibited by auranofin, implicating a need for reduced thioredoxin for the 3-MP induced elevation in respiration rate, we next evaluated if dihydrolipoic acid is also important to the 3-MP based increased respiration rate. We followed the same strategy as used to interrogate the thioredoxin dependant increase in respiration, now using 5-methoxy indole 2-carboxylic acid as an inhibitor of dihydrolipoamide dehydrogenase. Prior to the use of 5-methoxy indole 2-carboxylic acid on 3-MP mediated respiration rates we needed to confirm whether 5-methoxy indole 2-carboxylic acid has any off-target effects on respiration. We found that both 0.5 mM and 3 mM 5-methoxy indole 2-carboxylic acid inhibited state 3 respiration in the presence of succinate and rotenone (Fig. 4.7B) with 3 mM 5-methoxy indole 2-carboxylic acid also increasing non-phosphorylating respiration rates in either state 2 or 4o (Fig. 4.7A, C).

In a separate series of experiments mitochondria were incubated with succinate, rotenone and 3-MP in the presence or absence of 3 mM 5-methoxy indole 2-carboxylic acid. Under these conditions 5-methoxy indole 2-carboxylic acid did not alter respiration rates in non-phosphorylating conditions, state 2 (Fig 4.7D) or state 4o, the latter of which gave comparable results to the state 2 condition (data not shown). The addition of 5-methoxy indole 2-carboxylic acid to mitochondria respiring in state 3 with succinate and 3-MP led to a marked inhibition of respiration (Fig. 4.7E.). Since 5-methoxy indole 2-carboxylic acid inhibited respiration in the absence of 3-MP we adopted a somewhat different approach than used for auranofin, the latter of

which did not alter respiration in the absence of 3-MP. To evaluate the role dihydrolipoic acid may have as a reductant, we tested the degree of inhibition (%) for state 3 respiration by 5-methoxy indole 2-carboxylic acid alone while mitochondria were respiring strictly on succinate (Fig. 4.7B) relative to the degree of inhibition when mitochondria are oxidizing succinate and 3-MP (Fig. 4.7D). Although 3mM 5-methoxy indole 2-carboxylic acid inhibited both 3-MP and succinate mediated state 3 respiration rates, the inhibition was more pronounced in presence of 3-MP plus succinate than with succinate alone (Fig. 4.7F) suggesting 3-MP stimulated respiration is also sensitive to 5-methoxy indole 2-carboxylic acid.

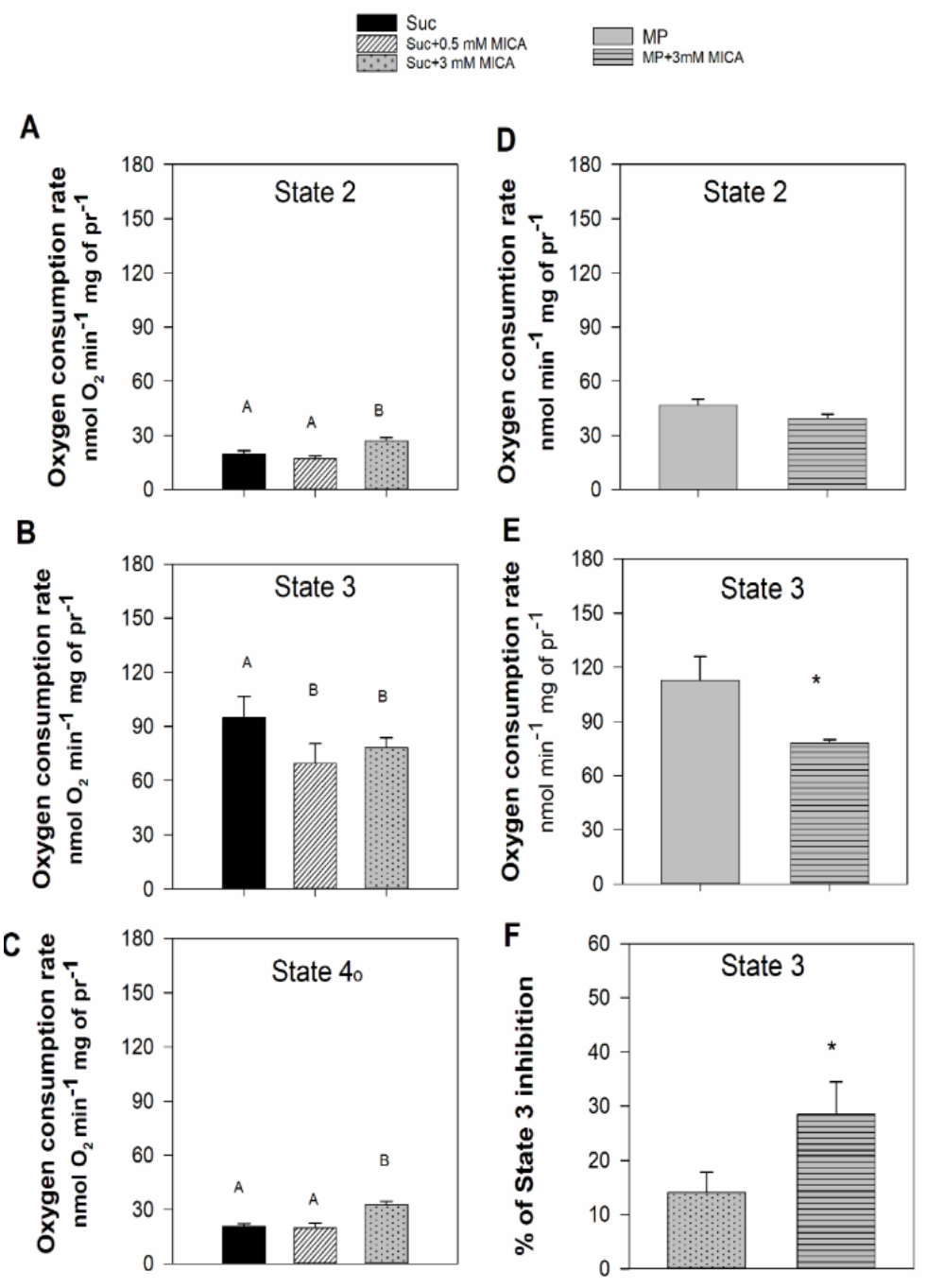

Fig. 4.7. **Effects of dihydrolipoic acid on MPST mediated respiration**. **A)** State 2 respiration was obtained with 10 mM succinate+2µM rotenone, either in presence or absence of 5-methoxy indole 2-carboxylic acid (MICA) **B)** State 3 respiration was obtained after addition of 500 µM of ADP added **C)** 1 µg/mL of oligomycin was added in each chamber which gave the state 4o respiration; , **D)** State 2 respiration was obtained in presence of 10 mM succinate +2µM Rotenone+100 µM of 3-MP. **E)** State 3 was estimated in presence of 500 µM of ADP **F)** percentage of state 3 inhibition was estimated in presence of 3mM MICA, while succinate or with succinate+3-MP were added. Data are presented as mean±SEM, n=3-4, (for boxes A-C, different letters represent $p<0.05$, paired t-test followed by a Bonferroni test; * signifies $p<0.05$ for boxes D-F, paired t-test)

Discussion

H_2S is gaining growing attention because of its numerous physiological functions [21]. Along with its antioxidant properties H_2S is involved in a number of cellular signalling processes such as activation of the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and Nrf-2 (Nuclear factor erythroid 2-related factor 2 (Nrf2)) pathways of which the first one is associated with inflammation whereas the second one is involved in cellular defence (reviewed in [1, 22]). Another important mechanism of H_2S is sulfhydration, by which a number of proteins and enzymes are modified [23]. In addition, H_2S metabolism in rat liver mitochondria enhances mitochondrial respiration rate [11].

The current study focused on the liver mitochondria of the rat to investigate the major H_2S producer(s) and explored whether the producers need any reductant to release maximal H_2S in a functional mitochondrion. A previous study reported that when ruptured mouse brain mitochondria isolates were incubated with 3-MP, H_2S production was only evident in the presence of dithiothreitol, thioredoxin or dihydrolipoic acid but not with reduced or oxidized GSH, NADPH, NADH or acetyl CoA [7]. Therefore, we used pharmacological inhibitors of regenerating the candidate biological reductant(s) of MPST, thioredoxin and dithiothreitol, to manipulate the 3-MP-dependant respiration in functional mitochondria, which can help to determine the role of those reductants (if any) in mitochondrial H_2S production *in vivo*. Of note, MPST does not supply electrons for oxidative phosphorylation by itself directly, rather MPST forms H_2S from 3-MP which is subsequently oxidized by SQR. From SQR, electrons are transferred to complex III of the electron transport chain which can then terminally reduce oxygen to water. Therefore, H_2S

production can induce oxygen consumption which can then be used as a proxy measure of H_2S formation.

It is important to note that the assays of H_2S formation were conducted under anoxic conditions to prevent H_2S oxidation and H_2S production capacity obtained from freeze-thawed ruptured liver mitochondria are not dependant on amino acid (cysteine, homocysteine, 3-MP) transporters that may contribute to H_2S production differently *in vivo*. In the current study, we first investigated what roles, if any, CBS or CGL might have on mitochondrial H_2S formation. We found that the isolated frozen liver mitochondria have the capacity to produce a substantial amount of H_2S only in presence of both supraphysiological levels of homocysteine and cysteine (10 mM) are present. Previous studies reported that H_2S production capacity is much lower while either cysteine or homocysteine are present alone in the assay medium, but the combination of these two substrates enhances H_2S production capacity to several fold higher [24-26]. For instance, a combination of cysteine and homocysteine enhance H_2S production capacity by ~30 and ~23 fold compared to cysteine itself in chicken liver and by recombinant human CBS, respectively [24, 25]. Mouse liver homogenate also showed a similar pattern, as H_2S production capacity was much higher in the presence of 20 mM cysteine and 20 mM homocysteine relative to the rate with either of the substrates alone [26]. In the current study, we used 10 mM of each of the substrates (homocysteine or cysteine) or in combination; therefore it is not quite certain how 20 mM of homocysteine or 20 mM of cysteine would have responded to H_2S production. A study of enzyme kinetics predicted that under substrate saturating conditions (maximal velocity, or V_{max}), recombinant human CBS produces H_2S from cysteine and homocysteine with a much higher specific activity than the cysteine alone (18.7±2.6 units/mg versus 0.4±0.08 units/mg respectively) [25]. While, CGL dependant H_2S production capacity was estimated it showed a different pattern; for instance under

V_{max} conditions, the homocysteine dependant H_2S production rate was 6.6±0.47 units/mg whereas the combination of cysteine and homocysteine generates H_2S at a 0.20±0.03 units/mg rate [27]. Given that, in current experiment combination of homocysteine and cysteine increase mitochondrial H_2S production capacity by ~20 fold (Fig. 4.2 A), it can be suggested that in isolated rat liver mitochondria at least at supraphysiological levels of cysteine and homocysteine (10 mM), CBS is the predominant H_2S producer. Of note, whether it is CBS or CGL, the Michaelis constant (K_m) value for either homocysteine, cysteine or both for H_2S forming substrates are varied from 2-12 mM depending on the ping-pong or ordered enzymatic reactions [25, 27].

In the presence of more physiological levels of homocysteine or cysteine, alone or in combination, H_2S formation was negligible (Fig 4.2). Therefore, we suggest that liver mitochondria might have homocysteine and cysteine dependant H_2S producing pathways, but their relevance to the physiological function is not clear. There is a possibility that these homocysteine and cysteine dependant pathways might activate in response to specific situations because previous studies demonstrated that MPST can be susceptible to oxidative stress [28] while both CBS and CGL derived H_2S production increase in response to oxidative stress [29, 30]. For instance, although CGL is primarily located in the cytosol, there have been studies suggesting that CGL moves from the cytosol to mitochondria during stress conditions [12]. Mitochondria obtained from smooth muscle cells only localizes CGL at the outer membrane of mitochondria while the cells are treated with stressors, i.e. A23187 (Ca^{+2} ionophore) but not in absence of A23187, suggesting H_2S may be produced from an alternative source during stress conditions [12]. Fu *et al*. further showed that A23187 treated mitochondria produces H_2S in the presence of cysteine (10 mM) which declined when a CGL inhibitor, propargylglycine is added [12].

Unlike the millimolar concentrations of homocysteine and cysteine required to produce substantial amounts of H_2S, the addition of 10 µM of 3-MP to isolated rat liver mitochondria produces measurable amounts of H_2S and the rate of production increases with increasing 3-MP concentration to at least five-folder higher than that seen with 10 mM cysteine and homocysteine combined (Fig. 4.2). One of the limitations of our experiment is in the H_2S producing assays we used frozen isolated liver mitochondria where we were unable to estimate inflow of H_2S from other possible sources that might be happening in an intact cell. Since we used isolated mitochondria, our study design will allow me to capture substrate dependant H_2S production from the producers which are already expressed in mitochondria. Despite the limitation in study, based on substrate dependant H_2S production it can be suggested that MPST has the greater capacity to produce H_2S in rat liver mitochondria.

It has been reported that cysteine enhances mitochondrial respiration in the presence of 10 mM succinate and it was suggested that from cysteine, MPST produces enough H_2S that in turn increase respiration rates [6]. Cysteine aminotransferase converts cysteine to 3-MP which could be used by MPST to generate pyruvate and H_2S [3]. Therefore, in the absence of a complex I inhibitor, it is difficult to tell whether the electrons are coming from H_2S or from pyruvate or alternative NADH producing pathways like the oxidation of glutamate. Glutamate is also formed in the CAT reaction with cysteine and the glutamate can be oxidized, forming NADH, by glutamate dehydrogenase which is very highly expressed in liver mitochondria [31, 32]. Glutamate oxidation would maintain intramitochondrial alpha-ketoglutarate levels which could help maintain CAT flux in the cysteine deamination direction. In addition, cysteine can also be a substrate of CBS and CGL and due to

debate in the literature over the mitochondrial content of CBS and CGL it is not known whether these two enzymes have any role on H_2S dependant respiration. In the respirometry experiments, we added rotenone to block electrons from complex I. This strategy will help to identify whether cysteine, homocysteine or 3-MP generates H_2S to enhance mitochondrial respiration.

Consistent with our results for H_2S production, we found that addition of physiological levels of cysteine or homocysteine to mitochondria respiring on succinate did not enhance mitochondrial respiration, indicating any H_2S formed by these substrates was insufficient to detect by increases in oxygen consumption rate. Similar to our findings, a previous study reported that homocysteine does not change oxygen consumption in rat liver mitochondria [33], however, on the contrary, a study reported that similar low levels of cysteine-increases respiration in the presence of succinate [6]. In the current study, we did note an increased respiration rate immediately following addition of cysteine, but this transient increase in oxygen consumption rate stabilizes to a lower steady-state rate within minutes (Supplementary Fig. 4.1). Conversely, 3-MP could lead to a stable increase in respiration rate. This further confirms that in rat liver mitochondria MPST has the substantial capacity to produce mitochondrial H_2S which donates electrons to the Q pool via SQR, therefore respiration rate increases. To note, a previous study reported that 3-MP only increases respiration at state 3 but not in state 2 or state 4o [11], however; along with state 3, we found that 100 µM of 3-MP increased state 2 and 4o respirations as well. The discrepancy between the increases in respiration in non-phosphorylating respiratory states in our study and the previous study is not clear. To evaluate how 3-MP increased respiration in my experiments, mitochondrial uncoupling or a combination of both we further investigated the 3-MP mediated increase respiration by simultaneously monitoring the relative mitochondrial membrane potential and

respiration rates. Based on typical expectations for mitochondrial respiratory control, since electrons are entering the electron transport chain independent of complex I, if the increased respiration rate was solely due to 3-MP increasing the substrate oxidation capacity then we would anticipate addition of 3-MP would increase the apparent membrane potential as well as the rate of respiration. Alternatively, increased respiration rate combined with a lower membrane potential, or even no change in membrane potential, would suggest that the addition of 3-MP was leading to some degree of mitochondrial uncoupling. In the current study, increased respiration with unaltered membrane potential raises the possibility that the mercaptopyruvate used (sodium mercaptopyruvate) may itself cause some measurable mitochondrial uncoupling or some other form of proton re-entry such as sodium/proton exchange. Nevertheless, even if some of the observed increase in mitochondria oxygen consumption was due to intrinsic uncoupling by 3-MP, our strategy was to evaluate if some component of this increased respiration rate may be sensitive to pharmacological manipulation of the availability of reduced thioredoxin or lipoamide.

Although the addition of 3-MP may be causing some degree of uncoupling, the increase in respiration rate can still be used to investigate whether MPST requires any reductants to H_2S production while *in situ* within the mitochondrion. Therefore, following assessing membrane potential, we focused on pharmacological tests of the potential physiological reductant(s) of mitochondrial MPST.

Auranofin is a potent inhibitor of thioredoxin reductase and it impairs the recycling process of reduced thioredoxin from the oxidized form [19, 34]. In the current study, we hypothesized that in isolated liver mitochondria MPST requires reduced thioredoxin to release H_2S. Therefore, it is crucial to confirm auranofin does not have any off-target effects on mitochondrial respiration. Although previous studies reported that up to 8 μM of auranofin does not inhibit succinate (5mM)

mediated non-phosphorylating respiration, nothing was mentioned about the phosphorylating state [34]. Hence, we investigated both phosphorylating and non-phosphorylating respiration rates and found that 2 μM of auranofin does not inhibit either states in presence of succinate (10 mM), rather it inhibits respiration while MPST is active due to 3-MP (Fig. 4.6A-C). This suggests that thioredoxin is a key player in releasing H_2S from the active site of MPST.

We followed similar strategies to identify another biological reductant dihydrolipoic acid, which might be required by MPST. 5-methoxy indole 2-carboxylic acid inhibits lipoamide dehydrogenase and thus impairs dihydrolipoic acid formation [20, 35]. Previous studies demonstrated that 1 to 3 mM 5-methoxy indole 2-carboxylic acid significantly impairs the reduction of α-lipoic acid to DHLA by inhibiting dihydrolipoamide dehydrogenase in rat liver mitochondria [20, 35], therefore these concentration were chosen to see whether this inhibitor has any off-target effect on respiration. Although, 5-methoxy indole 2-carboxylic acid inhibits succinate mediated state 3 respiration, the inhibition was doubled while 3-MP was also present along with the succinate (Fig. 4.7F). This suggests that MPST also requires dihydrolipoic acid to generate H_2S. When human recombinant MPST was incubated with lipoic acid, GSH and other acceptors, it was found that thioredoxin is the preferred persulfide acceptor [36]. Since addition of either auranofin or methoxy indole 2-carboxylic acid inhibited 3-MP mediated increases in respiration, it can be assumed that both thioredoxin and dihydrolipoic acid contribute to maintaining H_2S release from MPST. That both inhibitors suggest importance of their respective electron supplying pathways, our data also suggest that one pathway for supply of intramitochondrial reductants possibly cannot fully compensate for the inactivation/absence of the other pathway. It is important to note that the reductants possibly accept the MPST bound persulfide, therefore in the absence of those reductants activated persulfide might be a burden for

mitochondria and those persulfide can also influence negatively on mitochondrial respiration. Other reductant might also influence MPST mediated respiration; however, inhibitor(s) used to block that particular acceptor might interfere mitochondrial regular bioenergetics as we observed with methoxy indole 2-carboxylic acid. Therefore, caution should be taken while any inhibitor is being used.

In summary, we conclude that MPST is likely the predominant H_2S producer in liver mitochondria and that both thioredoxin and dihydrolipoic acid may contribute to the generation of H_2S by MPST when measuring H_2S release as an increase in respiration with intact mitochondria. In addition, we recommend that methoxy indole 2-carboxylic acid and auranofin can be potential pharmacological inhibitors of MPST mediated H_2S production via indirect inhibition of the supply of electrons via physiological reductants.

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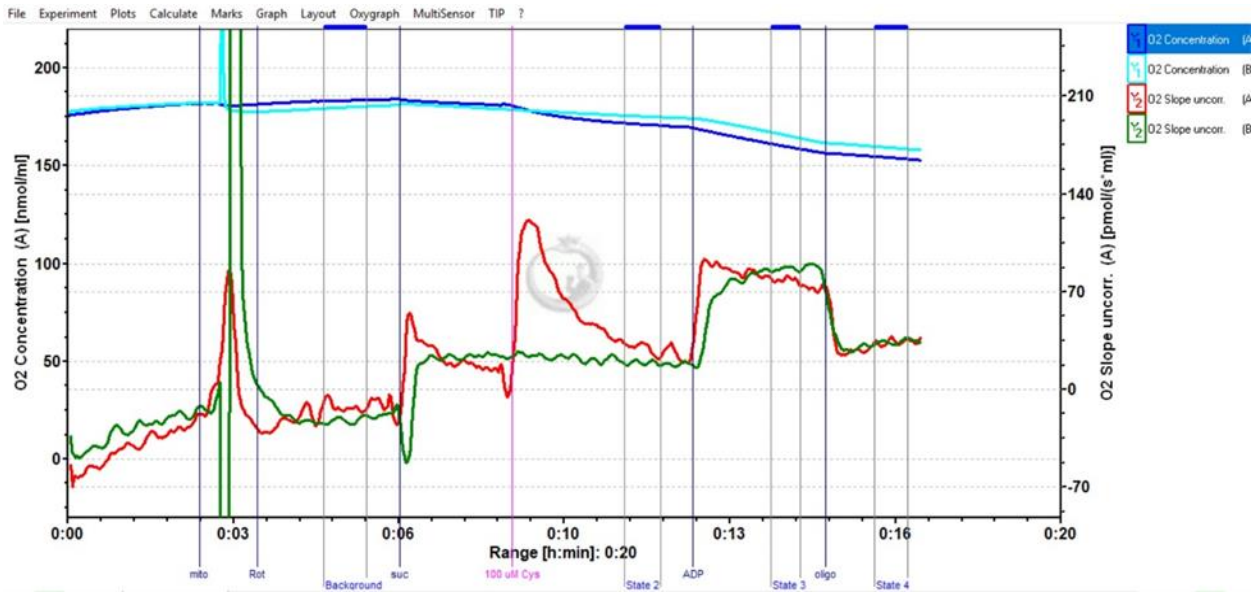

Supplementary Fig. 4.1. Cysteine mediated oxygen consumption rate. A typical experiment is illustrated. Data show how an isolated rat liver mitochondrion consume oxygen at different respiratory states in presence of substrates. Initially mitochondria and rotenone were added to respirometer chambers and the oxygen signal was allowed to stabilize. Once the background was stabilized, 10 mM of succinate was added in both chambers and then 100 µM of cysteine was added in chamber A, which instantly spiked up and gradually stabilized (red trace). Following the state 2, 500 µM of ADP was added to obtain state 3 and finally state 4o was estimated in presence of oligomycin (1µg/mL)

Chapter Five

General Conclusions

The objective of this thesis was to understand mechanisms involved in sulphur amino acid and H_2S metabolism in a mammal model. Compounds containing at least one thiol (-SH) group have significant contributions to cellular processes, from signalling to cell structure as well defence mechanisms. Methionine and cysteine are the two major sulphur containing amino acids which serve as precursors of other thiol molecules. Therefore, the availability of these amino acids in cells can affect overall thiol metabolism, as a result, specific thiol dependant pathways involved with the cellular antioxidant status and signalling capacity might be altered. Similar to other thiols, H_2S production also depends on the availability of sulphur amino acids and elevation of endogenous H_2S production capacity has been speculated to be a unifying mechanism in delaying aging process by different dietary restriction across taxa [1]. Additionally, H_2S has the capacity to alter mitochondrial bioenergetics in the presence of mitochondrial respiratory substrate(s) [2].

This thesis investigated the responses of different thiols to methionine restriction and also explored H_2S production at the whole tissue level and in isolated liver mitochondria. Specifically, this thesis was designed to address three separate but related research questions, each with a chapter dedicated to the following: i) how MR, a lifespan extension dietary regimen, elevates homocysteine levels and maintains H_2S production capacity, ii) does MR lead to activation of other antioxidants in response to low GSH levels and iii) which enzyme has the highest capacity to produce H_2S in liver mitochondria?

To investigate the first two research questions, Fischer-344 rats were fed a methionine restricted diet and data were obtained from same rats. Since, the same F-344 rats fed on same diet were used in chapter two and three, the variables studied between these two chapters are comparable. For instance, in chapter two and three we reported that methionine restriction decreases liver cysteine and GSH content respectively while these two variables are co-related. Although GSH is comprised of three amino acid, cysteine is often the limiting amino acid in GSH synthesis rather than glycine or glutamate [3]. Moreover, in methionine restricted animals, neither glutamate nor glycine should be limited because the animals show increased mass-specific food consumption compared to control fed animals [6,7]. On top of that, glutamate content was increased in the methionine restricted diet to make the diet isonitrogenous (see materials and method section in chapter two or three). While the chapter two and three are comparable, the third research chapter is distinct from chapter two and three in terms of diet formulation and strain used. To be specific, we used Sprague-Dawley rats in chapter 4 and they were fed on laboratory chow diet. Since, F-344 rats are well studied for methionine restricted research including documented effects of the diet on lifespan, therefore, for chapter two and three this strain was chosen so results would apply to the longevity model established for this strain. For chapter four, Sprague-Dawley rats were chosen for their calm nature of strain and specifically we studied this strain because they are well studied for biomedical research including toxicology and pharmacology as well as isolated mitochondrial work ([2, 4, 5]). Methionine restricted rats were fed AIN-76 semi-purified diet, so that methionine content in the diet can be varied, while Sprague-Dawley rats were fed laboratory chow diet as the objective was not associated with diet induced responses. Although in all three chapters thiol containing compounds were investigated, cautions should be taken while

comparisons are made among the chapters. Due to the nature of the diet (semi-purified versus chow) or strain difference (Fischer 344 versus Sprague-Dawley) may interfere the interpretation.

Our data suggests that H_2S production is not solely dependent on one pathway or enzyme and that the H_2S production capacity appears to differ depending on the dietary sulfur amino acid intake as well as the major enzymes involved, which can vary by cellular compartment (chapters two and four). In addition, we suggest that low GSH in tissues does not necessarily mean that those tissues are experiencing oxidative stress. There might be other pathways, i.e. elevated thioredoxin system, which can compensate for the low GSH (chapter three). Additionally, we showed that, based on pharmacological manipulation, mercaptopyruvate sulfurtransferase may require dihydrolipoic acid and reduced thioredoxin to maximally produce H_2S in functional mitochondria inferring a reductant role in H_2S production (chapter four). The findings of the three research chapters are summarized in Figure 5.1.

The responses induced by methionine restriction are not straightforward. In general, thiol dependant pathways appear to response in such a way that utilize the limited sulphur amino in different metabolic pathways instead of incorporating the amino acid in the body mass of the animal. While high homocysteine and low GSH raise the questions of utilizing the available sulphur amino acid in a beneficial way, simultaneously increased H_2S production and increased thioredoxin activity evidentiary benefits the organism in multiple ways. Whether hyperhomocysteinemia or low GSH is detrimental for methionine restricted animals are contentious, selection of sulphur amino acid utilization pathway as a whole seems to be beneficial for methionine restricted animals because previous studies reported that this diet improve both health and lifespan. One of the signalling molecules that is beneficial for both methionine restricted animals and other organism is H_2S. While the transsulfuration enzymes along with MPST all play

significant role in producing H_2S in cytosol, MPST seems the key H_2S producer in mitochondrial compartment. Mitochondria itself have the capacity to produce H_2S from MPST with the presence of biological reductants thioredoxin and lipoic acid, which comprise thiol groups. This further shows the importance of thiol containing molecules in cellular metabolism.

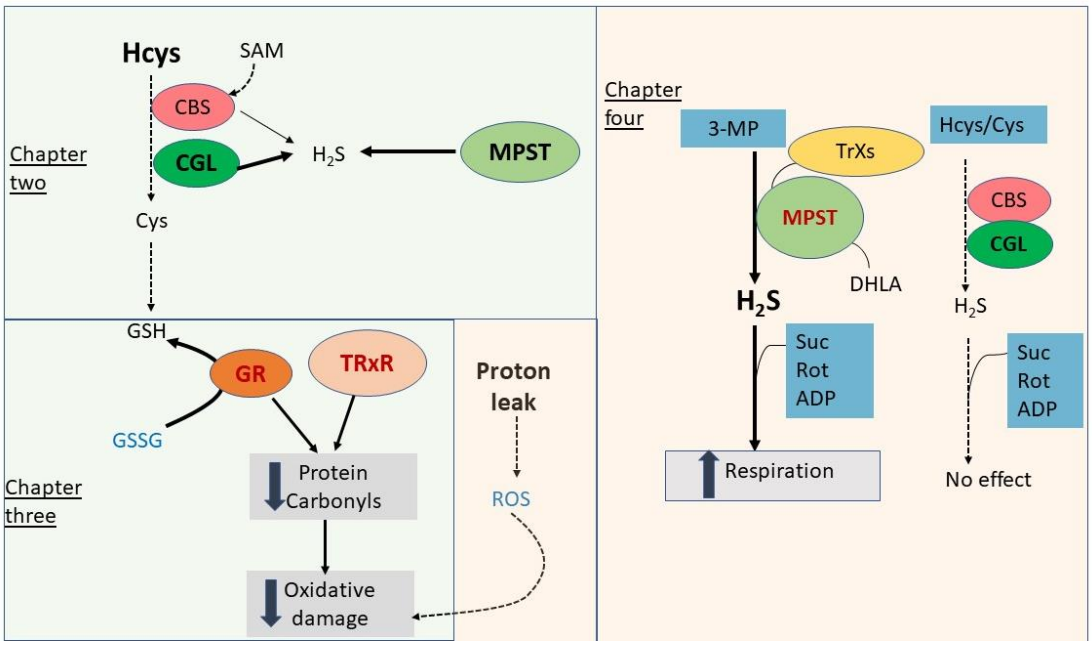

Figure 5.1 Summary of the three thesis research chapters. The light green box is used to indicate whole liver tissue while the light orange box represents liver mitochondria. Bold text and solid arrows represent either an increase of the metabolite, enzyme or a pathway whereas dashed arrows and black font represents a decrease. Block vertical arrows represent either a decrease or increase of a process or levels. While blue fonts represent unmeasured metabolites, aqua shaded boxes are used to indicate substrates. Low S-adenosyl-methionine (SAM) levels and cystathionine-β-synthase (CBS) activity impair homocysteine degradation, as a result hyperhomocysteinemia occurs during MR. Although, H_2S production via cystathionine-β-synthase may decline during MR, cystathionine-γ-lyase (CGL) and mercaptopyruvate-sulfurtransferase (MPST) enhance H_2S production capacity in liver during MR. Impaired transsulfuration leads to low cysteine levels which in turn affects tissue glutathione (GSH) levels. The activity of glutathione reductase (GR) increases the recycling of GSH from oxidized GSH (GSSG), although the levels of GSSG were not estimated. Increased GR and thioredoxin reductase (TrxR) probably reduce protein oxidative modifications (estimated via protein carbonyls) as well as oxidative damage. In liver mitochondria, proton leak was observed to increase with MR which may contribute to a lower ROS production

occurring during MR. In liver mitochondria, H_2S can be produced from either from 3-mercaptopyruvate (3-MP) or from homocysteine, cysteine or a combination of homocysteine and cysteine. However, H_2S production capacity is much higher with 3-MP and only oxidation of 3-MP can increase the respiration rate in the presence of mitochondrial respiratory substrates indicating that 3-MP alone can generate enough H_2S to measure sulfide quinone reductase (SQR) activity by oxygen consumption. 3-MP dependant mitochondrial respiration was decreased in presence of the pharmacological inhibitors thioredoxin (Trxs) and dihydrolipoic acid (DHLA) suggesting MPST requires these two biological reductants to maximize H_2S release.

Hyperhomocysteinemia and H_2S production by methionine restriction

Chapter two: objectives and summary

Diet manipulation has emerged as a key strategy to treat diseases as well as showing promise for extension of lifespan in laboratory animals. Methionine restriction extends lifespan in a number of species [6-12]. Although, MR enhances longevity, some of the phenotypes caused by MR in rodents are paradoxical to longevity of which elevation of hyperhomocysteinemia [13, 14] is one. Hyperhomocysteinemia induced by MR raised two questions: despite the limited availability of precursor methionine how MR can increase circulating homocysteine and, if hyperhomocysteinemia occurs with MR then how might the homocysteine metabolising pathway (transsulfuration) enhance H_2S production? In the second chapter, we concluded that the activity of the rate controlling enzyme of transsulfuration, cystathionine-β-synthase (CBS) was lower which contributes to hyperhomocysteinemia. Furthermore, the low CBS activity may be compounded *in vivo* from the limited availability of its allosteric modulator, S-adenosyl-methionine (SAM). Although transsulfuration flux was not estimated in the current study, low

CBS activity and low SAM levels suggest that transsulfuration pathway might be decreased by MR and therefore it seems inconsistent how elevated transsulfuration flux (as per [1]) would be consistent with increased H_2S production. This study found elevated activity of cystathionine-γ-lyase (CGL) and mercaptopyruvate-sulfurtransferase (MPST), which suggests H_2S production is enhanced or maintained by these alternative sources of H_2S during MR.

Significance

Elshorbagy *et al*. first reported that methionine restriction causes hyperhomocysteinemia [10] which received certain criticism [15]. Despite some early challenges to the first observation, further studies by Elshorbagy *et al*. [13] and Ables *et al*. [11] have shown that MR leads to hyperhomocysteinemia in F-344 rats and apolipoprotein deficient E mice (Apo-E), respectively. To our knowledge, we are the third research group to independently demonstrate that MR causes hyperhomocysteinemia. It is well known that high methionine leads to hyperhomocysteinemia therefore it was necessary to evaluate low methionine induced elevation of homocysteine. In addition, we investigated some of the mechanism(s) leading to hyperhomocysteinemia. Whether hyperhomocysteinemia might lead to cardiovascular disease is a matter of debate [16], but concomitant elevation of adiponectin and fibroblast growth factor 21 in plasma suggest that MR induces these adaptive responses to protect the heart from possible damage [14]. Both adiponectin and fibroblast growth factor 21 exert cardioprotective effects but via a different signalling cascade [17-20]. Therefore, elevation of these proteins might have other beneficial aspects to MR animals.

Metabolic pathways can be intricately connected and often impairment in one specific metabolite formation or accumulation can modulate the connected pathways either by activation or inhibition. In the case of MR, elevated CGL and MPST activity therefore may act as an example of alternate routes for the possible compensation of H_2S in response to MR. Given the importance of H_2S in

cell signalling and possibly in aging, increased H_2S production via transsulfuration is considered as a unifying mechanism in lifespan extension interventions [1]. To our knowledge, this is the first study demonstrating that MPST is also increased by MR, which emphasises that this enzyme should also be taken into account in other contexts addressing H_2S research.

Responses of antioxidants and oxidative damage by methionine restriction

Chapter three: objectives and summary

Depletion of glutathione (GSH) in several tissues is another trait observed in MR rodents that seems contradictory to increased longevity. It was anticipated that tissues with low GSH will be vulnerable to oxidative damage and if not, then MR might activate alternative responses to deal with oxidative stress. In the third chapter, low oxidative damage in whole liver protein, with no difference in lipid oxidation in F-344 rats suggests that MR does not lead to elevated oxidative damage at the whole tissue level. Elevated activity of the antioxidants glutathione reductase (GR) and thioredoxin reductase (TrxR) might contribute to the low oxidative damage observed. Mitochondria may be a significant producer of reactive oxygen species (ROS) and mild proton leak observed in this study suggests that the observation that MR lowers mitochondrial ROS production [21] may be facilitated by mild uncoupling (proton leak).

Significance

This chapter illustrates that a methionine limited diet leads to depletion of tissue GSH in the liver and demonstrates how this apparently pro-oxidant state may be compensating by switching to, or upregulating, other antioxidant mechanisms. These include activation of antioxidant enzymes at the whole tissue level and enhancement of proton leak as possible mechanisms of reducing ROS

production in mitochondria. These findings suggest that reducing oxidative stress is one of the strategies to extend lifespan in response to MR. Although this study was only 8 weeks long, the effect of this short term feeding study on oxidative stress can be long lasting. For instance, reductions in hepatic GSH 4 weeks following MR remains low throughout the dietary restriction (2-32 months) in rodents [6, 9, 22-26], which suggests that MR presumably maintains low oxidative damage by altering other antioxidants in response to low GSH as I have reported.

Major H_2S producer in mitochondria and its' correspondent reductant

Chapter four: objectives and summary

In fourth chapter, the major H_2S producing enzyme in liver mitochondria was investigated and from there it was deduced whether the enzyme(s) require any reductant for the maximal formation of H_2S. Both direct measurement of H_2S production via a conventional zinc trapping method [27] and indirect measurements through respiration suggest that MPST has a greater capacity to produce H_2S in liver mitochondria. Although liver mitochondria may contain other H_2S producers along with MPST, CBS being the likely other major potential contributor, it appears the H_2S production is only high at supraphysiological levels of substrates from the non-MPST sources examined. When pharmacological inhibitors of thioredoxin reductase and dihydrolipoamide dehydrogenase were used, they inhibited the MPST mediated increased respiration suggesting H_2S production is impaired by those inhibitors. This is to our knowledge the first time that pharmacological inhibition of thioredoxin and lipoic acid have been shown to be important to work in co-ordinance with MPST to produce H_2S in a functional mitochondrion.

Significance

In the final research chapter, auranofin and 5-methoxy indole 2-carboxylic acid (MICA) were used to inhibit MPST mediated respiration which are the inhibitors of thioredoxin reductase and lipoic acid dehydrogenase, respectively. Inhibiting the thioredoxin reductase or lipoamide acid dehydrogenase can affect cellular processes that require thioredoxins or DHLA. For instance, thioredoxin reductase is involved in a number of cellular functions including H_2O_2 detoxification. Similarly, DHLA makes several antioxidants reduced such as oxidized glutathione and oxidized thioredoxin [28]. Thereby, inhibiting thioredoxin reductase or DHLA can also affect the associated pathways. To our knowledge, to date there is no known pharmacological inhibitor of MPST which inhibits H_2S production [29, 30]. Given that, auranofin and MICA inhibited MPST mediated respiration, these two inhibitors can be recommended as potential pharmacological indirect inhibitors of MPST to H_2S formation. However, given the potential for off-target effects unrelated to H_2S formation, how feasible these inhibitors will be cannot be predicted.

Our work provided evidence on how MPST requires intramitochondrial reductants to exert its' maximum effect *ex vivo* using isolated but intact mitochondria. The current study can be helpful from the therapeutic purpose as well. For instance, salts of H_2S or H_2S producing substrates are gaining growing attention in therapeutic approaches, therefore there is a possibility that a combination of MPST producing substrates along with its biological reductant might have more pronounced effects on releasing H_2S.

Strengths and limitations

'Methionine restriction causes hyperhomocysteinemia', this finding can be a matter surprize for two reasons: despite a limited availability of precursor methionine how homocysteine is increased and while, hyperhomocysteinemia is considered as a marker of cardiovascular diseases then how

would methionine restricted animals live longer? Furthermore, while it was found that methionine restriction increases H_2S production capacity via the transsulfuration pathway, which is also unexpected as hyperhomocysteinemia might be a result of impaired transsulfuration flux. In our first objective, we found that MR leads to hyperhomocysteinemia because of the impaired transsulfuration and H_2S production is maintained by pathway other than transsulfuration. Similarly, in the second study we found that although the GSH levels were low in liver tissue, other antioxidant(s) activity increases which may compensate to low GSH. This suggests that, limited availability of methionine in the diet shuffles pathways/enzymes/proteins over other which are helping the animals to live longer. However, how increased homocysteine will be beneficial in delaying aging process is needed to be explored. Our study was done on a well-established rodent model which mimics many aspects of human metabolism. Therefore, results from this proof-of-concept study will allow to extend similar research in other model organisms before ultimate translation into human subjects. Although the study was short-term, it could suggest the possible outcome of a long term of methionine restriction diet and thus will be helpful in gerontological research. In the third objective, we used isolated mitochondria to investigate H_2S production. In an isolated mitochondrion, we had the advantage of studying specific substrate dependant respiration and we also could block pathways by using inhibitors to determine whether corresponding molecules are necessary for respiration. Sulphur amino acids, antioxidants and H_2S have emerging applications as supplements or as therapeutics. Therefore, knowing the pathways of these molecules will be helpful in pharmacological research as well as increasing lifespan extension. For instance, addition of thioredoxin or dihydrolipoic acid along with H_2S producing substrates (cysteine, 3-mercaptopyruvate) may enhance the efficacy of H_2S relevant signalling. While our studies have some potential strength, there are some limitations as well. For example, a

formulated methionine restricted diet gave the leverage of studying the responses of this diet on metabolism, however, it is not known whether same responses will be evident in the presence of a typical and complex diet mixture. To date, methionine restriction diet is gaining a considerable attention as a cancer therapy; however how much methionine restriction will be safe for human still needs to be investigated. Besides, In our experiment, we have not estimated metabolic flux i.e. transsulfuration flux for metabolite analysis or enzyme kinetics due to the complexity of experiments. Having those data would have provided better insight for *in vivo* metabolism. In addition, we did not have any pair-fed controls which would provide additional knowledge on diet restriction. For the mitochondrial assays, we found inhibitor usagee can be challenging since it interferes with multiple pathways. Selective inhibitors of MPST associated reductants would have provided better insights to the underlying biochemical mechanisms; however specific inhibitors still need to be developed. Since, in all experiments were done on rodents, it is difficult to tell whether the responses are similar in other organisms at this point. However, it can be speculated that similar changes in thiol metabolism as well as mitochondrial respiration at some extent may possibly take other mammalian organisms including human and the efficacy can be only confirmed by a clinical trial.

Future directions

It has been reported that MR increases lifespan in progeroid mice by reverting transcriptional alternations in inflammatory and DNA damage pathways [31]. In addition, it was found that MR modulates redox and nucleotide metabolism by altering flux through one carbon metabolism and thus ultimately produced therapeutic responses in two patient-derived xenograft models of chemotherapy-resistant RAS-driven colorectal cancer, and in a mouse model of autochthonous

soft-tissue which was resistant to radiation [32]. To date, auranofin and methotrexates are used as therapeutics for rheumatoid arthritis [33, 34] and higher doses of methotrexates are used as cancer therapies [35]. While auranofin is an inhibitor of thioredoxin reductase it delays the progression of synovitis (inflammation in joints) by inhibiting certain cytokines [36], whereas methotrexate competitively inhibits dihydrofolate reductase (DHFR) and thus affects folate cycle. It has been suggested that inhibition of DHFR is not involved in the anti-arthritic properties of methotrexate, instead inhibition of downstream process may regulate the mechanism of action [37]. Given that, MR have been shown to improve pathological conditions by altering one carbon flux and anti-inflammatory properties it will be worthwhile to see the progression of arthritis in presence of MR in rodents.

Methionine restriction increases lifespan in male rodents, however; whether it influences on the females at the same extent is not known. Given that, MR lowers body weight or maintains stable weight thorough the lifespan, how this will affect on the offspring is not known. Whether the offspring will be malnourished or will be healthy will be a part of the future studies. In addition, the cause of death followed by a lifelong MR diet needs to be addressed.

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