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EVALUATION OF MITOCHONDRIAL FUNCTION IN A MODEL OF DEVELOPMENTAL PROGRAMMING OF HYPERTENSION ASSOCIATED WITH TRANSIENT NEONATAL OXYGEN EXPOSURE

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RÉSUMÉ

UNE EXPOSITION NÉONATALE À L'OXYGÈNE MÈNE À DES MODIFICATIONS DE LA FONCTION MITOCHONDRIALE CHEZ LE RAT ADULTE

Introduction: L'exposition à l'oxygène (O_2) des ratons nouveau-nés a des conséquences à l'âge adulte dont une hypertension artérielle (HTA), une dysfonction vasculaire, une néphropénie et des indices de stress oxydant. En considérant que les reins sont encore en développement actif lors des premiers jours après la naissance chez les rats, jouent un rôle clé dans le développement de l'hypertension et qu'une dysfonction mitochondriale est associé à une augmentation du stress oxydant, nous postulons que les conditions délétères néonatales peuvent avoir un impact significatif au niveau rénal sur la modulation de l'expression de protéines clés du fonctionnement mitochondrial et une production mitochondriale excessive d'espèces réactives de l' O_2.

Méthodes: Des ratons Sprague-Dawley sont exposés à 80% d'O_2 (H) ou 21% O_2 (Ctrl) du 3^e au 10^e jr de vie. En considérant que plusieurs organes des rats sont encore en développement actif à la naissance, ces rongeurs sont un modèle reconnu pour étudier les complications d'une hyperoxie néonatale, comme celles liées à une naissance prématurée chez l'homme. À 4 et à 16 semaines, les reins sont prélevés et les mitochondries sont extraites suivant une méthode d'extraction standard, avec un tampon contenant du sucrose 0.32 M et différentes centrifugations. L'expression des protéines mitochondriales a été mesurée par Western blot, tandis que la production d' H_20_2 et les activités des enzymes clés du cycle de Krebs ont été évaluées par spectrophotométrie. Les résultats sont exprimés par la moyenne ± SD.

Résultats: Les rats mâles H de 16 semaines (n=6) présentent une activité de citrate synthase (considéré standard interne de l'expression protéique et de l'abondance mitochondriales) augmentée (12.4 ± 8.4 vs 4.1 ± 0.5 µmole/mL/min), une diminution de l'activité d'aconitase (enzyme sensible au redox mitochondrial) (0.11 ± 0.05 vs 0.20 ± 0.04 µmoles/min/mg mitochondrie), ainsi qu'une augmentation dans la production de H_20_2 (7.0 ± 1.3 vs 5.4 ± 0.8 ρmoles/mg protéines mitochondriales) comparativement au groupe Ctrl (n=6 mâles et 4 femelles). Le groupe H (vs Ctrl) présente également une diminution dans l'expression de peroxiredoxin-3

(Prx3) (H 0.61±0.06 vs. Ctrl 0.78±0.02 unité relative, -23%; p<0.05), une protéine impliquée dans l'élimination d' H_20_2, de l'expression du cytochrome C oxidase (Complexe IV) (H 1.02±0.04 vs. Ctrl 1.20±0.02 unité relative, -15%; p<0.05), une protéine de la chaine de respiration mitochondriale, tandis que l'expression de la protéine de découplage (uncoupling protein)-2 (UCP2), impliquée dans la dispersion du gradient proton, est significativement augmentée (H 1.05±0.02 vs. Ctrl 0.90±0.03 unité relative, +17%; p<0.05). Les femelles H (n=6) (vs Ctrl, n=6) de 16 semaines démontrent une augmentation significative de l'activité de l'aconitase (0.33±0.03 vs 0.17±0.02 μmoles/min/mg mitochondrie), de l'expression de l'ATP synthase sous unité β (H 0.73±0.02 vs. Ctrl 0.59±0.02 unité relative, +25%; p<0.05) et de l'expression de MnSOD (H 0.89±0.02 vs. Ctrl 0.74±0.03 unité relative, +20%; p<0.05) (superoxide dismutase mitochondriale, important antioxidant), tandis que l'expression de Prx3 est significativement réduite (H 1.1±0.07 vs. Ctrl 0.85±0.01 unité relative, -24%; p<0.05). À 4 semaines, les mâles H (vs Ctrl) présentent une augmentation significative de l'expression de Prx3 (H 0.72±0.03 vs. Ctrl 0.56±0.04 unité relative, +31%; p<0.05) et les femelles présentent une augmentation significative de l'expression d'UCP2 (H 1.22±0.05 vs. Ctrl 1.03±0.04 unité relative, +18%; p<0.05) et de l'expression de MnSOD (H 1.36±0.01 vs. 1.19±0.06 unité relative, +14%; p<0.05).

Conclusions: Une exposition néonatale à l'O_2 chez le rat adulte mène à des indices de dysfonction mitochondriale dans les reins adultes, associée à une augmentation dans la production d'espèces réactives de l'oxygène, suggérant que ces modifications mitochondriales pourraient jouer un rôle dans l'hypertension artérielle et d'un stress oxydant, et par conséquent, être un facteur possible dans la progression vers des maladies cardiovasculaires.

Mots-clés**: Mitochondries, Reins, Hypertension, Oxygène, Stress Oxydant, Programmation**

ABSTRACT

EVALUATION OF MITOCHONDRIAL FUNCTION IN A MODEL OF DEVELOPMENTAL PROGRAMMING OF HYPERTENSION ASSOCIATED WITH TRANSIENT NEONATAL OXYGEN EXPOSURE

Introduction: Rats exposed to oxygen (O_2) as newborns suffer complications in adulthood, including: arterial hypertension, vascular dysfunction, nephropenia and indices of oxidative stress. Although the rats are born at term, their organ development is equivalent to that of a preterm fetus, allowing organs of interest such as the kidney to be compared to premature infants. Given that impaired nephrogenesis or reduced nephron numbers has been shown to promote the development of hypertension and mitochondrial dysfunction is associated with increased oxidative stress, we hypothesised that exposure to high oxygen concentrations in the neonatal period would significantly impact the expression and activity of key proteins involved in renal mitochondrial function and lead to an excessive production of reactive oxygen species by the mitochondria.

Methods: Sprague-Dawley rat pups were exposed to 80% O_2 (Hyperoxic (H) group; O_2 exposed) or 21% O_2 (Control (Ctrl) group) from day 3 to day 10 of life. At 4 and 16 weeks of age, kidneys were rapidly excised and the mitochondria isolated following a standard protocol; with a buffer containing 0.32 M sucrose and differential centrifugations. Expression of mitochondrial proteins was assessed by Western blot, whereas the release of hydrogen peroxide (H_20_2), activities of key citric acid cycle enzymes and mitochondrial swelling were assessed by spectrophotometry. Results are expressed as the means ± SE. Both male and female offspring were studied.

Results: In male H rats at 16 weeks of age (n=6), citrate synthase activity (internal standard and measure of relative mitochondrial abundance) was significantly increased (12.4 ± 8.4 vs 4.1 ± 0.5 μmole/mL/min), whereas aconitase activity (sensitive to ROS) was significantly decreased (0.11 ± 0.05 vs 0.20 ± 0.04 μmoles/min/mg mitochondria) and H_20_2 release was significantly increased (7.0 ± 1.3 vs 5.4 ± 0.8 ρmoles/mg mitochondrial protein) compared to the controls (Ctrl, n=6 males and 4 females). The H group (vs Ctrl) also demonstrated a reduction in the expression of

peroxiredoxin-3 (Prx3) (H 0.61±0.06 vs. Ctrl 0.78±0.02 relative units, -23%; p<0.05), a protein involved in the elimination of H_20_2 and in the expression of cytochrome C oxidase (Complex IV) (H 1.02±0.04 vs. Ctrl 1.20±0.02 relative units, -15%; p<0.05), a protein in the mitochondrial respiratory chain, whereas the expression of uncoupling protein-2 (UCP2), a protein involved in dissipating the proton gradient, was significantly increased (H 1.05±0.02 vs. Ctrl 0.90±0.03 relative units, +17%; p<0.05). Female H rats (n=6) (vs Ctrl, n=6) at 16 weeks of age demonstrated a significant increase in aconitase activity (0.33±0.03 vs 0.17±0.02 μmoles/min/mg mitochondria), in the expression of ATP synthase β subunit (H 0.73±0.02 vs. Ctrl 0.59±0.02 relative units, +25%; p<0.05) (involved in ATP production) and in the expression of MnSOD (H 0.89±0.02 vs. Ctrl 0.74±0.03 relative units, +20%; p<0.05) (mitochondrial antioxidant involved in scavenging superoxide), whereas Prx3 expression was significantly reduced (H 1.1±0.07 vs. Ctrl 0.85±0.01 relative units, -24%; p<0.05). In male H rats (vs Ctrl) at 4 weeks of age, the expression of Prx3 was significantly increased (H 0.72±0.03 vs. Ctrl 0.56±0.04 relative units, +31%; p<0.05). Female H rats (vs Ctrl) at 4 weeks of age demonstrated a significant increase in the expression of UCP2 (H 1.22±0.05 vs. Ctrl 1.03±0.04 relative units, +18%; p<0.05) and in the expression of MnSOD (H 1.36±0.01 vs. 1.19±0.06 relative units, +14%; p<0.05).

Conclusion: The findings of this study demonstrate that transient oxygen exposure in the neonatal rat modifies protein expression, enzymatic activity and leads to indices of mitochondrial dysfunction (increase in ROS) in the adult kidney; these adverse changes in the mitochondria were more pronounced in adult males than in females. Overall, these findings, suggest that impaired mitochondrial function is associated with and could play a role in the development of arterial hypertension, oxidative stress and cardiovascular disease associated with transient neonatal hyperoxic stress.

Keywords**: Mitochondria, Hypertension, Kidneys, Developmental Programming, Oxygen, ROS**

LIST OF ABBREVIATIONS

Δp	Proton-motive force
ACON	Aconitase
ADP	Adesonine diphosphate
ANT	Adenine nucleotide translocase
ATP	Adesonine triphosphate
BDP	Bronchopulmonary dysplasia
BH_4	Tetrahydrobiopterin
CAT	Catalase
CLD	Chronic lung disease
CO_2	Carbon dioxide
CoQ	Quinone
CoQH	Semi-quinone
$CoQH_2$	Quinol
COX	Cyclooxygenase
CS	Citrate synthase
Ctrl	Control
Cu/Zn SOD	Copper/Zinc superoxide dismutase
Cyt c	Cytochrome c
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
ETC	Electron transport chain
$FAD^+/FADH_2$	Flavin adenine dinucleotide oxidized/reduced
FCCP	Trifluorocarbonylcyanide phenylhydrazone

FT-NSVD	Full-term normal spontaneous vaginal delivery
GPX	Glutathione peroxidase
GS/GSH	Glutathione oxidized/reduced
GTP	Guanosine triphosphate
H	Hyperoxic group
HIF	Hypoxia-inducible factor
H_2O_2	Hydrogen peroxide
IMT	Intima-media thickness
IUGR	Intrauterine growth restriction
LPT	Larger preterm infants
MnSOD	Manganese-dependent superoxide dismutase
MOMP	Mitochondrial outer membrane permeabilization
MRC	Mitochondrial respiratory chain
mtDNA	Mitochondrial DNA
$NAD^+/NADH+H^+$	Nicotinamide adenine dinucleotide oxidized/reduced
NEC	Necrotizing enterocolitis
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Diatomic oxygen
O_2-	Superoxide anion
OH-	Hydroxyl radical
OXPHOS	Oxidative-phosphorylation system
PPROM	Premature pre-labour rupture of membranes (PPROM)
Prx	Peroxiredoxin
PT-NSVD	Preterm spontaneous vaginal delivery

PTP	Permeability transition pore
RNS	Reactive nitrogen species
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SPT	Smaller preterm infants
TCA cycle	Citric acid cycle
UCPs	Uncoupling proteins
VDAC	Voltage Dependant Anion Channel
4-HNE	4-hydroxy-2-nonenal

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1.0 INTRODUCTION

It is now well recognized that low birth weight can significantly impact adult health and disease, particularly the cardiovascular system[1-3]; this concept is termed 'developmental programming'. Low birth weight (classically defined as <2500g) can result from intrauterine growth restriction and/or premature birth. Human and experimental studies have shown that developmental programming of hypertension is associated with vascular dysfunction, vascular oxidative stress and impaired nephrogenesis. The exact mechanisms underlying programming of elevated blood pressure are only partly understood. Among the many factors implicated in adverse perinatal conditions and developmental programming of hypertension, oxidative stress seems an important common denominator. Prematurely born infants are at high risk of sustaining oxidative stress because of their immature antioxidant defenses and the exposure to prooxidant conditions (increase in arterial pO_2 upon birth, exposure to infection, inflammation and supplemental oxygen). The current master's studies will examine in a rodent model of developmental programming of hypertension associated with neonatal exposure to hyperoxic stress (as a model of prematurity associated prooxidant conditions), whether renal mitochondria function is impaired, and contribute to enhance oxidative stress later in life.

Clinical relevance of current studies:

1.1 Prematurity

Prematurity is defined as the birth of a child prior to 37 completed weeks of gestational age[4]. Infants born before 32 weeks are defined as 'very preterm' and those born before 28 weeks as 'extremely preterm'. Prematurity affects about 8% of births in Canada[5] and almost 13% in the United States[6].

There are many conditions associated with preterm birth, such as spontaneous preterm labour, premature pre-labour rupture of membranes (PPROM), infection (both overt and subclinical such as bacterial vaginosis and periodontal disease), multiple gestations, pre-eclampsia, intrauterine growth restriction, antepartum haemorrhage, cervical insufficiency, and uterine malformations[6].

The incidence of preterm birth has increased over recent decades[8]. Preterm birth is a global phenomenon with varying prevalence according to differences in health care throughout the world. For example, the prevalence of preterm births exceeds 12% in the United States[6] with an even higher prevalence amongst certain ethnic backgrounds (highest risk in black and Indigenous women, lowest risk in Hispanic and east Asian women[11]). These high rates are comparable to those found in some third world countries and significantly exceed rates in Europe and the Scandinavian countries[9]. The preterm birth rate remained stable in Canada between 2004 and 2008, fluctuating between 7.7% and 8.2%, with an average of 7.9%[5].

Preterm birth is the leading cause of perinatal morbidity and mortality[9]. The most prominent short-term complications of preterm birth include brain injury, bronchopulmonary dysplasia and retinopathy of prematurity[9]. Technological advances in prenatal care have led to an increase in survival rates into young adulthood of these prematurely born infants[9], causing long term consequences of preterm birth to become more clinically evident. In a national cohort examined by Moster *et al.* which included children who were born at a wide range of gestational ages and who were followed until adulthood, the risk of severe medical disabilities increased sharply with decreasing gestational age at birth[12]. Impact of preterm birth on neurodevelopment has been extensively studied[12, 13]; however, long term impact on other organs/systems is much less known.

1.2 Long-term consequences of preterm birth

As mentioned, the concept that many adult conditions or diseases can have their origins traced back to fetal and early postnatal life has been termed 'developmental programming'[15]. There are multiple insults or factors that can affect a developing fetus leading to the programming of adult diseases such as nutrition, oxidative stress and inflammation, glucocorticoids, fetal hypoxia, and epigenetic changes[16]. Revolutionary studies spanning several decades, with David Barker as the pioneering figure, demonstrated that low birth weight (assimilated to impaired fetal growth in most studies) is associated with raised blood pressure[17], hypertension[17] and cardiovascular mortality later in life in both males and females[18]. Not only is cardiovascular health affected in the

long-term but also other markers of metabolic syndrome, including: type 2 diabetes or insulin resistance[19-21], dyslipidemia[22, 23] and obesity[21, 24], as well as chronic kidney diseases[25]. The 'fetal or developmental origins/programming of disease' concept is now well accepted but the mechanisms of programming remain poorly understood[26, 27]. Developmental studies taking into account gestational age, and therefore prematurity, into their analyses are fewer and examine younger populations of individuals.

Recently, a number of studies have shown an increased risk of developing cardiovascular disease in children and adults born preterm[28-31]. Teenagers and young adults born preterm showed a 5 to 6 mmHg greater mean systolic blood pressure (SBP) compared to those born at term, even after taking (restricted) fetal growth into account[32]. Similarly, Vohr *et al*.[33] demonstrated higher systolic blood pressure and higher rates of systolic prehypertension and hypertension in former preterm 16-year-old adolescents compared to term controls. A study by Skilton *et al.*, demonstrated that maximum aortic thickness, measured as maximum aortic intima-media thickness (IMT) was significantly higher in babies with intrauterine growth restriction (810 µm [SD 113]) than in those without (743 µm [76])[34]. An increased aortic thickness increases the probability of developing atherosclerosis and consequently cardiovascular risk. This follows the trend that birth weight is highly important with regards to the onset of diseases in adulthood. In addition, a study by Jiang *et al.* found that 9 year-old children (both male and female) who had weighed less at birth (after adjusting for current body size and maternal pre-pregnancy size) had significantly smaller total coronary artery diameter, aortic root diameter and left ventricular outflow diameter[35]. A smaller coronary artery diameter has been linked to a higher prevalence of atherosclerotic lesions[36], and a poorer outcome after cardiac interventions or invasive procedures such as coronary bypass surgery or angioplasty[37]. An important element in the development of hypertension and cardiovascular disease is endothelial dysfunction which precedes the disease outcome and therefore may be an important target for prevention strategies[38]. In 17-28 years old prematurely born adults (⍰1000g), endothelial function (evaluated by brachial artery flow mediated vasodilation) is attenuated and correlated with gestational age at birth[39]. These cardiovascular system dysfunctions may also correspond to the observed increased risk of cardiovascular events.

Renal development and function has also been shown to be impaired as a result of preterm birth[25, 40, 41]. In the human fetus, nephrogenesis reaches completion at approximately 34-36 weeks of gestation with more than 60% of nephrons being formed during the last trimester[9, 42]. In infants born prior to 36 weeks of gestation, nephrogenesis is still ongoing making them highly susceptible to a reduced nephron number, be it through intrauterine stress or prenatal or postnatal perturbations[43]. Lackland *et al*.[44] reported an inverse relationship between birth weight and renal failure from all causes of ESRD in Caucasians and African Americans in both males and females in the south-eastern United States. In addition a Norwegian cohort comprised of 526 subjects examined with 38 years of follow-up by Vikse *et al*[45] revealed that low birth weight conferred a 70% increased risk for end-stage renal disease (ESRD) in those born with a birth weight 10th percentile compared within the 10th to 90th percentiles. Similarly, the outcome was observed in both men and women and persisted after adjustments for other birth-related variables. Brenner *et al.*[46] proposed a mechanism for this observed phenomenon, suggesting that impaired or retarded fetal growth leads to a reduced number of nephrons, which in turn leads to increased hydrostatic pressure in the glomerular capillaries, glomerular hyperfiltration, and the development of glomerular sclerosis. This sclerosis can lead to further loss of nephrons as the glomerular hypertension and hyperfiltration worsen[46], ultimately contributing to elevation and maintenance of high blood pressure.

1.3 Reactive Oxygen Species

Reactive oxygen species (ROS) is a term used to describe a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. The superoxide anion (O_2-); the product of a one-electron reduction of oxygen is central to ROS chemistry as it is the precursor of most ROS and a mediator in oxidative chain ractions[47, 48]. Dismutation of O_2 (either spontaneously or through a reaction catalyzed by superoxide dismutases) produces hydrogen peroxide (H_20_2), which in turn may be fully reduced to water or partially reduced to hydroxyl radical (OH-), one of the strongest oxidants in nature[49]. The formation of OH- is catalyzed

by reduced transition metals, which can be reduced again by O_2-, driving this process. 'Oxidative stress' is an expression used to describe an imbalance between pro-oxidants (excessive formation of ROS and/or reactive nitrogen species) and limited antioxidant defences as a result of various deleterious processes[50] or immature in development.

Reactive oxygen species are one of the major contributors of oxidative stress and are also essential signalling molecules required by the cell for normal cellular function. However, when there is an imbalance between ROS production and antioxidant levels, ROS act as oxidizing agents that can potentially trigger cell death via the induction of apoptosis or necrosis[51]. Excessive ROS production is thought to underpin many pathologies associated with neurological degenerative diseases (e.g. Alzheimer & Parkinson diseases), obesity, diabetes (types 1 and 2), metabolic syndrome, cardiovascular disease and ageing[52].

ROS include free radicals such as superoxide anion (O_2-), hydroxyl radical (OH), lipid radicals (ROO-) and nitric oxide (NO). Other reactive oxygen species, hydrogen peroxide (H_20_2), peroxynitrite (ONOO-) and hypochlorous acid (HOCl), although they are not free radicals, have oxidizing effects that contribute to oxidative stress[53]. ROS has been implicated in cell damage, necrosis and cell apoptosis due to its direct oxidizing effects on macromolecules such as lipids, proteins and DNA[48].

1.3.1 Intracellular Sources of Reactive Oxygen Species

1.3.1.1 NADH/NADPH oxidase system

NADH/NADPH oxidases are membrane-associated enzymes that catalyse the 1-electron reduction of oxygen using NADH or NADPH as the electron donor (Figure 1). NADH/NADPH oxidases, initially described in neutrophils, are the most important oxidases in vascular tissue and in cardiac cells[54], are important sources of endovascular ROS and are also integral to the generation of ROS in phagocytic cells[48, 55].

NADH/NADPH activity is regulated by a number of factors known to be involved in the pathogenesis of cardiovascular disease including cytokines, hormones, local metabolic changes and haemodynamic forces[56]. Shear stress (of vascular wall) can have variable effects on NADPH oxidase, be it a transient elevation or a sustained increase[48]. NADH/NADPH-dependent oxidase activity and consequently NADH/NADPH driven O_2- production are also increased in vascular smooth muscle cells by stimulation with angiotensin II[48].

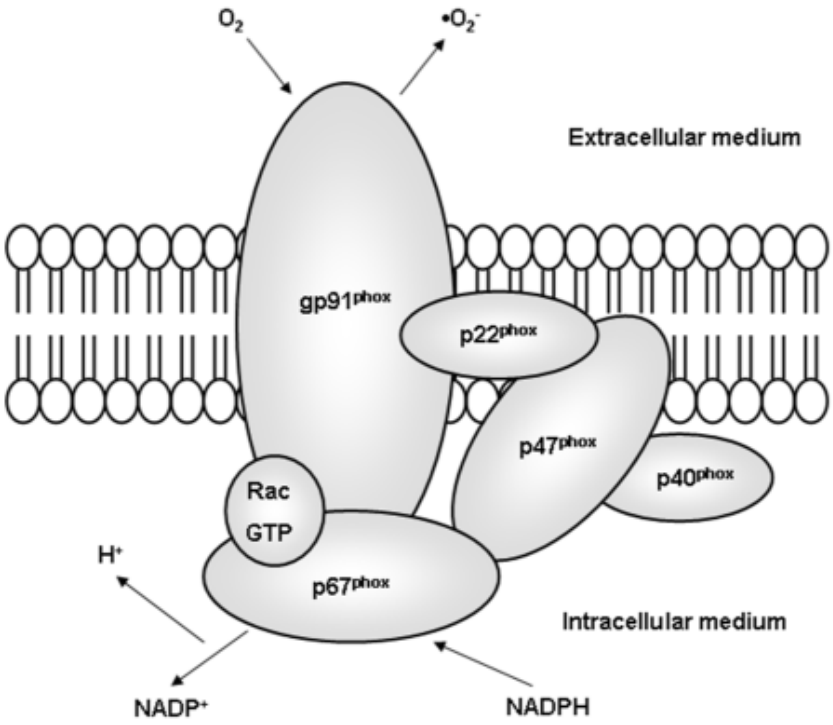

Figure 1: Structure of NAD(P)H oxidase (Reprinted from Rabelo *et al*.[57]). NADPH oxidase consists of the membrane-bound $gp91^{phox}$ and $p22^{phox}$, the cytosolic proteins p47 and p67, and a low molecular-weight G protein (rac).

1.3.1.2 Xanthine oxido-reductase system

Xanthine oxido-reductase exists in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase[58]. The first form reduces NAD+ whereas the latter produces superoxide anion and hydrogen peroxide via a reaction with O_2[48, 59]. The dual role means that it is an important regulator of cellular redox state. Xanthine oxidase generates ROS via purine metabolism pathway and is involved in causing endothelial dysfunction in patients with coronary disease and contractile dysfunction in heart failure[48]. Studies have shown that xanthine oxidase expression and activity are increased in cardiomyocytes isolated from failing hearts[60-63].

1.3.1.3 Nitric oxide synthase uncoupling

Recent studies have demonstrated the crucial role of endothelial NOS (eNOS) as a ROS producing enzyme; resulting in vascular endothelial dysfunction[48, 64]. When tetrahydrobiopterin (BH_4) is limited, electron transfer becomes uncoupled to L-arginine oxidation, leading to the dissociation of the ferrous dioxygen complex and superoxide is produced[64]. Endothelial NOS can produce both NO via its oxygenase function and superoxide through its reductase function (dependent on NADPH)[48]. The product of the reaction between NO and O_2- can oxidize BH_4 which may lead to further eNOS uncoupling. Imbalance between endothelial NO and ROS production is one of the major contributors of endothelial dysfunction which plays an important part in the development of atherosclerosis and cardiovascular disease[48].

1.3.1.4 Mitochondrial electron transport chain

The major source of ROS is considered to be the mitochondrial electron transport chain in the inner mitochondrial membrane, with respiratory complexes I and III being the major sites of superoxide formation in the electron transport chain[65] (Figure 2).

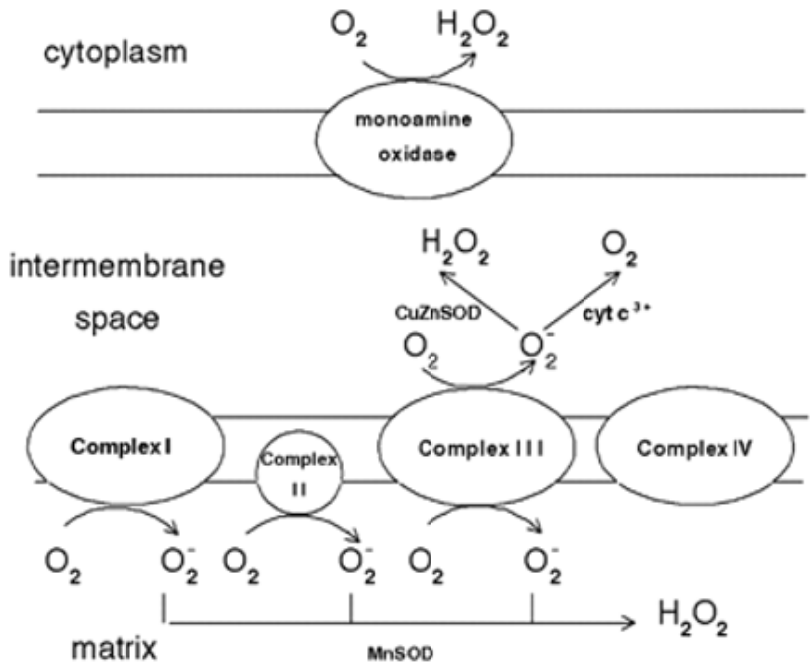

Figure 2: Sites of superoxide formation in the electron transport chain (Reprinted from Turrens *et al.*[66]) Various electron transport chain complexes leak electrons to oxygen producing mainly superoxide anion (O_2-). Superoxide can then reduce cytochrome c (in the intermembrane space), or be converted to hydrogen peroxide (H_2O_2) (in both the matrix and the intermembrane space). If superoxide concentrations increase to a high enough level, they may reduce transition metals via fenton reactions producing hydroxyl radicals (OH) or may react with nitric oxide (NO) to form peroxynitrite. Both hydroxyl radicals and peroxynitrite are very strong oxidants which react with, and cause damage to nucleic acids, lipids and proteins[66].

In the vasculature and the kidneys, ROS derive mainly from the mitochondrial electron transport chain, NAD(P)H oxidases and uncoupled nitric oxide synthase (NOS)[67]. For the current research, we will focus on the impact of mitochondria (dys)function. The roles of NAD(P)H oxidase and of (uncoupled) NOS-derived ROS in vascular dysfunction after neonatal hyperoxic stress were examined in a series of recent studies from the laboratory.

1.4 Oxidative Stress

Oxidative stress is mainly caused by an imbalance between the activity of endogenous pro-oxidative enzymes (such as NADPH oxidase, xanthine oxidase, uncoupled NO synthase or the mitochondrial respiratory chain) and antioxidant enzymes (such as superoxide dismutase, glutathione peroxidase, heme oxygenase, thioredoxin peroxidase/peroxiredoxin, catalase and paraoxonase)[68].

Under oxidative stress, excessive superoxide also releases free iron from iron-containing molecules, which further generate highly reactive hydroxyl radicals (OH) by reacting with hydrogen peroxide via the Fenton reaction[69]. ROS can also induce the opening of the mitochondria membrane permeability transition pore (PTP) and cause a release in cytochrome c and other factors that can lead to apoptosis-mediated cell death[48].

Under physiological conditions, cells increase activities of antioxidant enzymes and other antioxidant defences in order to counter-act the occurrence of oxidative stress. These antioxidants include Copper/Zinc superoxide dismutase (Cu/Zn SOD), manganese-dependent superoxide dismutase (MnSOD), glutathione peroxidase, glutathione reductase and catalase (CAT). MnSOD and Cu/Zn SOD convert O_2- to hydrogen peroxide, which is then transformed to water by glutathione peroxidase, catalase or peroxiredoxin[48, 70]. The thioredoxin system is composed of several proteins including peroxiredoxin, that are important in redox homeostasis due to their ROS scavenging ability[71]. Other antioxidant defences include radical scavengers such as vitamin E, beta carotene and vitamin C[48]. (Figure 3)

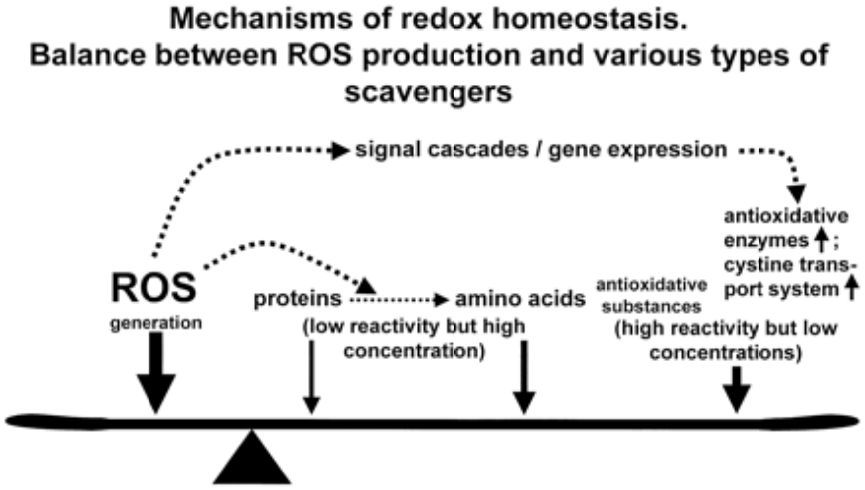

Figure 3: Redox homeostasis (Reprinted from Droge[70]). Balance between ROS production and scavengers. The steady-state levels of ROS are determined by the rate of production and clearance by the various scavenger mechanisms. Antioxidative enzymes include superoxide dismutase (SOD), glutathione peroxidase, catalase and thioredoxin.

1.5 Preterm- high oxygen after birth

The human embryo develops in an oxygen-poor environment compared with later stages of fetal life[72]. This is thought to be responsible for the switch from embryonic to fetal hemoglobin, and may protect the developing embryo from the deleterious effects of high oxygen levels and free radicals[72]. A study by Fischer *et al*. revealed that the mean oxygen tension in the female reproductive tract of rhesus monkeys, hamsters and rabbits was only 40% or less of atmospheric O_2 (atmospheric O_2 is approximately 21%)[73]. Preterm infants therefore face a number of difficulties that must be overcome, including: difficulties in gas exchange resulting from surfactant deficiency, incomplete lung development, inadequate respiratory drive, and poor clearance of lung fluid[74]. Among the most common treatments in neonatal intensive care is the use of supplemental oxygen, and finding the optimum oxygen concentration whereby growth and development is promoted, while minimizing the risks of hyperoxia is of utmost importance[75]. A study by Vento *et*

al. found that initiating resuscitation with 30% versus 90% oxygen for infants 28 weeks' gestation or less significantly reduced the risk of developing bronchopulmonary dysplasia[74, 76]. Even brief exposure to high levels of oxygen induces oxidative stress, and the subsequent production of oxygen free radicals can be extremely dangerous, particularly for preterm infants who lack adequate protection from indigenous antioxidants[75]. Since fetal life takes place in a relatively hypoxic environment, preterm birth results in the sudden and premature exposure to comparatively high levels of oxygen and oxidative stress results from the lack of protection (underdeveloped antioxidant system) coupled with the necessary supplemental oxygen therapy to treat this population. This increased risk of oxygen toxicity poses a significant threat of tissue damage in response to reactive oxygen species and excess free radicals[75].

The vast spectrum of disease evident in the neonatal period includes those that stem from the interruption of normal organogenesis in the vascular tree, pancreas, lung and kidney[9]. Of particular relevance for the current Master thesis, kidney development (nephrogenesis) begins at approximately day 30 of gestation, with the majority of nephrons being formed between week 20 until 34 to 36 weeks of gestation[14]. Given that nephrogenesis is still ongoing prior to 34 weeks of gestation, it is very likely that neonates born at less than 34 weeks of gestation will experience alterations in the structural development of the kidney[14]. In addition, the extrauterine environment is suboptimal for organogenesis due to the exposure to a number of insults, including high oxygen concentrations[14]. Oxidative stress has emerged as a likely promoter of several pregnancy-related disorders, such as spontaneous abortions, embryopathies, preeclampsia, fetal growth restriction, preterm labor and low birth weight[77].

Saugstad *et al*.[78] described the implications of oxidative stress in a number of conditions affecting premature infants, including: bronchopulmonary dysplasia (BPD) or chronic lung disease (CLD), retinopathy of prematurity (ROP), necrotizing enterocolitis (NEC) and patent ductus arteriosus. A number of studies indicate that the development of CLD is related to oxidative stress detected by the augmentation of peroxidation products in the very first days of life[79, 80]. Retinopathy of prematurity (ROP) is thought to occur through the frequent hyperoxygenation of the retina and subsequent oxidative damage due to the limited antioxidant defense of the retina in the preterm

infant[81]. A study by Papp *et al*. discovered that the ratio of oxidized to reduced glutathione more than doubled in patients with ROP compared with controls, and may be used as a method of identifying infants at risk of developing ROP[82].

Newborn infants possess an underdeveloped antioxidative capacity, and when exposed to a greater percent oxygen concentration relative to the intrauterine milieu[83], they are at a significantly higher risk of oxidative stress; a fact supported by many studies in both human and animal models[84-86]. A study by Lee *et al*.[87] showed that smaller preterm infants (SPT) had significantly lower levels of GSH and NADPH ratio compared to larger preterm infants (LPT). GSH and the NADPH ratio are believed to be important indices of the cellular redox state[88] and when impaired can lead to ROS generation[48]. It was further demonstrated that preterms had increased glucose-6-phosphate dehydrogenase (G6PD) activity compared to full term appropriate-for-age infants, suggesting a compensatory mechanism to combat an adverse environment[87], as patients with G6PD deficiency often have lower antioxidant capacities[89]. Georgeson *et al*.[90] reported significantly higher catalase (CAT), glutathion peroxidase (GPX), and Cu/Zn-SOD activity in healthy neonates, in the full-term normal spontaneous vaginal delivery (FT-NSVD) category compared to preterm normal spontaneous vaginal delivery (PT-NSVD)[90]. Of further importance for the current work is the role of oxidative stress and free radicals in renal injury. Studies by Vento et al.[91] and Perrone et al.[92], showed elevated concentrations of N-acetyl-glucosaminidase in the days following birth in preterm babies (a reliable marker for detecting renal tubular damage after neonatal anoxia). This increase in N-acetyl-glucosaminidase concentration demonstrates the correlation between indices of oxidative stress and kidney damage[92]. These results clearly demonstrate that preterm neonates possess an impaired antioxidant capacity relative to their term counterparts which may compromise their ability to deal with oxidative stress, predisposing them to the diseases discussed above.

1.6 Oxidative Stress and its role in programming

Oxidative stress both during fetal and neonatal life is considered a key element in programming of long-term cardiovascular consequences[93-95] (see Figure 4). Regarding the kidney, Yzydorczyk *et al*.[83] explored the relationship between neonatal O_2 exposure and long-term renal damage. A significant reduction in nephron number was observed in both male and female rats exposed to 80% oxygen (hyperoxic group) from day 3 to day 10 of life compared to the control (room air) and examined at 25-35 weeks of age (which corresponds to an adult in humans). Nephrogenesis in rats proceeds until 5 to 8 days postnatally[83] compared to 32-36 weeks in humans[96], allowing the kidney to be compared to that of a preterm infant. Since nephron number is decreased in adult patients with primary hypertension[97], and reduced nephron endowment is a function of impaired renal growth, the kidney is an important organ to explore in the development (programming) of hypertension. Furthermore, oxygen tension has been shown to possess a regulatory role in nephrogenesis through hypoxia-inducible factor (HIF)[98] which regulates expression of genes involved in angiogenesis and rat organogenesis appears to be enhanced in low (1-3%) O_2 concentration compared to the standard (21%) O_2 concentration[83]. All of these factors clearly indicate that the kidney is adversely affected under high oxygen concentrations and since mitochondria are known to be the primary producers of ROS[65], it is important to investigate possible effects of oxidative stress and programming of adult disease on mitochondria function in the kidney.

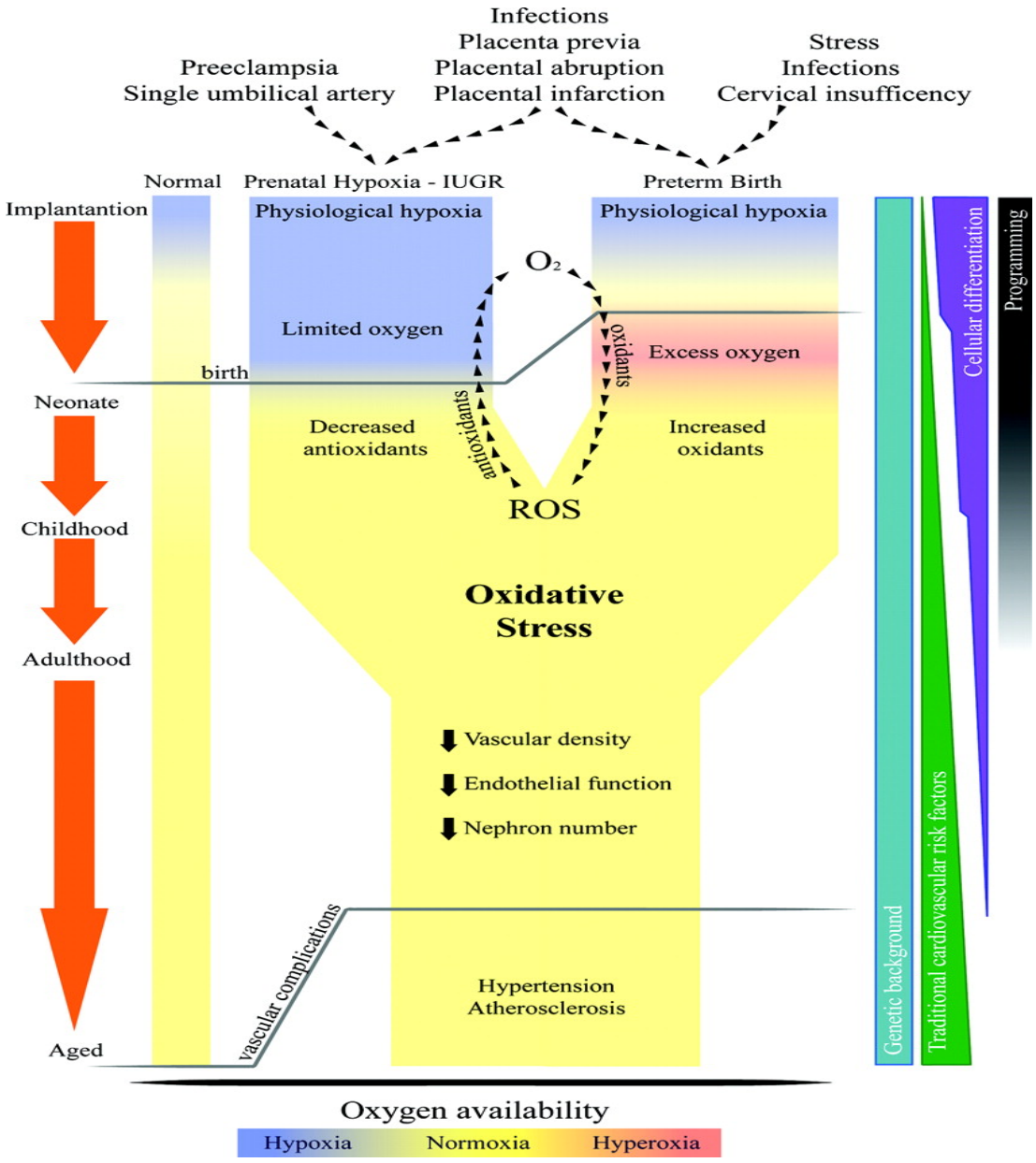

Figure 4: Representation of potential common pathways in the 'fetal origins of adult disease' theory (Reprinted from Davidge *et al*.[99]).

1.7 Mitochondria

Mitochondria (shown in Figure 5) are surrounded by a double-membrane system, consisting of inner and outer mitochondrial membranes separated by an intermembrane space. The inner membrane forms numerous folds (cristae), which extend into the interior (or matrix) of the organelle and provide a greater surface area for the production of ATP. Each component is responsible for distinct functional roles, with the matrix and inner membrane accounting for the vast majority of mitochondrial work and ATP production[100].

Mitochondria also contain their own genome, which is separate and distinct from the nuclear genome of the cell. Mitochondrial DNA (mtDNA) follows maternal inheritance and is termed 'naked' DNA since it lacks histone protection and has a weak repair capacity, making it more vulnerable to damage than nuclear DNA[101]. The level of oxidized bases in mtDNA is 10- to 20-fold higher than in nuclear DNA[102].

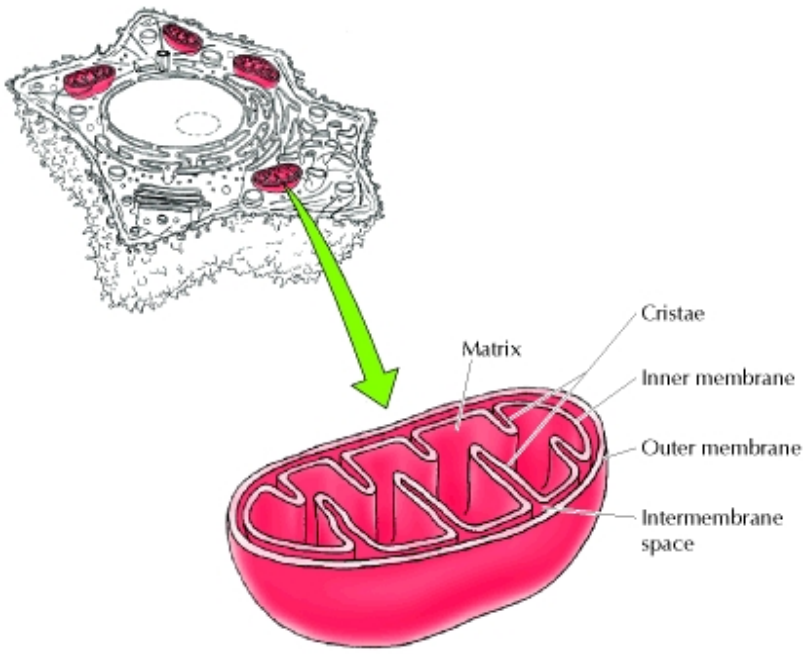

Figure 5: Structure of a mitochondrion (Reprinted from Cooper *et al*.[100]).

1.7.1 Mitochondrial Function

Mitochondria are the primary energy-generating system in most eukaryotic cells and play a crucial role in energy metabolism through their involvement in ATP production by oxidative phosphorylation[103]. Apart from energy conversion, mitochondria take part in a number of other processes including calcium homeostasis, nucleotide precursor biosynthesis, cofactor biosynthesis, and apoptosis[104]. Given that mitochondria are involved in so many important and well-established functions, mitochondrial dysfunction has a multitude of effects in multicellular organisms, some of which remain poorly understood[103]. Importantly, dysfunction of the mitochondria has been linked to a wide range of human disorders, including, cancer, neurodegenerative diseases, Type 2 Diabetes and hypertension [105, 106] and at a cellular level can contribute to inflammation, ageing, apoptosis and alterations in cellular calcium handling[104].

1.7.2 The Citric Acid Cycle

The citric acid cycle is located in the mitochondrial matrix and plays a crucial role in regulating cell metabolism through the production of reducing equivalents in the form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$)[107, 108]. It is through the rate of delivery of these reducing equivalents to the electron transport chain, along with adenosine diphosphate that the citric acid cycle is able to regulate oxidative phosphorylation. This flux through the citric acid cycle is dependent upon the supply of acetyl units, activation of the three non-equilibrium reactions within the citric acid cycle and the total concentration of the citric acid cycle intermediates[107]. The cycle (dependent on oxygen) is also an important source of precursors, not only for the storage forms of fuels, but also for the building blocks of many other molecules such as amino acids, nucleotide bases, cholesterol, and porphyrin (the organic component of heme)[109].

The citric acid cycle includes a series of oxidation-reduction reactions that result in the oxidation of an acetyl group to two molecules of carbon dioxide; transforming fuel molecules into ATP via the electron transport chain[109]. The overall pathway of the citric acid cycle is shown in Figure 6.

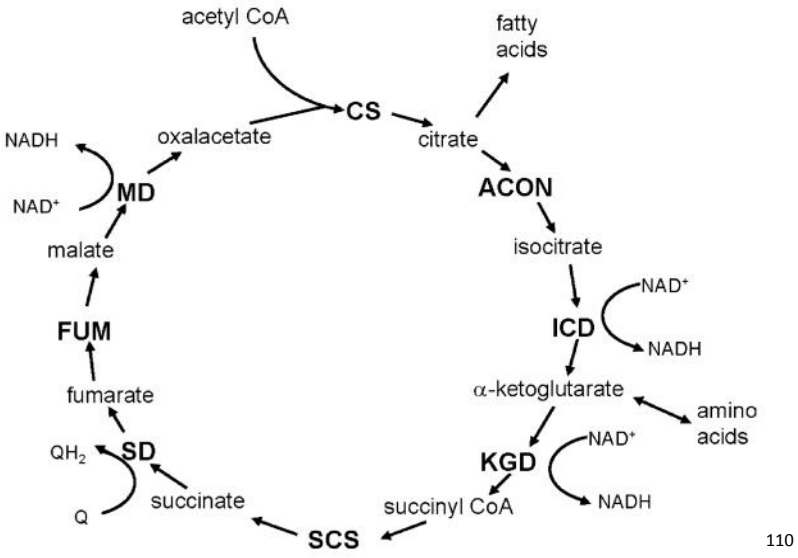

Figure 6: Overview of the Citric Acid Cycle (Reprinted from Berg *et al.*[109]) The citric acid cycle oxidizes two-carbon units, producing two molecules of CO_2, one molecule of GTP, and high-energy electrons in the form of NADH and $FADH_2$. A four-carbon compound (oxaloacetate) condenses with a two-carbon acetyl unit to yield a six-carbon tricarboxylic acid (citrate), which undergoes a series of decarboxylations producing succinate. Oxaloacetate is then regenerated from succinate. Two carbon atoms enter the cycle as an acetyl unit and two carbon atoms leave the cycle in the form of two molecules of carbon dioxide. Three hydride ions (six electrons) are transferred to three molecules of nicotinamide adenine dinucleotide (NAD+), whereas one pair of hydrogen atoms (two electrons) is transferred to one molecule of flavin adenine dinucleotide (FAD). The function of the citric acid cycle is the harvesting of high-energy electrons from carbon fuels[109]. CS, citrate synthase; ACON, aconitase; ICD, isocitrate dehydrogenase; KGD, α-ketoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SD, succinate dehydrogenase; FUM, fumarase; MD, malate dehydrogrenase.

Aconitase (mitochondrial, m-aconitase) is localized in the mitochondrial matrix. It catalyzes conversion of citrate to isocitrate as part of the citric acid cycle. The enzyme is inactivated upon oxidation of its iron-sulfur cluster by superoxide. Upon inactivation, isolated aconitase induces production of hydroxyl radical, most likely mediated by the release of Fe^{2+}. [111]

Experimental studies have shown that specific enzymes of the citric acid cycle are susceptible to oxidation[112-114]. Aconitase exhibits the most significant age-associated decline in activity and is known to be highly susceptible to oxidative damage[110]. Citrate is a key intermediate in the citric acid cycle and in fatty acid synthesis, and increased citrate synthase activity may contribute to progressive renal injury[115].

1.7.3 Electron Transport Chain

The respiratory chain, located in the inner mitochondrial membrane, consists of five multimeric protein complexes[116] (Figure 8). Mitochondrial ATP production relies on the electron transport chain, composed of respiratory chain complexes I-IV, which transfer electrons in a stepwise fashion until they finally reduce oxygen to form water[117]. Mitochondria contain their own genome, 13 polypeptides, all of them structural subunits of the oxidative phosphorylation complexes I, III, IV and V are encoded[118]. As explained by Mailloux *et al*.[119], NADH: ubiquinone oxidoreductase (complex I) is composed of approximately 45 subunits and is the site of NADH oxidation. The flavin mononucleotide of complex I accepts the electrons from NADH and passes them through a series of eight iron-sulfur clusters to ubiquinone. Ubiquinol: cytochrome c oxidoreductase (complex III) has 11 subunits and contains three hemes and a Fe-S cluster center. Complex III plays a crucial role in funneling electrons from the ubiquinol generated by complexes I and II to cytochrome c. Upon binding to the Qo site of complex III, one electron from ubiquinol is ferried through the Rieske Fe-S cluster protein to the electron acceptor, cytochrome c. The resulting unstable semiquinone then donates the remaining electron to the cytochrome b hemes (cytochrome bL and bH). The electron in cytochrome b is then used to re-reduce ubiquinone in the Qi site to produce ubiquinol. Two electrons from semiquinones in the Qo are required for the reduction of ubiquinone to ubiquinol in the Qi site. This is referred to as the Q-cycle (Figure 7) because lone electrons remaining in semiquinone are reused to reduce ubiquinone back to ubiquinol. This electron cycling mechanism

is crucial for preventing the use of these lone electrons for univalent reduction of O_2 to ROS. Complex III is very well regarded as the major source of ROS from the respiratory chain[27, 120]. The ability to generate ROS on either side of the mitochondrial inner membrane can be attributed to the build-up of semiquinone radicals in the Qo site[119].

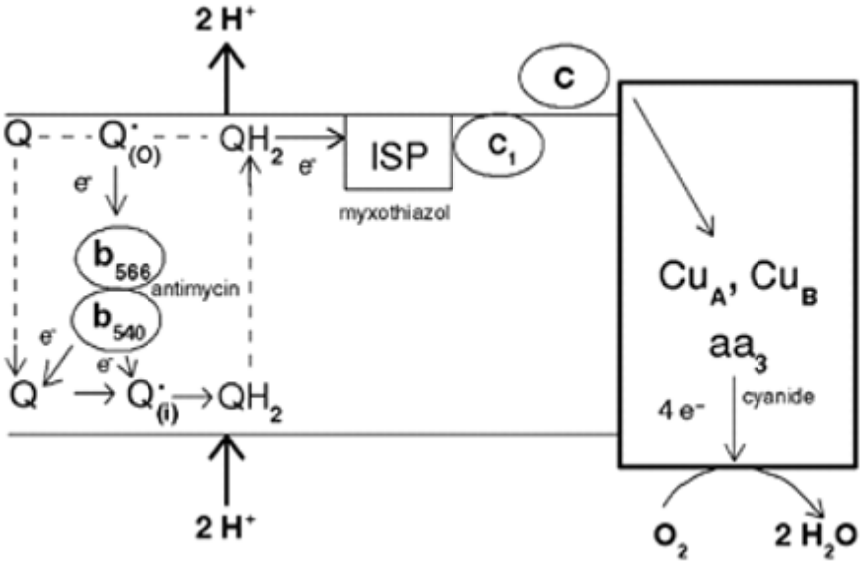

Figure 7: Mechanism of electron flow in the Q-cycle in Complex III (Reprinted from Turrens *et al*.[66]). Ubiquinone (Q) is reduced either by complex I or II or by electrons transferred from cytochrome b on the inner side of the membrane (i), producing ubiquinol (QH_2). Ubiquinol then transfers one electron to oxygen via the Rieske iron-sulfur protein (ISP), cytochrome C_1 and cytochrome C. The terminal oxidase is reduced by four electrons which in turn are transferred to oxygen producing water (H_20)[66].

1.7.4 Mitochondrial ATP production

Mitochondria play a central role in energy metabolism through their significant contribution to adenosine triphosphate (ATP) production; this process depends on oxygen. When oxygen is limited, the less efficient process of anaerobic respiration metabolizes glycolytic products directly in the cytosol[121, 122]. NADH and $FADH_2$ are energy rich molecules formed in glycolysis, fatty-acid oxidation and the citric acid cycle that donate electrons to the ETC, establishing an electrochemical gradient[122]. Electrons move toward compounds with more positive oxidative potentials and the incremental release of energy during the electron transfer is used to pump protons (H^+) into the intramembrane space. As described in a recent review by Bratic and Trifunovic (2010)[121] complexes I, III and IV function as H^+ pumps that are driven by the free energy of coupled oxidation reactions. During the electron transfer, protons pumped from the mitochondrial matrix to the intermembrane space produce a potential of around 150-180 mV[121, 123]. The proton gradient generates a chemiosmotic potential, also known as the proton motive force, which drives the ADP phosphorylation via the ATP synthase (F_0F_1-ATPase-Complex V). The rate of mitochondrial respiration depends on the phosphorylation potential expressed as [ATP]/[ADP][Pi] ratio across the inner mitochondrial membrane that is regulated by the adenine nucleotide translocase (ANT). In the case of increased cellular energy demand, when the phosphorylation potential is decreased and more ADP is available, a respiration rate is increased leading to an increased ATP synthesis. Tight coupling between the electron transport and the ATP synthesis and an inhibition of ATP synthase will therefore also inhibit the electron transport and cellular respiration. Under certain conditions, protons can re-enter into mitochondrial matrix without contributing to the ATP synthesis and the energy of proton electrochemical gradient will be released as heat. This process, known as proton leak or mitochondrial uncoupling could be mediated by protonophores (such as FCCP) and uncoupling proteins (UCPs)[121].

Mitochondrial ATP production is fuelled by the chemical energy stored within the carbon-to-carbon bonds of various energy substrates and relies on the great oxidative power of diatomic oxygen (O_2). After the oxidation of substrates in the citric acid cycle, the resulting electron carriers, NADH and $FADH_2$, are oxidized by complexes I and II respectively. The liberated electrons/reducing equivalents are then transferred through the respiratory complexes III and IV via prosthetic groups arranged in increasing redox potential to the terminal electron acceptor, O_2. This generates a

proton-motive force (Δp) across the mitochondrial inner membrane. This stored potential energy is then tapped by the F_0F_1-ATP synthase as protons move through it back to the matrix and drive ATP formation from ADP and Pi. This process is referred to as coupled respiration because the energy derived from the transfer of electrons to the terminal electron acceptor O_2 is completely harnessed to drive ATP production. However, the reduction of dioxygen to water during aerobic respiration is accompanied by variable extents of reactive intermediate formation, because the two outermost electrons in molecular oxygen occupy separate orbitals. Consequently, the univalent reduction of O_2 results in the formation of various singlet-electron-containing intermediates that can damage the cell[119] (see Figure 8).

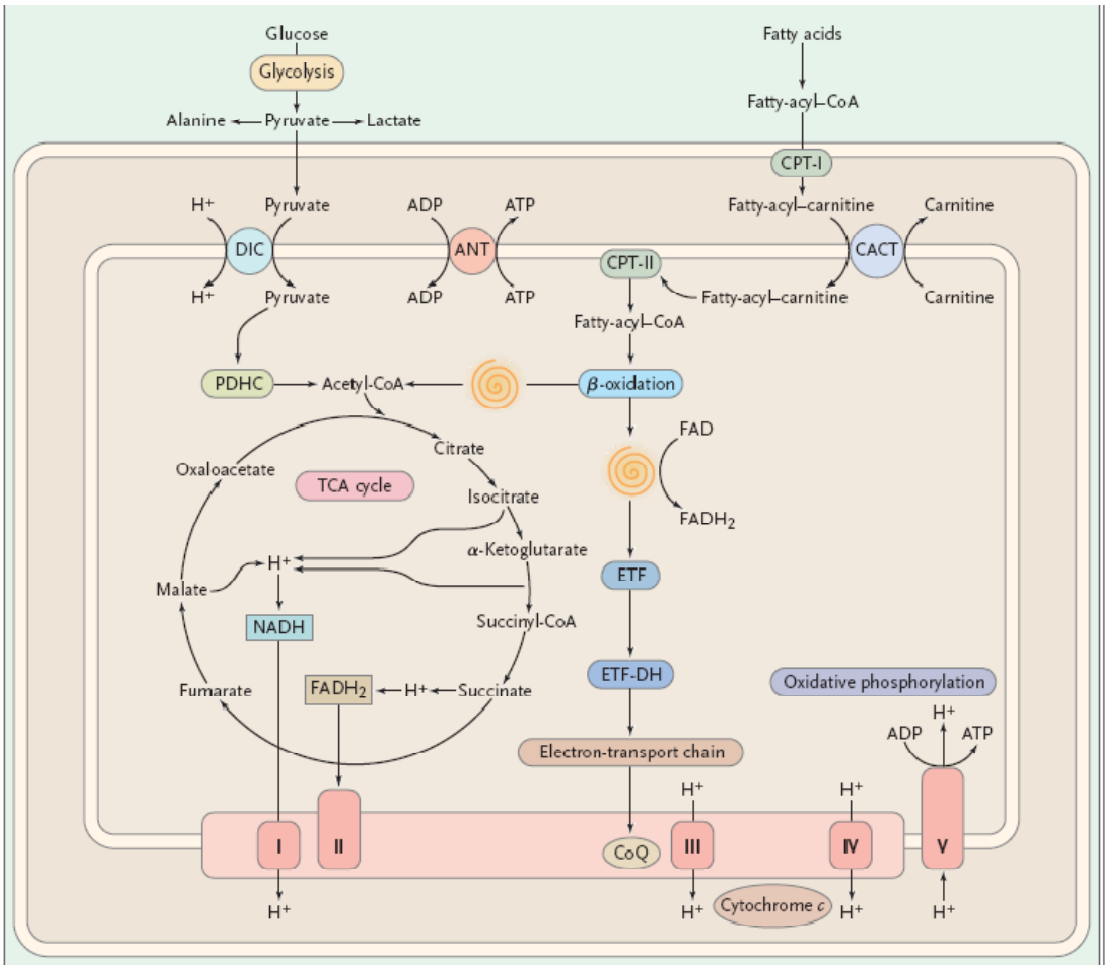

Figure 8: Mitochondrial energy metabolism (Reprinted from DiMauro *et al.*[116]). Overview of the numerous tasks performed by mitochondria, including : pyruvate oxidation, the citric acid (TCA) cycle, metabolism of amino acids, fatty acids, and steroids, but the most important is the generation of adenosine triphosphate (ATP) via the electron-transport chain and the oxidative-phosphorylation system (the 'respiratory chain')[116]. ADP, adenosine diphosphate; ANT, adenine nucleotide translocator; CACT, carnitine-acylcarnitine translocase; CoQ, coenzyme Q; CPT, carnitine palmitoyltransferase; DIC, dicarboxylate carrier; ETF, electron-transfer flavoprotein; ETF-DH, electron-transfer dehydrogenase; FAD, flavin adenine dinucleotide; $FADH_2$, reduced FAD; NADH, reduced nicotinamide adenine dinucleotide; PDHC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid; I, complex I; II, complex II; III, complex III; IV, complex IV; V, complex V.

1.7.5 ROS production and handling in the mitochondria

Approximately 90% of cellular oxygen is consumed within the mitochondria, accounting for nearly 90% of the total ROS produced in the cell[121, 124]. ROS have dual roles within aerobic cells, whereby they may act as signalling molecules in a wide range of normal cellular processes or as oxidizing agents that can potentially trigger cell death[119]. The mitochondrial electron transport chain is the major ROS production site but the mitochondria are also a prime target for oxidative damage. With accumulating oxidative damage, such as with aging or in disease states, mitochondria become larger and less numerous with age, accumulating vacuoles, cristae abnormalities and intra-mitochondrial paracrystalline inclusions[125]. Mitochondrial respiratory chain enzymes activities decrease, as well as mitochondrial membrane potential (on which the production of ATP is dependent); the amount of oxidative damage to proteins and mtDNA increases, with an associated accumulation in the quantity of mtDNA mutations[124, 126]. Age-associated decline in the mitochondrial respiratory chain capacity has also been reported in various tissues[121], including the kidney[127].

Approximately 1-2% of the O_2 consumed in the mitochondria is reduced (univalently) to superoxide (O_2-), the main molecule of ROS signalling and damage[102, 119]. Superoxide is then usually converted into H_20_2 by the manganese-superoxide dismutase or copper/zinc-superoxide dismutase, found in the mitochondrial matrix and in the intermembrane space, respectively[128]. The high amount of O_2 and the low concentration of superoxide, makes superoxide formation a very energetically favorable process[102, 129]. Superoxide can also react with nitric oxide radical (NO) to generate peroxynitrite (ONOO), a lipid soluble and highly toxic reactive nitrogen species. Of greater importance, superoxide production by the respiratory chain can increase significantly when the mitochondrial membrane potential is high (e.g. when ATP production is low)[130, 131]. Unlike superoxide, H_20_2 is more stable and is able to oxidize low-molecular weight thiolating

agents such as glutathione (GSH). This stability allows H_20_2 to play a key role as a mitochondrial signalling molecule. It is when H_20_2 is produced in an uncontrolled fashion that it can damage the cell by generating hydroxyl radical through Fenton or Haber-Weiss reactions[132].

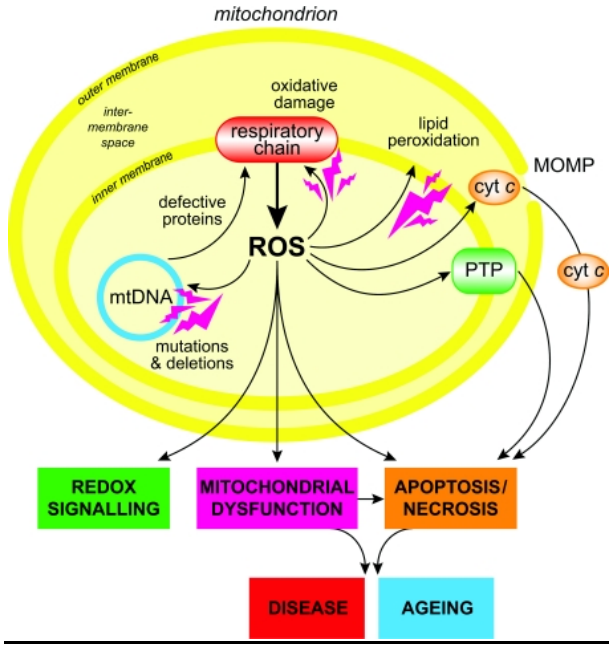

Figure 9: Overview of mitochondrial ROS production (Reprinted from Murphy[133]). ROS production by mitochondria can impair their ability to synthesize ATP and to carry out normal cellular functions, including the citric acid cycle, fatty acid oxidation, the urea cycle, amino acid metabolism, heme synthesis and FeS centre assembly. Mitochondrial oxidative damage can also increase the tendency of mitochondria to release proteins such as cytochrome c (cyt c) to the cytosol via the mitochondrial outer membrane permeabilization, thereby activating the cell's apoptotic machinery. In addition, mitochondrial ROS can affect redox signalling and lead to the induction of the mitochondrial permeability transition pore (PTP), rendering the inner membrane permeable to small solutes in situations such as ischemia/reperfusion[133].

1.7.6 Antioxidant Defense Systems

The balance between oxidants and antioxidants is commonly referred to as the 'redox state'[48]. Antioxidant defense is comprised of a large set of enzymes and low-molecular weight compounds that sequester the excess ROS molecules generated by aerobic respiration. Cells engage in a complex array of short and long term preventative measures such as the mild uncoupling of the oxidative phosphorylation system in the short term and possible upregulation of antioxidant enzyme expression in the long term. Some antioxidative systems can be energetically costly because they are reliant on both ATP and NADPH[119].

The peroxiredoxin (Prx) and GSH/glutaredoxin (Grx)/glutathione peroxidase (GPx) systems are the most efficient at eliminating H_20_2 in the mitochondria and cytosol because of their low Km values and abundance[134-136]. The presence of catalase in the mitochondria is also strongly debated because it seems to be localized primarily in peroxisomes and to a lesser extent the cytosol. For example, Kirkinezos *et al*.[137], describe mitochondria as being catalase free, whereas Radi *et al*.[138], demonstrate the presence of catalase in rat hearts. The antioxidative defense system is rejuvenated by the reductive power of NADPH (requires energy)[119]. They require ATP to synthesize small-molecule antioxidants and molecules for the sequestration of ROS and ROS by-products. The reactivation of H_20_2-scavenging system requires the reductive power of NADPH (requires energy).

The second layer of ROS defenses is formed by enzymes dealing with the primary ROS generated in mitochondria, superoxide radical and hydrogen peroxide. Superoxide is a substrate for mitochondrial manganese-containing superoxide dismutase (MnSOD, also known as SOD2). This enzyme is localized exclusively to the mitochondrial matrix, and its only function is to facilitate dismutation of superoxide radical to H_20_2, thereby protecting mitochondrial iron-sulfur cluster containing enzymes from superoxide attack[111]. MnSOD does not require any cofactors so the efficiency of this system is determined solely by the amount of enzyme present.

Catalase is an antioxidant enzyme (ubiquitously expressed by aerobic cells containing the cytochrome system and is localized to the peroxisome, the nucleus, or mitochondria) that converts H_20_2 into O_2 and H_20[137]. Two isoforms of peroxiredoxins (Prx3 and Prx5) are found in mammalian mitochondria. Prx3 (or SP-22) is ubiquitously present in various rat tissues, including the kidney[139],

whereas Prx5 is mainly expressed in bovine tissues, with the highest level found in testis[111]. Importantly, Prx3 gene expression is known to be induced by oxidative stress[111].

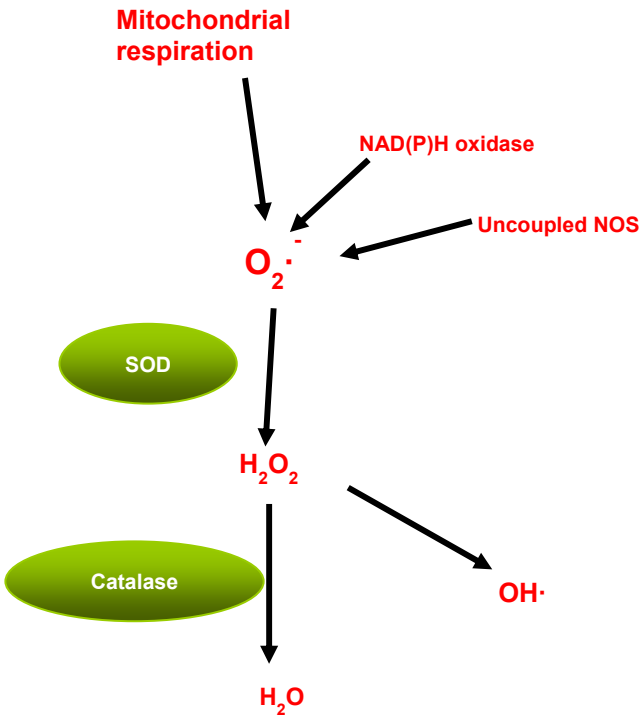

Figure 10: Selected pathway of ROS formation and neutralization

1.7.7 Mitochondria and Uncoupling Proteins

Mild uncoupling of mitochondrial oxidative phosphorylation is effectively short circuiting the OXPHOS system due to leak of protons from the intermembrane space to the matrix, which means that the protons bypass ATP synthase and cause a decrease in the mitochondrial inner membrane potential[119]. A strong correlation exists between mitochondrial membrane potential and ROS production; small increases in the membrane potential induce ROS formation whereas slight decreases can substantially diminish the production of these reactive intermediates. Although the mechanisms responsible for the mitochondrial proton leak are still debated[140-144], the proteins thought to be involved include adenine nucleotide translocase (ANT) and the uncoupling proteins, UCPs 1-3. ROS and ROS derivatives (specifically superoxide and 4-hydroxy-2-nonenal) have been described as activators of UCPs 1-3. Negative feedback mechanism has been suggested in which increased ROS induce uncoupling to decrease the formation of these toxic species. It is generally thought that the two major sites of mitochondrial ROS production are complexes I and III of the respiratory chain[119].

UCPs are mitochondrial transporters present in the inner mitochondrial membrane and are found in all mammals[145]. Uncoupling proteins function as proton channels to allow proton leakage back across the inner mitochondrial membrane without creating ATP. It has been shown that O_2- can activate UCP2[146], possibly as a protective mechanism against excessive mitochondrial ROS formation[52].

High proton motive force comes with an additional cost, the production of ROS. Because ROS production is highly dependent on the proton motive force, proton leak might help to limit the oxidative damage. There are presently 3 identified isoforms of UCPs (UCP1, UCP2, UCP3). UCP2 and UCP3 are recently identified UCP1 homologues but their function in normal cellular physiology is still unclear[145, 147]. UCP2 and UCP3 proteins might be involved in the proton conductance only upon activation with certain activators (such as ubiquinone, superoxide, reactive alkenals and other alkenal analogues)[121].

ANT and UCPs 1-3 contribute to inducible proton leak. However, only UCPs 2 and 3 have been found to catalyze a proton leak that decreases ROS emission from mitochondria. UCP2 is widely expressed in a number of cells and tissues including the spleen, thymus, macrophages,

hypothalamus, pancreatic β-cells, stomach and the kidney[148]. UCP3 is expressed almost exclusively in skeletal muscle and brown adipose tissue and to a lesser extent in the heart[148]. In several studies Brand and colleagues[149, 150] provided empirical evidence that superoxide and derivatives of ROS, namely 4-HNE, activated proton leak through UCPs 1-3. In this mechanism, the overproduction of ROS by the respiratory chain activates proton leak through the UCPs, thereby creating a negative feedback loop that regulates ROS formation by the mitochondria[119].

1.7.8 Mitochondrial Permeability Transition Pore

Apart from energy conversion, mitochondria take part in a number of other processes including calcium homeostasis and apoptosis[151]. It is well documented that an increased permeability of the mitochondrial membranes is a key event in controlling apoptotic pathways, with the permeability transition pore (PTP) thought to play a major role[152-156]. Prolonged opening of the PTP, a nonspecific high-conductance proteinaceous channel of the inner membrane, leads to collapse of the membrane potential, mitochondrial matrix swelling, rupture of the outer mitochondrial membrane and release of cytochrome C [157, 158], which can ultimately lead to energy failure of the cell.

1.8 Hyperoxia exposure and mitochondria

A study by Clerch *et al*.[159], found that neonatal rats exposed to ≥ 95% O_2 (hyperoxia) for 48h exhibited a fall in lung activity of MnSOD. This suggests that the increased production of superoxide during hyperoxia results in the observed decrease in MnSOD activity which worsens the effect of the enhanced formation of superoxide and thereby contributes in a major way to the loss of mitochondrial function during hyperoxia[159]. Further studies have shown that oxidative damage to mitochondrial enzymes plays a significant role in oxygen toxicity. Schoonen *et al*.[160], found that exposure of Chinese hamster ovary cells to hyperoxia resulted in the inactivation of three key mitochondrial enzymes (NADH dehydrogenase, succinate dehydrogenase, and α-

ketoglutarate), a 2.5-fold decrease in cellular ATP, and cell death[159, 160]. Fischer *et al*.[161] found reduced oxidative metabolism and a fall in ATP concentration in pulmonary macrophages following a 72-hour oxygen exposure (≥ 95% O_2). Campian *et al*.[27] reported that hyperoxia tolerant HeLa-80 cells, as compared to wild-type HeLa-20 cells generate substantially less ROS through modification of cytochrome C oxidase activity and a tighter coupling of the electron transport chain (ETC). This was assessed by the oxidation of three fluorescent probes, the hyperoxia-mediated inactivation of aconitase, and the accumulation of mitochondrial protein carbonyls under hyperoxic conditions. Li *et al*.[162] demonstrated that the absence of MnSOD rapidly leads to impairment of mitochondrial function in several organs, with the heart suffering the most pronounced consequences, followed by the liver and the brain. Impaired fatty acid metabolism was also discovered through accumulation of lipid in the liver and skeletal muscle, metabolic acidosis and ketosis. Interestingly, no evidence of mtDNA deletions or of structural damage to the mitochondria were found[162]. Schriner *et al*.[163] demonstrate that catalase over expression increases lifespan in the mouse. Furthermore, several studies[164-166] have shown decreased levels of SOD, GPx and catalase in newborns and small for gestational age infants exposed to various toxins (alcohol, tobacco, etc.) in *utero*. In addition, Yarian *et al*.[110] report that among the citric acid cycle enzymes, aconitase exhibits the most significant age-associated decline in activity, while NADPH :NADP+ ratio also declined with age (important for redox balance) in mitochondria isolated from rat kidneys. Maintaining redox homeostasis by regulation of antioxidant defense systems or ROS production is therefore one of the key mechanisms for tissues to adapt to hyperoxia-induced oxidative stress[167]. An altered neonatal environment could have lasting effects on antioxidant enzymes and result in the observed disease phenotype. Currently not much is known about the precise mechanisms involved, making it especially important to determine the long-term effects.

1.9 Hypothesis, Aims and Study Design

1.9.1 Hypothesis

It was hypothesised that a transient neonatal hyperoxic stress would lead to chronic alterations in cellular oxidant/antioxidant coupling through the modulation of the expression of key proteins involved in mitochondrial function, enzymatic activity of key citric acid cycle enzymes and an excessive production of ROS.

1.9.2 Aims

To evaluate the expression and function of key proteins and enzymes participating in mitochondrial function and antioxidant capacity in the kidney of adult rats exposed to high oxygen as newborns; at 4 and 16 weeks of age.

1.9.3 Study Design

To address the aims of this study, Sprague-Dawley rat pups were exposed to 80% O_2 from day 3 to day 10 of life and studied at 4 and 16 weeks. Although the rats are born at term, their organ development is equivalent to that of a human preterm fetus, allowing organs of interest such as the kidney to be compared to premature infants[99]. Therefore the rat is an ideal model to examine the effects of neonatal hyperoxia exposure on mitochondrial function in the developing kidney, especially since they also contain a large number of mitochondria due to their high aerobic capacity and their dysfunction is important in cardiovascular disease and hypertension[52].

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2.0 MANUSCRIPT

EVALUATION OF MITOCHONDRIAL FUNCTION IN A MODEL OF DEVELOPMENTAL PROGRAMMING OF HYPERTENSION ASSOCIATED WITH TRANSIENT NEONATAL OXYGEN EXPOSURE

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ABSTRACT

Introduction: Rats exposed to oxygen (O_2) as newborns suffer complications in adulthood, including: arterial hypertension, vascular dysfunction, nephropenia and indices of oxidative stress. Although the rats are born at term, their organ development is equivalent to that of a preterm fetus, allowing organs of interest such as the kidney to be compared to premature infants. Given that impaired nephrogenesis or reduced nephron numbers has been shown to promote the development of hypertension and mitochondrial dysfunction is associated with increased oxidative stress, we hypothesised that exposure to high oxygen concentrations in the neonatal period would significantly impact the expression and activity of key proteins involved in renal mitochondrial function and lead to an excessive production of reactive oxygen species by the mitochondria.

Methods: Sprague-Dawley rat pups were exposed to 80% O_2 (Hyperoxic (H) group; O_2 exposed) or 21% O_2 (Control (Ctrl) group) from day 3 to day 10 of life. At 4 and 16 weeks of age, kidneys were rapidly excised and the mitochondria isolated following a standard protocol; with a buffer containing 0.32 M sucrose and differential centrifugations. Expression of mitochondrial proteins was assessed by Western blot, whereas the release of hydrogen peroxide (H_20_2), activities of key citric acid cycle enzymes and mitochondrial swelling were assessed by spectrophotometry. Results are expressed as the means ± SE. Both male and female offspring were studied.

Results: In male H rats at 16 weeks of age, citrate synthase activity (used as an internal standard and a measure of relative mitochondrial abundance) was significantly increased (12.4 ± 8.4 vs 4.1 ± 0.5), whereas aconitase activity (sensitive to ROS) was significantly decreased (0.11 ± 0.05 vs 0.20 ± 0.04) and H_20_2 release was significantly increased (7.0 ± 1.3 vs 5.4 ± 0.8) compared to the controls (Ctrl). The H group (vs Ctrl) also demonstrated a reduction in the expression of peroxiredoxin-3 (Prx3) (-23%), a protein involved in the elimination of H_20_2 and in the expression of cytochrome C oxidase (Complex IV) (-15%), a protein in the mitochondrial respiratory chain, whereas the expression of uncoupling protein-2 (UCP2), a protein involved in dissipating the proton gradient, was significantly increased (+17%). Female H rats (vs Ctrl) at 16 weeks of age demonstrated a significant increase in aconitase activity (0.33±0.03 vs 0.17±0.02), in the expression of ATP synthase β subunit (+25%) (involved in ATP production) and in the expression of MnSOD (+20%) (mitochondrial antioxidant involved in scavenging superoxide), whereas Prx3

expression was significantly reduced (-24%). In male H rats (vs Ctrl) at 4 weeks of age, the expression of Prx3 was significantly increased (+31%). Female H rats (vs Ctrl) at 4 weeks of age demonstrated a significant increase in the expression of UCP2 (+18%) and in the expression of MnSOD (+14%).

Conclusion: The findings of this study demonstrate that transient oxygen exposure in the neonatal rat modifies protein expression, enzymatic activity and leads to indices of mitochondrial dysfunction (increase in ROS) in the adult kidney; these adverse changes in the mitochondria were more pronounced in adult males than in females. Overall, these findings, suggest that impaired mitochondrial function is associated with and could play a role in the development of arterial hypertension, oxidative stress and cardiovascular disease associated with transient neonatal hyperoxic stress.

INTRODUCTION

Infants born prematurely at less than 37 weeks' gestation account for over 8% of all births in Canada (up to 12% in the United States) and over 80% of infants weighing less than 2500 grams at birth (low birth weight infants)[9, 83]. Preterm neonates are exposed at birth to a high oxygen (O_2) concentration relative to the intrauterine milieu[83]. Of concern, the preterm infants possess a diminished and under-developed antioxidant capacity and are therefore more susceptible to the effects of reactive oxygen species (ROS)[75, 83]. This concept of premature newborns being more susceptible to oxidative tissue damage has been well established, with oxidative stress known to contribute to common pathologies such as retinopathy of prematurity and bronchopulmonary dysplasia[9, 81]. However the long term consequences of neonatal oxidative injury have only recently begun to be unravelled, and the mechanisms leading to long-term disease are to date not well understood[83].

The concept that many adult conditions or diseases can have their origins traced back to fetal and early postnatal life has been termed 'developmental programming'[15]. There are multiple insults or factors that can affect a developing fetus leading to the programming of adult diseases such as nutrition, oxidative stress and inflammation, glucocorticoid exposures, fetal hypoxia and epigenetic changes[16]. Revolutionary studies spanning several decades, with David Barker as the pioneering figure, demonstrated that impaired fetal growth is associated with raised blood pressure[17], hypertension[17] and cardiovascular mortality later in life in both male and female subjects[168]. This 'fetal or developmental origins/programming of disease' concept is now well accepted but the 'programming' mechanisms remain poorly understood[26].

Kidney development (nephrogenesis) begins at approximately day 30 of gestation, with the majority of nephrons being formed around week 20 until 34 to 36 weeks of gestation[14]. Given that nephrogenesis is still ongoing prior to 34 weeks of gestation, it is very likely that neonates born at less than 34 weeks of gestation will experience alterations in the structural development of the kidney[14]. In addition, the extrauterine environment is suboptimal for organogenesis due to the exposure to a number of insults, including high oxygen concentrations[14]. A previous study from our laboratory[83] demonstrated that rats exposed to hyperoxia in the neonatal period led to a reduced nephron number, hypertension and vascular dysfunction in adulthood.

As mitochondria are also a major source of oxygen-derived free radicals, generated mainly as by-products of mitochondrial respiration, mitochondria are thought to be the primary target of oxidative damage[169, 170]. Mitochondria play a crucial role in energy metabolism through their involvement in ATP production by oxidative phosphorylation, and are thus often referred to as the powerhouse of the cell. After the oxidation of substrates in the citric acid cycle or Krebs cycle, the resulting electron carriers, NADH and $FADH_2$, are oxidized by complexes I and II, respectively, which generates a proton gradient and ultimately drives the synthesis of ATP[119, 170]. Experimental studies have shown that specific enzymes of the citric acid cycle are susceptible to oxidation[112, 171, 172]. Aconitase exhibits the most significant age-associated decline in activity and is known to be highly susceptible to oxidation[110] and increased citrate synthase activity has been suggested to contribute to progressive renal injury[115]. Apart from energy conversion, mitochondria take part in a number of other processes including calcium homeostasis and apoptosis[151]. It is well documented that an increased permeability of the mitochondrial membranes is a key event in controlling apoptotic pathways, with the permeability transition pore (PTP) thought to play a major role[173-176]. Prolonged opening of the PTP, a nonspecific high-conductance proteinaceous channel of the inner membrane, leads to collapse of the membrane potential, mitochondrial matrix swelling, and rupture of the outer mitochondrial membrane[157, 158].

Given the susceptibility of the mitochondria to oxidative stress, we hypothesised that exposure to high oxygen concentrations in the neonatal period would significantly impact the expression of key proteins involved in mitochondrial function and lead to an excessive production of reactive oxygen species by the mitochondria. The kidneys contain a large number of mitochondria due to their high aerobic capacity, they are important in cardiovascular disease, and hypertension is closely associated with progressive kidney dysfunction[52]. Therefore, the aims of the current study were to assess markers of mitochondrial protein expression and function in the kidney using a neonatal hyperoxic rat model. We examined the kidneys of rats exposed to hyperoxia (from P3 to P10) and compared them to normoxic controls at both 4 and 16 weeks of age.

METHODS

Animals

All experiments on animals were performed in accordance to the guidelines approved by the Animal Care Committee of Sainte-Justine University Hospital Center and conform with the objectives of the Canadian Council on Animal Care.

Sprague-Dawley rat pups (Charles River, St-Constant, Québec, Canada) were exposed to 80% O_2 (mixture of medical-grade 100% O_2 and room air; ProOx oxygen controller, A820CV, Biospherix) from day 3 to day 10 of life (Hyperoxic (H) group; O_2 exposed). To avoid maternal mortality associated with high O_2 exposure, the dam was alternated every 12 hours with a surrogate mother of a different litter. Control litters were kept in room air, and the dam was not interchanged (Control (Ctrl) group). After P10, all litters were returned to room air. Pups were weaned at 4 weeks of age to regular chow. Both male and female offspring were studied at 4 and 16 weeks. No more than 2 animals per gender per litter were used for each series of studies with a total of 3 litters.

Mitochondrial isolation

Kidney mitochondria were isolated according to a protocol adapted from Fernandez-Vizarra et al.[118] Animals were anaesthetized via inhaled isoflurane (2- :1.5 O_2) and then euthanized by guillotine. Kidneys were rapidly excised, medullas removed, weighed and the cortex immersed into ice cold isolation buffer (0.32 M sucrose, 1mM EDTA, 10 mM Tris, pH 7.4). All steps were performed on ice and centrifugations were performed at 4^{o}C. Kidney tissue was homogenized using a Potter-Elvehjem tissue grinder on maximum speed setting (for approximately 10 s). The homogenate was centrifuged at 1000g for 10 mins, followed by the supernatant being decanted and again centrifuged as above. The resultant supernatant was then centrifuged at 18 000g for 15 mins. The supernatant was discarded and the pellet was resuspended in 5 mL fresh isolation buffer, and centrifuged at 18 000g for 15 mins. The final mitochondrial pellets were resuspended in 2 mL isolation buffer and protein determinations were performed by the Bradford assay method with BSA (1.42 µg/µL) used as the standard.

Western Blot

Precast 4-12% NUPAGE Bis Tris Gels (Invitrogen) containing 40 µg of protein per well were transferred overnight to polyvinylidine fluoride (PVDF) membranes. Membranes were blocked for 1 hr at room temperature with 5% TBST/milk solution. Membranes were then incubated with primary monoclonal antibodies for 1.5 hrs. All primary antibodies were diluted to 1 :1000 unless otherwise noted : (UCP2-calbiochem, Billerica, MA), Peroxiredoxin-3 (Abcam), ATP synthase β (1 :3000, MitoSciences), cytochrome c oxidase subunit 1 (complex 4)-(MitoSciences), ubiquinol cytochrome c oxidoreductase core 2 subunit (Mitosciences), LDH (Abcam), anti-Cox IV antibody (internal reference protein, Abcam), anti-catalase (Abcam), MnSOD (1 :10 000, Stressgen). The secondary antibodies goat anti-rabbit and goat anti-mouse (1 : 15 000, Santa Cruz) were used according to the primary antibodies. Bands were visualized using an enhanced chemiluminescence method, ECL plus (Perkin Elmer, MA, USA). Films were scanned using a flatbed scanner, and images analyzed with Gel Pro analyser 3.1 (Media Cybernetics, MD, USA). Results were normalized to anti-COX IV (1 :1000, Abcam) or voltage-dependent anion channel (VDAC, 1 :10 000, Enzo Life Sciences) for loading variations.

Citrate Synthase Enzymatic Activity Assays

Total protein content was determined using a Bradford assay. For the assay, 100 µg of total protein was used per well. Enzymatic activity was determined using the citrate synthase assay kit (Sigma, St. Louis, Missouri) with slight modifications. The Absorbance readings at 412 nm were taken using an Envision 2104 Multilabel Reader spectrophotometer (Perkin Elmer, Waltham, Massachusetts) with 9 readings over the 1.5 min time span. The difference between baseline and oxaloacetate (OAA)-treated samples was obtained and used to calculate total citrate synthase activity according to the formula provided by the manufacturer.

Aconitase Activity

Aconitase activity kit (Mitosciences (MS745), Eugene, Oregon) was used. Aconitase activity is measured by following the conversion of isocitrate to cis aconitate through the increase of absorbance at 240 nm. Samples were diluted to a concentration of 5 µg/µL and assessed at 60 second intervals for 30 minutes.

H_2O_2 production

Mitochondrial H_20_2 production was determined by absorbance at 560 nm (Envision 2104 Multilabel Reader; Perkin Elmer) using Amplex red (Molecular Probes, Eugene, OR). Amplex red reacts with H_2O_2 in a 1:1 stoichiometry to produce highly coloured resorufin. Briefly, 100 µM Amplex red reagent and 0.2 U/ml horseradish peroxidase were added to the mitochondrial sample (50 µl) or H_2O_2 standard solution, and the sample was incubated for 30 mins in a 96-well microplate in the dark at room temperature. H_2O_2 concentrations were calculated on the basis of an H_2O_2 standard curve generated using H_2O_2 and Amplex red.

Mitochondrial Swelling (PTP opening)

Mitochondrial swelling was estimated through changes in OD_{540} nm recorded using an Envision 2104 Multilabel Reader (Perkin Elmer) spectrophotometer using a protocol adapted from Gogvadze *et al*[177] in Current Protocols in Cell Biology. The gradual decrease in OD_{540} nm due to mitochondrial swelling was analyzed at 1 minute intervals for 1 hr following the addition of calcium and other reagents to the mitochondrial suspension concentrated at 10 µg/µL. All measurements were performed at 25^{o}C.

Statistical Analysis

Data is expressed as the means ± SE. Differences between groups were compared using two-tailed Student's t-test. Mitochondrial swelling was assessed by the two-way repeated-measures ANOVA with the factors hyperoxia exposure (p_H), time point of assessment (p_T) and their interaction (p_{HxT}). Statistical analyses were performed using GraphPad Prism version 5.03 for Windows (CA, U.S.A). A difference of $P \leq 0.05$ was considered significant.

RESULTS

Enzymatic Activity of Citrate Synthase

The activity of citrate synthase was significantly increased in male rats exposed to hyperoxia (H) compared to the controls (Ctrl, p□0.05) at 16 weeks (Figure 1A). There was no significant difference between female experimental groups at 16 weeks (Figure 1B). No significant difference was observed between experimental groups in either males or females at 4 weeks of age (Figure 1C-D).

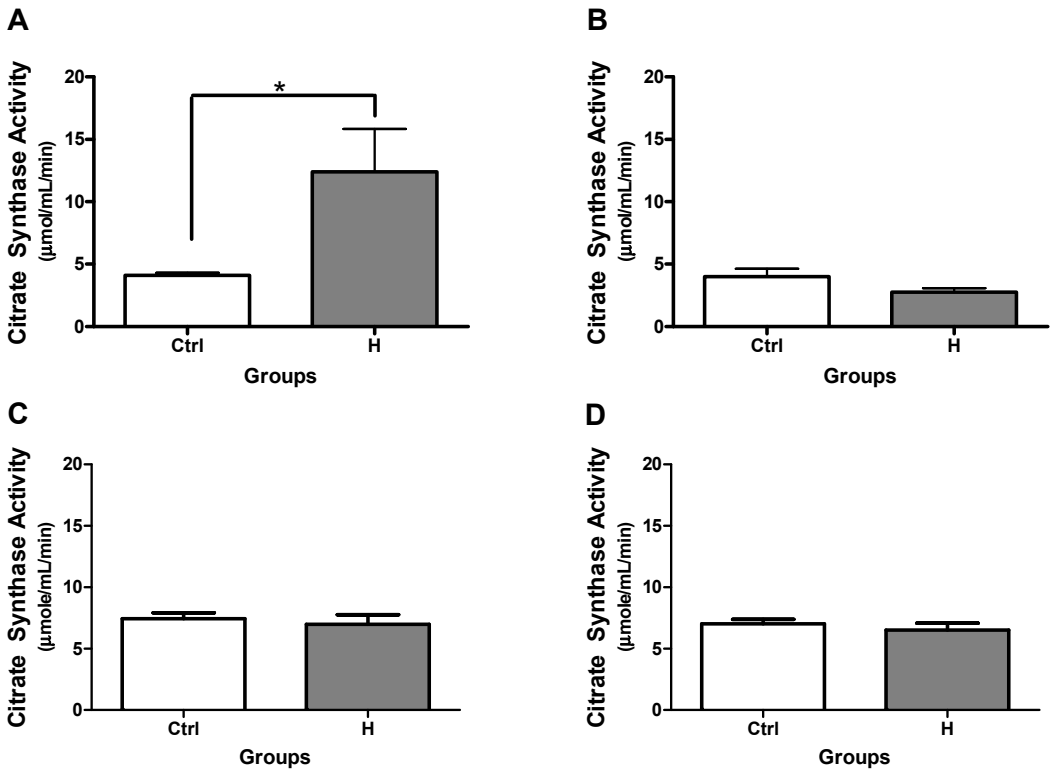

Figure 1: Citrate Synthase Activity. Citrate synthase activity (µmol/mL/min) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 per group). *=p≤ 0.05

Enzymatic Activity of Aconitase

The activity of aconitase was significantly decreased in male rats exposed to hyperoxia compared to the controls (p□0.05) at 16 weeks (Figure 2A). Conversely, aconitase activity was significantly increased in females exposed to hyperoxia, at 16 weeks, compared to the controls (p□0.01; Figure 2B). No significant difference in aconitase activity was observed between experimental groups in either males or females at 4 weeks of age (Figure 2C-D).

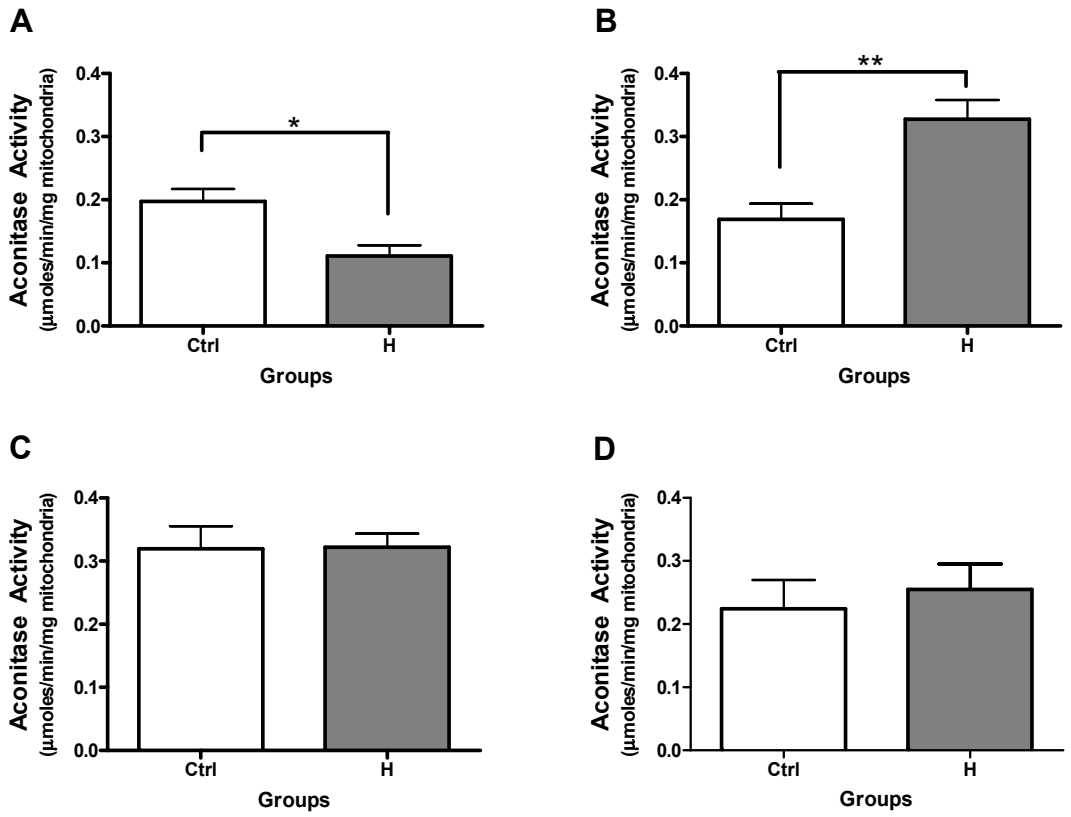

Figure 2: Aconitase Activity. Aconitase activity (µmol/min/mg mitochondria) in kidneys from 16 week old adult male (A) and female (B) rats , and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 per group). *=p≤ 0.05; **=p≤ 0.01

Protein Expression of Complex 3

No significant difference in the protein expression of complex 3 was observed between experimental groups in either males or females at 16 weeks (Figure 3A-B) or at 4 weeks of age (Figure 3C-D).

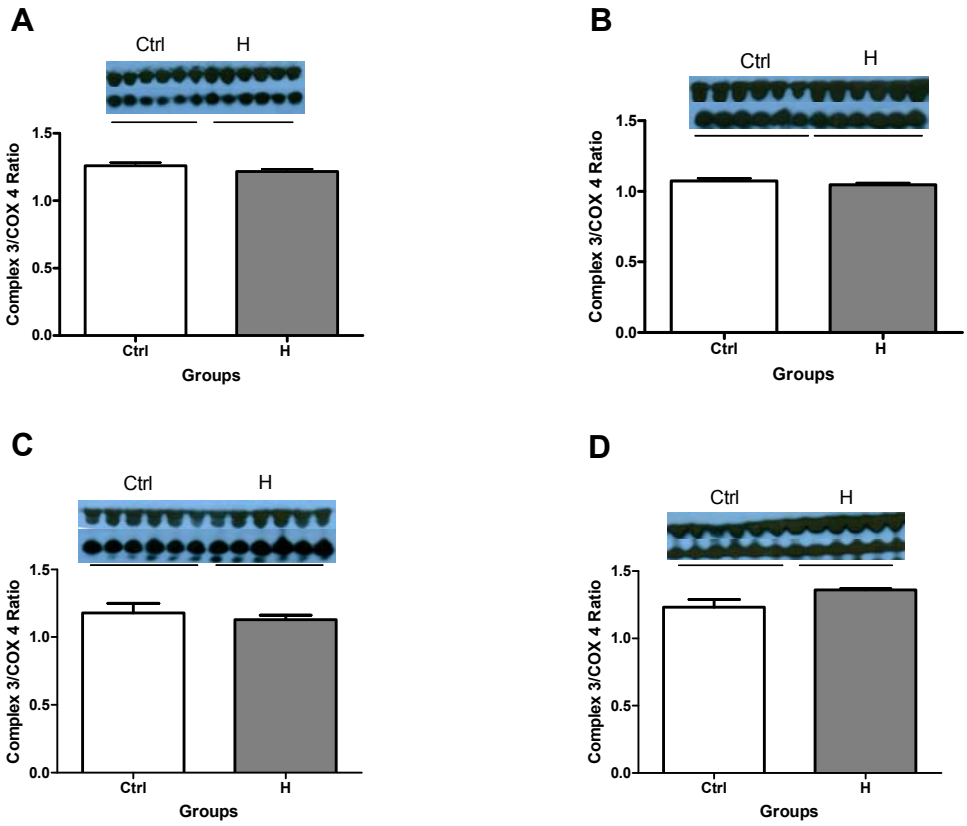

Figure 3: Complex 3 Protein Expression. Complex 3 protein expression (assessed by Western blotting and expressed as a ratio of COX4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs room air controls (Ctrl, n=6 per group).

Protein Expression of Complex 4

The expression of complex 4 was found to be significantly decreased in male rats exposed to hyperoxia compared to the controls (p□0.01) at 16 weeks of age (Figure 4A). In females at 16 weeks of age (Figure 4B) and in both males and females at 4 weeks of age (Figure 4C-D), no significant difference was observed between groups.

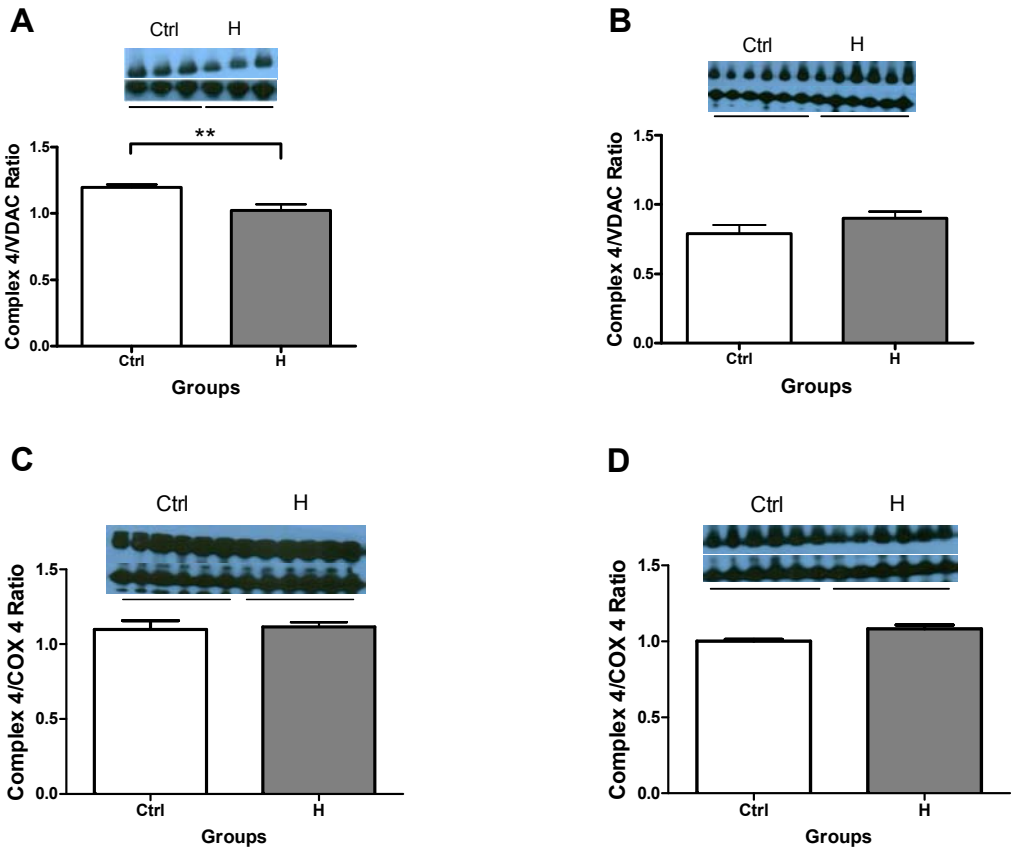

Figure 4: Complex 4 Protein Expression. Complex 4 protein expression (assessed by Western blotting and expressed as a ratio of VDAC or COX 4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=7 males and n=9 females) vs. room air controls (Ctrl, n=7 per group). **=p≤ 0.01

Protein Expression of ATP Synthase β subunit

The expression of ATP synthase β was found to be significantly increased in female rats exposed to hyperoxia compared to the controls (p□0.01) at 16 weeks of age (Figure 5B). In males at 16 weeks of age (Figure 5A), and both males and females at 4 weeks of age (Figure 5C-D), no significant difference was observed between groups.

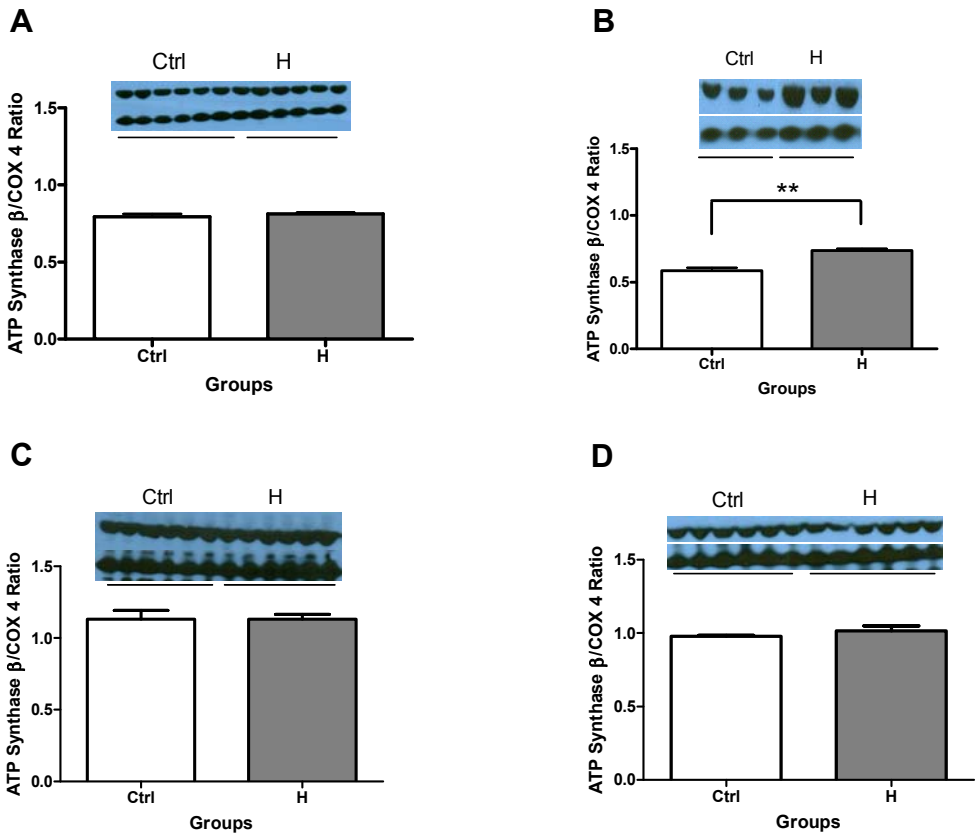

Figure 5: ATP synthase β subunit Protein Expression. ATP synthase β subunit protein expression (assessed by Western blotting and expressed as a ratio of COX4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 per group). **=p≤ 0.01

Protein Expression of Uncoupling Protein-2

At 16 weeks of age, uncoupling protein-2 (UCP2) expression was significantly increased in male hyperoxia-exposed rats compared to the controls (p□0.01) (Figure 6A), while no significant difference was observed between groups in females (Figure 6B). At 4 weeks of age, UCP2 expression was significantly increased in female hyperoxia-exposed rats compared to the controls (p□0.05; Figure 6D), while no significant difference was observed between the experimental groups in males (Figure 6C).

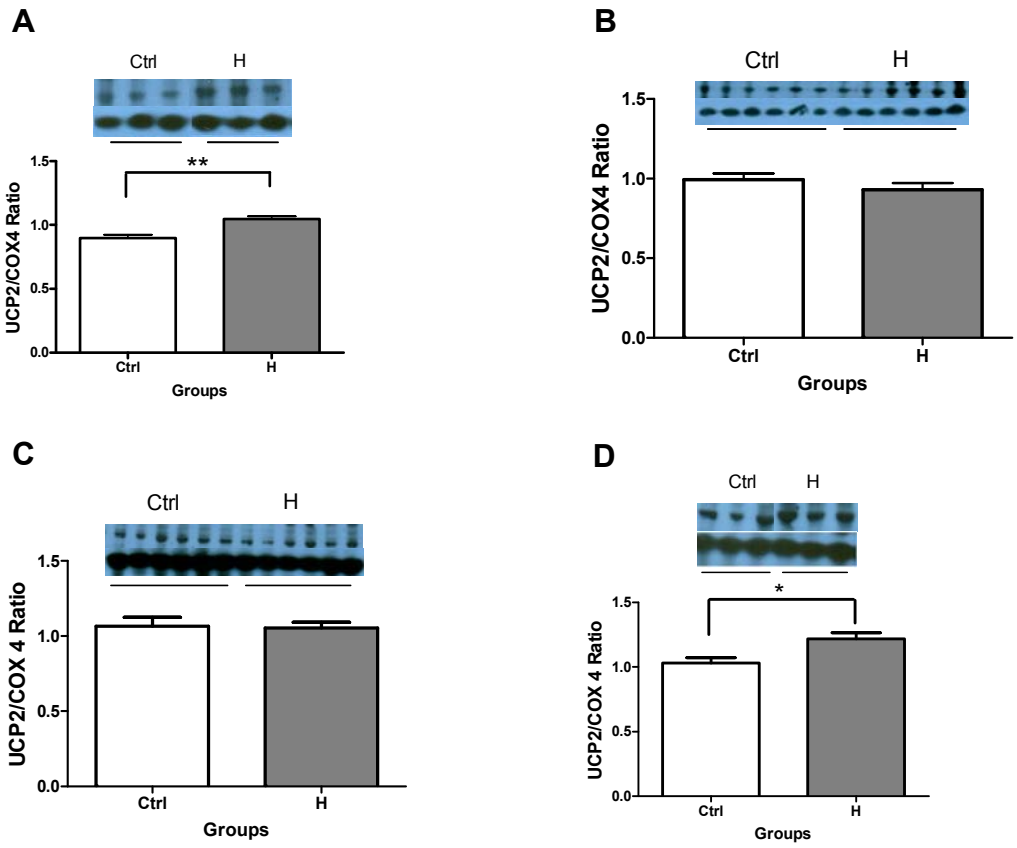

Figure 6: Uncoupling Protein 2 (UCP2) Protein Expression. Uncoupling Protein-2 (UCP2) protein expression (assessed by Western blotting and expressed as a ratio of COX4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 per group). *=p≤ 0.05; **=p≤0.01

Protein Expression of Manganese Superoxide Dismutase

Protein expression of mitochondrial manganese superoxide dismutase MnSOD was found to be significantly increased in females exposed to hyperoxia at both 16 weeks of age (p□0.01; Figure 7B) and 4 weeks of age (p□0.05; Figure 7D) compared to the controls. No significant difference was observed between the groups in males at both 16 and 4 weeks of age (Figure 7A, C).

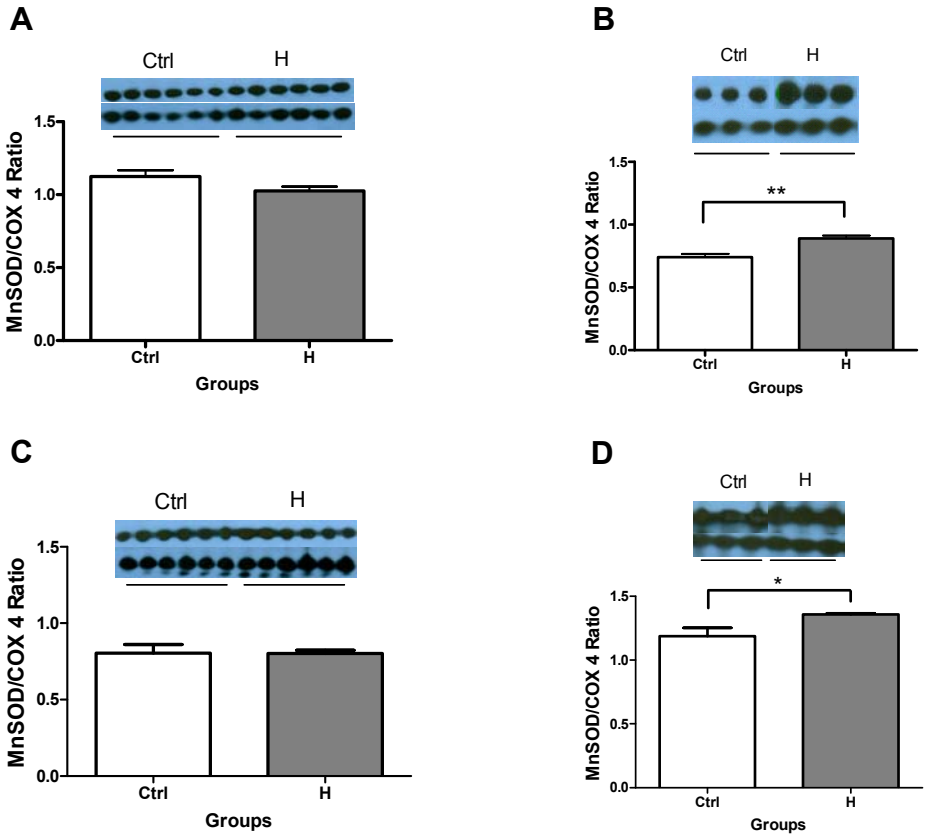

Figure 7: MnSOD Protein Expression. Manganese superoxide dismutase (MnSOD) protein expression (assessed by Western blotting and expressed as a ratio of COX4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 per group). *=p≤ 0.05; **=p≤ 0.01

Protein Expression of Catalase

No significant difference was observed between experimental groups in the protein expression of catalase, in either males or females, at 16 weeks (Figure 8A-B) or 4 weeks of age (Figure 8C-D).

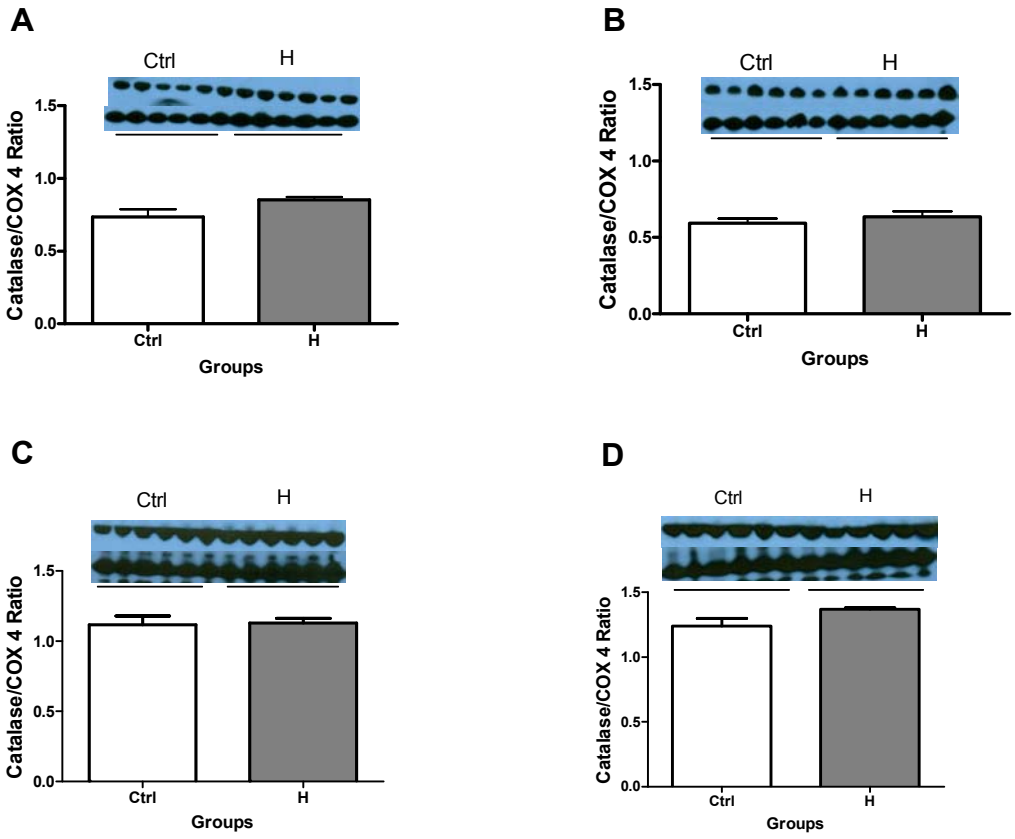

Figure 8: Catalase Protein Expression. Catalase protein expression (assessed by Western blotting and expressed as a ratio of COX4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 per group).

Protein Expression of Peroxiredoxin-3

At 16 weeks of age, the protein expression of peroxiredoxin-3 (Prx3) was found to be significantly decreased in the groups exposed to hyperoxia compared to the control groups (p□0.05), in both males (Figure 9A) and females (Figure 9B). At 4 weeks of age, Prx3 expression was significantly increased in males exposed to hyperoxia (p≤0.05; Figure 9C), while no difference was observed between the groups in females (Figure 9D).

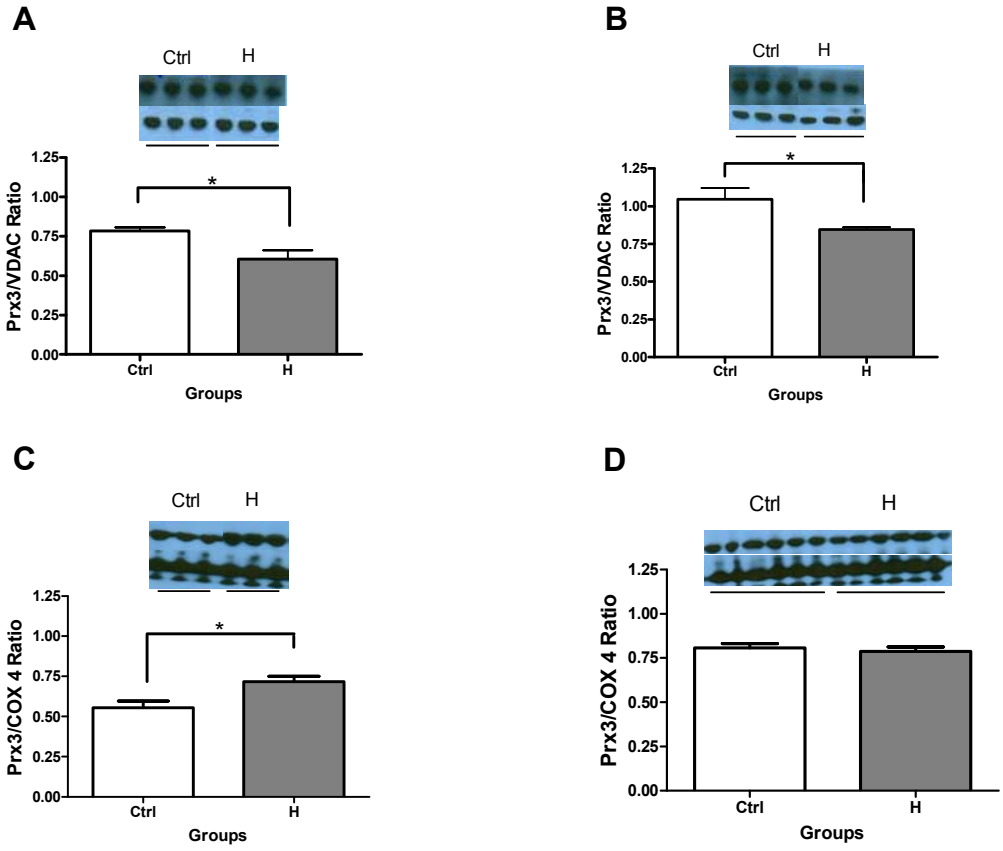

Figure 9: Peroxiredoxin-3 (Prx3) Protein Expression at 16 weeks. Peroxiredoxin-3 (Prx3) protein expression (assessed by Western blotting and expressed as a ratio of VDAC or COX 4 expression; see Methods) in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=6 per group) vs. room air controls (Ctrl, n=6 males and 4 females). *=p≤ 0.05

Levels of Hydrogen Peroxide (H_2O_2) Release

Hydrogen peroxide release was significantly increased in hyperoxia-exposed males compared to the controls (p□0.05) at 16 weeks of age (Figure 10A), whereas females showed a trend for an increase that did not quite reach statistical significance (Figure 10B). At 4 weeks of age, no significant difference between the groups was observed in either males or females (Figure 10C-D).

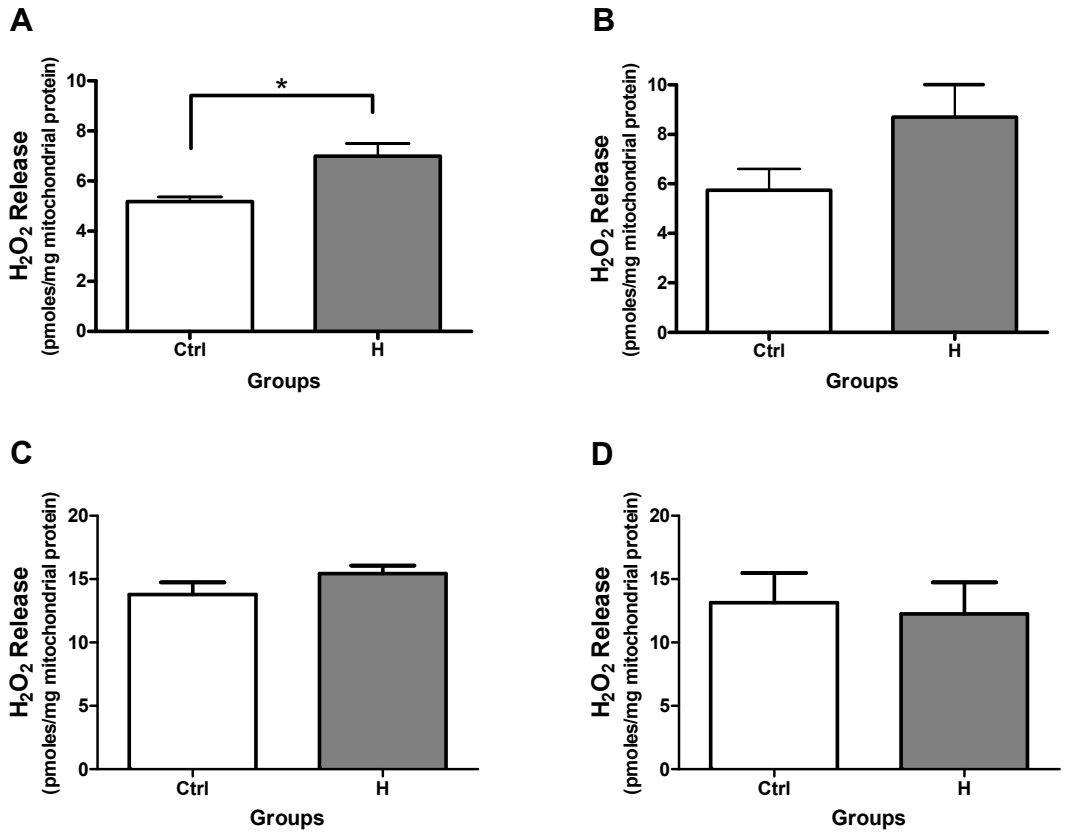

Figure 10: H_2O_2 Release (Amplex Red). Hydrogen peroxide (H_2O_2) release in kidneys from 16 week old adult male (A) and female (B) rats, and 4 week old male (C) and female (D) rats exposed to high oxygen (80% O_2) from day 3 to 10 of life (H, n=7 per group) vs. room air controls (Ctrl, n=6 males and 4 females). *=p≤ 0.05

Mitochondrial Permeability Transition Pore Opening

Opening of the mitochondrial permeability transition pore was induced with the addition of Ca^{2+}, and specrophotometrically monitored at 540 nm for 20 min. In mitochondria isolated from 4 week old male rat kidneys, the addition of Ca^{2+} resulted in a significant decrease in absorbance which is indicative of mitochondrial swelling (Figure 11). There was no significant difference in mitochondrial swelling between hyperoxia-exposed and control animals at any timepoint.

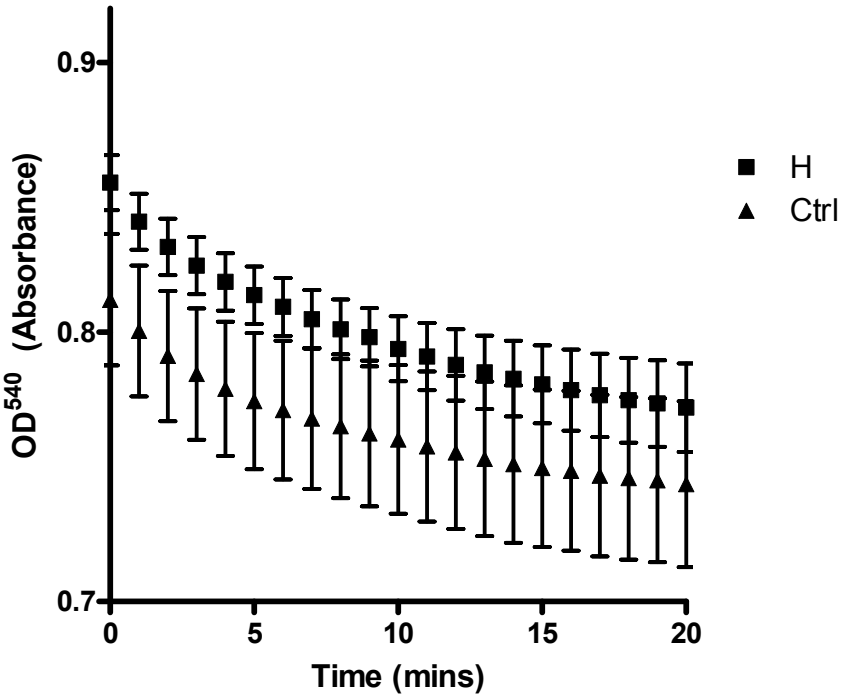

Figure 11: Mitochondrial Swelling at 4 weeks. Squares: Hyperoxic group (H; n=5). Triangles: Control group (Ctrl; n=6). Factors assessed using two-way ANOVA with repeated measures were time ($pT<0.0001$), hyperoxia exposure ($pH=0.31$) and their interaction ($pTxH=0.49$). Bonferroni post-hoc analysis showed no significant differences between hyperoxia (H) and control (Ctrl) groups at any individual timepoint.

DISCUSSION

Results of this study indicate that hyperoxic exposure of newborns modifies protein expression, enzymatic activity and leads to indices of mitochondrial dysfunction (increase in ROS) in the adult kidney; these adverse changes in the mitochondria were more pronounced in adult males than in females. Previous findings in our rodent model demonstrate that transient neonatal hyperoxia exposure leads to reduced nephron number, vascular dysfunction, and the development of hypertension in adulthood[46]. The mechanisms leading to the programming of hypertension in this model are unknown, but it was hypothesised that alterations in mitochondrial function would be a contributing factor. Hyperoxia has been shown in several previous studies to adversely affect mitochondrial metabolism. Citric acid cycle enzymes important in mitochondrial function have been shown to decline in activity, with aconitase exhibiting the most significant age-associated decline in activity[110]. Electron transport chain components have also been shown to be affected, along with a decrease in cellular ATP[159, 160]. In addition, the antioxidants MnSOD, GPx and catalase have all been shown to have decreased levels when exposed to neonatal hyperoxia or other toxins[157, 164-166]. Consistent with these previous studies, the current findings demonstrate that mitochondrial function is impaired in the adult rat kidney following neonatal oxygen exposure; components of the citric acid cycle, electron transport chain, and antioxidant levels are all adversely affected.

In males at 16 weeks of age enzymatic assays of both citrate synthase activity and aconitase activity demonstrated significant differences between groups. To further assess protein targets of the ageing process in mitochondria, enzymatic activity of citrate synthase was determined. Citrate synthase activity was found to be significantly increased in the hyperoxia exposed group compared to the control, whereas aconitase activity was found to be significantly decreased. A decrease in aconitase activity is likely to adversely affect the remaining portions of the citric acid cycle, thereby decreasing its overall efficiency (see Figure 6). Furthermore, aconitase has been shown to be highly sensitive to ROS, indicating levels of dysfunction in mitochondrial metabolism[110]. Normally intermediates produced in the citric acid cycle do not accumulate in the mitochondria, and are exported to the cytosol. The accumulation of citrate in the cytosol would likely activate fatty acid synthesis[110]. The activation of fatty acid synthesis can lead to reduced insulin sensitivity, cardiovascular disease and type 2 diabetes; all characteristics of

metabolic syndrome[178]. In contrast to the findings in males, females at 16 weeks of age demonstrated a significant increase in the activity of aconitase in the hyperoxia exposed group compared to the control suggesting that there is a protective effect in females. No significant difference in enzymatic activity was observed at 4 weeks of age between groups in either males or females, indicating that this may be a process related to ageing as hyperoxia-exposed animals are more susceptible to accelerated ageing.

At 16 weeks of age several differences were observed in the level of protein expression in various components of the electron transport chain and respiratory chain. Males showed a significant decrease in complex 4 expression in the hyperoxia exposed group compared to the control, whereas a significant increase in protein expression of UCP2 was observed. It is possible that reduction in complex 4 protein or subunit content contributes to adverse effects on energy production, and complex activities by impairing supercomplex formation. This is similar to what is seen in people with a dietary copper (Cu) deficiency[179]. The increase in UCP2 expression can potentially be attributed to ROS, which have been shown to increase proton conductance over the mitochondrial membrane by activation of UCPs as a possible mechanism to decrease prevalent free radicals and their effects in the cell[180]. No significant differences were observed in the expression of complex 3 or the ATP synthase β subunit between male groups. Females, however, showed an increase in the expression of ATP synthase β subunit at 16 weeks which suggests a possible increase in energy metabolism in the group exposed to hyperoxia. No significant differences were observed in protein expression levels of complex 3, complex 4 and UCP2 in females at 16 weeks of age. UCP2 expression in females exposed to hyperoxia was found to be significantly increased compared to the control group at 4 weeks of age. The reason for the differences in expression between 4 and 16 weeks is unclear, however, it may in part be due to a short-term protective effect to dissipate ATP production and by doing so, limit ROS production following oxidative insult, thus stabilizing long-term function. No other significant differences were observed in the assessed proteins in males or females at 4 weeks of age.

At 16 weeks in males the present study demonstrates a significant difference in ROS production, measured as H_20_2, between groups. The group exposed to hyperoxia was shown to have markedly increased ROS production, in tandem with decreased Prx3 expression demonstrating indices of dysfunction and a reduced capacity to eliminate the excess ROS. This may potentially

cause a vicious cycle, leading to amplified effects and endangering cell function. In females, MnSOD antioxidant expression was significantly increased at 4 weeks of age and even more so at 16 weeks of age in the group exposed to hyperoxia, suggesting that females are better able to deal with ROS than males, as hydrogen peroxide levels in females showed no significant difference between the groups. Prx3 expression, however, was found to be significantly decreased in females at 16 weeks of age compared to the control, while no significant difference was observed at 4 weeks. Interestingly, in males, Prx3 expression was found to be significantly increased at 4 weeks of age and significantly decreased at 16 weeks of age. A possible explanation for this may be that in the short term, oxidative stress induces Prx3 expression and in the long term it becomes depleted. No significant difference was observed in the protein expression of catalase in males or females at either timepoint. Since catalase is present in very low concentrations in mitochondria[181] it is not surprising that no significant difference was observed between groups. No other significant differences were observed between groups in the assessed antioxidants and ROS levels.

Previous studies have shown that males have an increased sensitivity to oxidative stress compared to females[182-185]. Females have been shown to have a lower level of oxidative damage to DNA than males[182]. Possible explanations include the antioxidant properties of estrogen although the exact mechanism remains unknown[186]. Other antioxidant-related factors are also thought to contribute to gender-related differences in antioxidant protection; however, nothing concrete has yet been determined[182]. Our findings demonstrate an increase in the quantity of MnSOD (an antioxidant enzyme specific to the mitochondria responsible for neutralizing superoxide free radical) in females exposed to hyperoxia compared to the control, demonstrating a protective effect which is in accordance with previous studies showing increased activity of antioxidant systems in females[186-188]. The precise mechanisms for the observed difference in renal antioxidant capacity would require more experiments, as presently the mechanism remains unknown.

As described by Nuyt *et al*.[16], programming occurs when the normal pattern of development is disrupted by an abnormal stimulus or 'insult' applied at a critical time-point. This leads to an adaptive structural or functional response in the fetus/neonate that promotes short-term survival, but which may manifest in adult disease (such as diabetes or hypertension) in later life.

Importantly, Yzydorczyk *et al*.[83] demonstrated a reduced nephron number and hypertension in adult rats following a transient neonatal hyperoxic insult (at a time when renal development was still ongoing), however the mechanisms underlying the programming of hypertension in this model are unknown. It was hypothesised that alterations in mitochondrial function may be a contributing factor in the development of hypertension; hyperoxia is known to lead to mitochondrial dysfunction[189] which in turn may adversely affect mitochondrial metabolism (increased levels of oxidative stress and decreased levels of antioxidant enzymes) and result in hypertension[189]. In the study by Yzydorczyk *et al*.[83], hypertension was only evident in adult animals at 8 weeks of age; therefore, to evaluate the role of mitochondria in the development of hypertension, in the current study it was important to look at timepoints both prior to the onset of hypertension (4 weeks) and after the onset of hypertension (16 weeks). If an effect is present at 4 weeks (early-onset), it may precede and potentially contribute to the development of hypertension and likely occurred as a direct consequence of hyperoxia exposure, whereas, if an effect is only present at 16 weeks (after the onset of hypertension), it may not be a contributing factor and it is difficult to tell if the effect is a consequence of hyperoxia exposure, or due to the later complications (such as hypertension or ageing). In assessing males and females at 4 and 16 weeks of age, we were better able to deduce if the changes in mitochondrial metabolism were due to programming or injury. In females, there seems to be both a programming aspect and a possible injury phenomenon that could affect mtDNA. The expression of MnSOD, which is a mitochondrial antioxidant, is significantly increased at 4 weeks of age (preceding the onset of hypertension) in the hyperoxia exposed group compared to control and even more so at 16 weeks of age (after the onset of hypertension/age), demonstrating a potential protective programming effect. In addition, the significant increase in UCP2 expression at 4 weeks of age in the hyperoxia exposed group may potentially serve to minimize ROS damage in older females, as no significant difference was observed at 16 weeks of age between groups. However, the expression of Prx3 seems to be brought about through injury instead of by programming, since Prx3 expression (a ROS scavenger) is significantly decreased at 16 weeks of age (in the group exposed to hyperoxia) but no significant difference was observed at 4 weeks of age, indicating a possible age-related or hypertension-related onset. In addition, aconitase activity is significantly increased at 16 weeks of age in the hyperoxia exposed group, suggesting a protective effect. This increase was not seen at 4 weeks of age (possible change

after onset of hypertension/ageing). Finally, ATP synthase β subunit (involved in ATP production) expression was found to be significantly increased at 16 weeks of age but not at 4 weeks of age, suggesting the possible role of injury to mitochondria instead of a programming effect. Therefore, in females, there seems to be evidence for both a programming effect and an effect possibly due to injury as a secondary effect to the development of hypertension. In males, the results seem to indicate more of an injury effect rather than a programming effect. Citric acid cycle enzymatic activity was significantly affected at 16 weeks of age, compared to no significant difference between groups at 4 weeks of age. This trend was also observed for complex 4 and UCP2 protein expression and for in males. However, Prx3 expression seems to follow a programming effect. In males (exposed to hyperoxia) at 4 weeks of age, Prx3 expression was significantly increased as a possible short-term compensatory mechanism to deal with oxidative insult and significantly decreased at 16 weeks of age as Prx3 potentially becomes depleted.

In conclusion, this study demonstrates that there appears to be differences in mitochondrial metabolism in the kidneys between males and females following oxidative insult with the potential dual roles of programming (following the neonatal hyperoxia exposure) and injury (following the onset of hypertension) as a possible mechanism for the observed disease phenotype. Furthermore, the observed sex difference indicates that females may be better able to cope with oxidative insult than males, which is a recognized fact in the neonatal period. Overall, the findings of this study have shown that neonatal hyperoxia exposure alters renal mitochondrial function in adulthood; however, future studies are required in order to deduce the mechanism responsible for the disease phenotype, and what exact role mitochondria may play in the programming of renal dysfunction and hypertension.

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3.0 DISCUSSION

The findings of this study demonstrate that transient oxygen exposure in the neonatal rat modifies protein expression, enzymatic activity and leads to indices of mitochondrial dysfunction (increase in ROS) in the adult kidney; these adverse changes in the mitochondria were more pronounced in adult males than in females. Males exposed to hyperoxia (vs Ctrl) demonstrated greater levels of mitochondrial dysfunction (adversely affected citric acid cycle enzymatic activity, significantly higher levels of H_2O_2) at 16 weeks compared to males at 4 weeks and compared to females at both ages. On the other hand, females exposed to hyperoxia (vs Ctrl), showed significantly higher levels of MnSOD antioxidant expression at 4 weeks, with an even greater expression at 16 weeks. Overall, these findings, suggest a possible role of impaired mitochondrial function following hyperoxia exposure in the development of arterial hypertension, accelerated ageing and cardiovascular disease.

Previous findings in our rodent model demonstrate that transient neonatal hyperoxia exposure leads to reduced nephron number, vascular dysfunction, and the development of hypertension in adulthood[46]. The mechanisms leading to the programming of hypertension in this model are incompletely known, but it was hypothesised that alterations in mitochondrial function would be a contributing factor. Hyperoxia has been shown in several previous studies to adversely affect mitochondrial metabolism. Citric acid cycle enzymes important in mitochondrial function have been shown to decline in activity, with aconitase exhibiting the most significant age-associated decline in activity, and the NADPH :$NADP^+$ ratio also declines with age[110]. Electron transport chain components have also been shown to be affected by hyperoxia, along with a decrease in cellular ATP[159, 160]. In addition, the antioxidants MnSOD, GPx and catalase have all been shown to have decreased levels when exposed to neonatal hyperoxia or other toxins, in erythrocytes, in the brain and in the liver [157, 164-166]. Consistent with these previous studies, the current findings reported in this thesis demonstrate that mitochondrial function is impaired in the adult rat kidney following neonatal oxygen exposure; components of the citric acid cycle, electron transport chain, and antioxidant levels are all adversely affected. This research will therefore go some way to further

understand this organelle's potential role in the development of hypertension and cardiovascular disease.

3.1 Summary of Main Findings

3.1.1 Citric Acid Cycle

In males at 16 weeks of age enzymatic assays of both citrate synthase activity and aconitase activity demonstrated significant differences between groups. To further assess protein targets of the ageing process in mitochondria, enzymatic activity of citrate synthase was determined. Citrate synthase activity was found to be significantly increased in the hyperoxia exposed group compared to the control, whereas aconitase activity was found to be significantly decreased. A decrease in aconitase activity is likely to adversely affect the remaining portions of the citric acid cycle, thereby decreasing its overall efficiency (see Figure 6). Furthermore, aconitase has been shown to be highly sensitive to ROS, indicating levels of dysfunction in mitochondrial metabolism[110]. Normally intermediates produced in the citric acid cycle do not accumulate in the mitochondria, and are exported to the cytosol. The accumulation of citrate in the cytosol would likely activate fatty acid synthesis[110]. The activation of fatty acid synthesis can lead to reduced insulin sensitivity, cardiovascular disease and type 2 diabetes; all characteristics of metabolic syndrome[178]. In contrast to the findings in males, females at 16 weeks of age demonstrated a significant increase in the activity of aconitase in the hyperoxia exposed group compared to the control suggesting that there is a protective effect in females. No significant difference in enzymatic activity was observed at 4 weeks of age between groups in either males or females, indicating that this may be a process related to ageing as hyperoxia-exposed animals are more susceptible to accelerated ageing or related to the elevated blood pressure, present at 16 weeks but not at 4 weeks[83].

3.1.2 Electron Transport Chain (ATP/Energy)

At 16 weeks of age several differences were observed in the level of protein expression in various components of the electron transport chain and respiratory chain. Males showed a significant decrease in complex IV expression in the hyperoxia exposed group compared to the control, whereas a significant increase in protein expression of UCP2 was observed. It is possible that reduction in complex IV protein or subunit content contributes to adverse effects on energy production, and complex activities by impairing supercomplex formation. This is similar to what is seen in people with a dietary copper (Cu) deficiency[179]. The increase in UCP2 expression can potentially be attributed to ROS, which have been shown to increase proton conductance over the mitochondrial membrane by activation of UCPs as a possible mechanism to decrease prevalent free radicals and their effects in the cell[180]. No significant differences were observed in the expression of complex III or the ATP synthase β subunit between male groups. Females, however, showed an increase in the expression of ATP synthase β subunit at 16 weeks which suggests a possible increase in energy metabolism in the group exposed to hyperoxia. No significant differences were observed in protein expression levels of complex III, complex IV and UCP2 in females at 16 weeks of age. However at 4 weeks of age, UCP2 expression in females exposed to hyperoxia was found to be significantly increased compared to the control group. The current studies cannot provide an explanation for the differences in expression observed between 4 and 16 weeks, however, it may in part be due to a short-term protective effect to dissipate ATP production and by doing so, limit ROS production following oxidative insult, thus stabilizing long-term function. No other significant differences were observed in the assessed proteins in males or females at 4 weeks of age.

3.1.3 Antioxidants/Oxidants

At 16 weeks in males the present study demonstrates a significant difference in ROS production, measured as H_20_2, between groups. The group exposed to hyperoxia was shown to have markedly increased H_20_2 levels, in tandem with decreased Prx3 expression demonstrating indices of dysfunction and a reduced capacity to eliminate the excess ROS. This may potentially cause a vicious cycle, leading to amplified effects and endangering cell function. In females, MnSOD

antioxidant expression was significantly increased at 4 weeks of age and even more so at 16 weeks of age in the group exposed to hyperoxia, suggesting that females are better able to deal with ROS than males, as hydrogen peroxide levels in females showed no significant difference between the groups. Prx3 expression, however, was found to be significantly decreased in females at 16 weeks of age compared to the control, while no significant difference was observed at 4 weeks. Interestingly, in males, Prx3 expression was found to be significantly increased at 4 weeks of age and significantly decreased at 16 weeks of age. A possible explanation for this may be that in the short term, oxidative stress induces Prx3 expression and in the long term it becomes depleted. No significant difference was observed in the protein expression of catalase in males or females at either timepoint. Since catalase is present in very low concentrations in mitochondria[181] it is not surprising that no significant difference was observed between groups with the method of detection used for the current studies. No other significant differences were observed between groups in the assessed antioxidants (MnSOD, Prx3, catalase) and H_20_2 levels.

3.1.4 NADPH/NADP+ ratio

NADPH/NADP+ ratio is an important indicator of the redox balance of the cell. A decrease in this ratio has been shown to favor a pro-oxidant state in kidney mitochondria[110]. Unfortunately, there was a problem with the company which supplied the protocol for this assay and the results were unable to be interpreted. This is an important test for evaluating mitochondrial function and will be repeated in future studies.

3.1.5 Mitochondrial swelling (PTP)

Mitochondrial swelling following calcium-induced opening of the PTP, a measure of mitochondrial dysfunction, was assessed in males at 4 weeks of age. Contrary to our hypothesis, no significant difference was observed between groups which indicates that the sensitivity of the PTP to calcium was not altered following hyperoxia exposure. It is possible that the animals studied were still relatively young and therefore that overt mitochondria dysfunction was not (yet) detectable.

Similarly, it could be hypothesized that PTP opening is normal in baseline conditions but becomes impaired in situation of additional challenge such as more pronounced hypertension, inflammation, etc.

3.2 Male and female differences

Previous studies have shown that males have an increased sensitivity to oxidative stress compared to females[182-185]. Females have been shown to have a lower level of oxidative damage to DNA than males[182]. Possible explanations include the antioxidant properties of estrogen although the exact mechanism remains unknown[186]. Other antioxidant-related factors are also thought to contribute to gender-related differences in antioxidant protection; however, nothing concrete has yet been determined[182]. Our findings demonstrate an increase in the quantity of MnSOD (an antioxidant enzyme specific to the mitochondria responsible for neutralizing superoxide free radical) in females exposed to hyperoxia compared to the control, demonstrating a protective effect which is in accordance with previous studies showing increased activity of antioxidant systems in females[186-188]. The precise mechanisms for the observed difference in renal antioxidant capacity would require more experiments, as presently the mechanism remains incompletely known.

3.3 Relation to programming of hypertension

As described by Nuyt *et al*.[16], programming occurs when the normal pattern of development is disrupted by an abnormal stimulus or 'insult' applied at a critical time-point. This leads to an adaptive structural or functional response in the fetus/neonate that promotes short-term survival, but which may manifest in adult disease (such as diabetes or hypertension) in later life. Importantly, Yzydorczyk *et al*.[83] demonstrated a reduced nephron number and hypertension in adult rats following a transient neonatal hyperoxic insult (at a time when renal development was still ongoing), however the mechanisms underlying the programming of hypertension in this model are unknown. It was hypothesised that alterations in mitochondrial function may be a contributing factor in the development of hypertension; hyperoxia is known to lead to

mitochondrial dysfunction[189] which in turn may adversely affect mitochondrial metabolism (increased levels of oxidative stress and decreased levels of antioxidant enzymes) and result in hypertension[189]. In the study by Yzydorczyk *et al*.[83], hypertension was only evident in adult animals at 8 weeks of age; therefore, to evaluate the role of mitochondria in the development of hypertension, in the current study it was important to look at timepoints both prior to the onset of hypertension (4 weeks) and after the onset of hypertension (16 weeks). If an effect is present at 4 weeks (early-onset), it may precede and potentially contribute to the development of hypertension and likely occurred as a direct consequence of hyperoxia exposure, whereas, if an effect is only present at 16 weeks (after the onset of hypertension), it may not be a contributing factor and it is difficult to interpret whether the effect is a consequence of hyperoxia exposure, or due to the later complications (such as hypertension or ageing). In assessing males and females at 4 and 16 weeks of age, we were better able to deduce if the changes in mitochondrial metabolism were due to programming or injury. In females, there seems to be both a programming aspect and a possible injury phenomenon that could affect mtDNA. The expression of MnSOD, which is a mitochondrial antioxidant, is significantly increased at 4 weeks of age (preceding the onset of hypertension) in the hyperoxia exposed group compared to control and even more so at 16 weeks of age (after the onset of hypertension/age), demonstrating a potential protective programming effect. In addition, the significant increase in UCP2 expression at 4 weeks of age in the hyperoxia exposed group may potentially serve to minimize ROS damage in older females, as no significant difference was observed at 16 weeks of age between groups. However, the expression of Prx3 seems to be brought about through injury instead of by programming, since Prx3 expression (a ROS scavenger) is significantly decreased at 16 weeks of age (in the group exposed to hyperoxia) but no significant difference was observed at 4 weeks of age, indicating a possible age-related or hypertension-related alteration in its expression. In addition, aconitase activity is significantly increased at 16 weeks of age in the hyperoxia exposed group, suggesting a protective effect. This increase was not seen at 4 weeks of age (possible change after onset of hypertension/ageing). Finally, ATP synthase β subunit (involved in ATP production) expression was found to be significantly increased at 16 weeks of age but not at 4 weeks of age, suggesting the possible role of secondary injury to mitochondria instead of a programming effect. Therefore, in females, there

seems to be evidence for both a programming effect and an effect possibly due to injury as a secondary effect to the development of hypertension. In males, the results seem to indicate more of an injury effect rather than a programming effect. Citric acid cycle enzymatic activity was significantly affected at 16 weeks of age, compared to no significant difference between groups at 4 weeks of age. This trend was also observed for complex 4 and UCP2 protein expression in males. However, Prx3 expression seems to follow a programming effect. In males (exposed to hyperoxia) at 4 weeks of age, Prx3 expression was significantly increased as a possible short-term compensatory mechanism to deal with oxidative insult and significantly decreased at 16 weeks of age as Prx3 potentially becomes depleted or exhausted.

3.4 Future Directions

This study has demonstrated novel findings with regards to parameters of mitochondrial function in the kidney following neonatal oxygen exposure, including : ROS production, enzymatic activity and expression of key proteins involved in ATP production and antioxidant defense at 4 and 16 weeks of age in both male and female rats. However, there still remains important studies to be done in order to fully describe and understand the effects of transient neonatal hyperoxia exposure on later life renal mitochondrial function.

Future studies would include further tests to examine mitochondrial function (respiration/ATP production) and completing the evaluation of mitochondrial swelling (PTP opening) in females at 4 weeks of age and in both genders at 16 weeks of age and repeating the NADPH/NADP+ assay. This study has assessed various aspects of mitochondrial metabolism in the kidney, however, it would be important to evaluate mitochondria in other tissues including the heart and brain, as mitochondria have been shown to respond differently in different tissues. It is especially important in the heart considering its role in hypertension and cardiovascular disease. In addition, evaluating mtDNA would be of interest since mtDNA has been shown to be reduced in the reprogramming of mitochondrial function in the development of type 2 diabetes[190] and mtDNA has been shown to be sensitive to ROS damage[191]. Furthermore, future studies would include assessing animals at older

ages (12-24 months) to demonstrate long-term effects. Finally, evaluating the female cycle and the effects of estrogen could potentially allow different interpretation of the observed male/female differences.

4.0 CONCLUSION

Parameters of mitochondrial function in the kidney following a neonatal hyperoxic insult and its influence on young adults (4 weeks) and older adults (16 weeks) were evaluated throughout these studies. To our knowledge, this study is the first to comprehensively examine mitochondrial metabolism following transient neonatal hyperoxia exposure in male and female rats, both prior to and following the observed rise in blood pressure in this model. Importantly, we have shown that exposure to hyperoxia in the neonatal period results in mitochondrial dysfunction in the kidney in adulthood, with findings of altered enzymatic activity in the citric acid cycle, altered expression of antioxidants and of proteins involved in ATP production, as well as increased levels of H_20_2. Preterm babies possess a diminished antioxidative capacity and are exposed upon birth to high oxygen concentrations relative to the intrauterine environment. Given the findings of the current study, this vulnerability to oxidative insults may also impact a vulnerability to mitochondrial dysfunction in individuals born preterm. Previous studies have demonstrated evidence for the possibility of ROS being a major contributor to developmental programming of adult diseases, such as the high blood pressure and renal disease observed in children and adults born preterm; however, the mechanisms remain unknown. Mitochondria are the major contributor of cellular ROS, and are involved in many processes (including ATP production and apoptosis) that have been shown to be adversely affected by ageing, indicating that they may play a role in the programming of adult diseases. The originality of this study is to also show for the first time, that there appears to be differences in mitochondrial metabolism in the kidneys between males and females following oxidative insult with the potential dual roles of programming (following the neonatal hyperoxia exposure) and injury (following the onset of hypertension) as a possible mechanism for the observed disease phenotype. Furthermore, the observed sex difference indicates that females may be better able to cope with oxidative insult than males,

which is a recognized fact in the neonatal period. Overall, the findings of this study have shown that neonatal hyperoxia exposure alters renal mitochondrial function in adulthood; however, future studies are required in order to deduce the mechanism responsible for the disease phenotype, and what exact role mitochondria may play in the programming of renal dysfunction and hypertension.

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