Impact of High Fat Dietary Treatment and Nox2-Deficiency on Prooxidant and Antioxidant Enzyme Expression in the Heart

Rich, Hannes Samuel

Abstract: Over the last decades obesity has become a major global health problem and its prevalence is still increasing in both western and developing countries [1-3]. Obesity is associated with a state of low-grade systemic inflammation and increased levels of oxidative stress, which both contribute to the development of cardiovascular disease [4-9]. However, up to now little is known about cardiac redox balance and the potential contribution of free radicals to cardiac disease in obesity. In this study, the effects of 15 weeks of dietary treatment with either a low fat diet (LFD), a high fat diet (HFD) or a very high fat diet (VHFD) on steady state messenger RNA (mRNA) expression levels of pro- and antioxidant genes in the heart of C57BL/6 mice were examined. In addition, the impact of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase subunit Nox2-deficiency combined with a low fat diet (LFD-N) or a high fat diet (HFD-N) were investigated. RNA was isolated from the heart tissue, reverse transcribed and quantified using real-time polymerase chain reaction. While mRNA expression levels of NAD(P)H oxidase subunits Nox2 and Nox4, nitric oxide synthase-2 and -3, superoxide dismutase-3 and glutathione peroxidase-1 were not affected by 15 weeks of high fat dietary treatment, reduced mRNA expression levels of NAD(P)H oxidase subunit p22phox (P=0.02 vs. LFD, P=0.03 vs. HFD) as well as reduced mRNA expression levels of superoxide dismutase-1 (P=0.01 vs. LFD, P=0.01 vs. HFD) and antioxidant-1 (P=0.004 vs. LFD, P=0.007 vs. HFD) were found in mice fed a VHFD. In Nox2-deficient mice, HFD lowered expression levels of p22phox mRNA (P=0.05 vs. LFD-N) and of superoxide dismutase-3 (P=0.03 vs. HFD). The mRNA expression levels of the other genes investigated were not affected by Nox2-deficiency. In conclusion, the present study demonstrates that in C57BL/6 mice neither high fat dietary intake nor Nox2-deficiency lead to major alterations in cardiac mRNA expression of the redox genes investigated. However, high fat dietary treatment may lead to down-regulation of superoxide dismutase-1 mRNA expression in the heart, thus possibly contributing to impairment of cardiac defense mechanisms against oxidative stress and promoting cardiac disease.
Impact of High Fat Dietary Treatment and Nox2-Deficiency on Prooxidant and Antioxidant Enzyme Expression in the Heart
Dedicated to my parents
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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ACEI</td>
<td>Angiotensin-converting-enzyme-inhibitor</td>
</tr>
<tr>
<td>Atox1</td>
<td>Antioxidant-1</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>°C</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>CCS</td>
<td>Copper Chaperone for SOD1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>Efficiency</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
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<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HFD-N</td>
<td>Nox2-deficient mice assigned to high fat diet</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>kcal</td>
<td>Kilocalorie</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>l</td>
<td>Liter</td>
</tr>
<tr>
<td>LFD</td>
<td>Low fat diet</td>
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<tr>
<td>LFD-N</td>
<td>Nox-2 deficient mice assigned to low fat diet</td>
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<tr>
<td>LOOH</td>
<td>Lipid hydroperoxides</td>
</tr>
<tr>
<td>m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Square meter</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>μl</td>
<td>Microliter</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeters of mercury</td>
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<tr>
<td>mmol</td>
<td>Millimole</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>Nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>ONOO^-</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>P</td>
<td>P-value</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticicus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TYRO3</td>
<td>Protein tyrosine kinase 3</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VHFD</td>
<td>Very high fat diet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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</table>
1 Abstract

Over the last decades obesity has become a major global health problem and its prevalence is still increasing in both western and developing countries [1-3]. Obesity is associated with a state of low-grade systemic inflammation and increased levels of oxidative stress, which both contribute to the development of cardiovascular disease [4-9]. However, up to now little is known about cardiac redox balance and the potential contribution of free radicals to cardiac disease in obesity. In this study, the effects of 15 weeks of dietary treatment with either a low fat diet (LFD), a high fat diet (HFD) or a very high fat diet (VHFD) on steady state messenger RNA (mRNA) expression levels of pro- and antioxidant genes in the heart of C57BL/6 mice were examined. In addition, the impact of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase subunit Nox2-deficiency combined with a low fat diet (LFD-N) or a high fat diet (HFD-N) were investigated. RNA was isolated from the heart tissue, reverse transcribed and quantified using real-time polymerase chain reaction. While mRNA expression levels of NAD(P)H oxidase subunits Nox2 and Nox4, nitric oxide synthase-2 and -3, superoxide dismutase-3 and glutathione peroxidase-1 were not affected by 15 weeks of high fat dietary treatment, reduced mRNA expression levels of NAD(P)H oxidase subunit p22phox ($P=0.02$ vs. LFD, $P=0.03$ vs. HFD) as well as reduced mRNA expression levels of superoxide dismutase-1 ($P=0.01$ vs. LFD, $P=0.01$ vs. HFD) and antioxidant-1 ($P=0.004$ vs. LFD, $P=0.007$ vs. HFD) were found in mice fed a VHFD. In Nox2-deficient mice, HFD lowered expression levels of p22phox mRNA ($P=0.05$ vs. LFD-N) and of superoxide dismutase-3 ($P=0.03$ vs. HFD). The mRNA expression levels of the other genes investigated were not affected by Nox2-deficiency. In conclusion, the present study demonstrates that in C57BL/6 mice neither high dietary fat intake nor Nox2-deficiency lead to major alterations in cardiac mRNA expression of the redox genes investigated. However, high fat dietary
treatment may lead to down-regulation of superoxide dismutase-1 mRNA expression in the heart, thus possibly contributing to impairment of cardiac defense mechanisms against oxidative stress and promoting cardiac disease.
2 Introduction

2.1 Obesity

2.1.1 The Prevalence of Obesity

Over the last few decades the prevalence of obesity showed a significant increase in both adults and children throughout the world [1-3]. At least 1 billion people worldwide are estimated to be overweight or obese and at least 300 million people are estimated to be obese [10]. In most studies on the subject the body mass index (BMI) is used for classification and comparison of the degree of obesity, which is a widely accepted measure of obesity in research and clinical work. The BMI is defined as the weight in kilograms divided by the square of the height in meters (kg/m²) and therefore allows a height-independent measure of body mass and a rough estimate of body fatness. Normal weight is defined by the World Health Organization (WHO) as a BMI between 18.5 and 24.9. Overweight is classified as a BMI of 25 or more, obesity as a BMI of 30 or more [11]. However, the BMI may not exactly correspond to the same ratio of lean and fat tissue in different populations, due to the differences in body proportion between ethnic groups [11].

Worldwide, the highest rates of obesity are found in the Pacific Islands, the lowest rates in the lesser developed parts of Asia [12]. The rates are generally high in Europe and North America, but vary remarkably between different countries and regions. A review by Berghöfer et al. analyzed data collected between 1980 and 2005 in Europe and found the prevalence of obesity in European countries to be ranging from 4.0% to 28.3% in men and from 6.2% to 36.5% in women. The prevalences were especially high in Eastern Europe and the Mediterranean countries, while they were lower in countries located in Western and Northern Europe [13]. The prevalence of obesity among adults in the United States increased from 12% in the year 1991 to 19.8% in 2000, which reflects a dramatic raise by 74% [14].
According to the data of the NHANES (National Health and Nutrition Examination Survey, Centers for Disease Control and Prevention; USA) – which is conducted in 2-year cycles – the prevalence of obesity among US adults (aged 20 years or older) in the years 1999-2000 even amounted to 27.5% for men and 33.4% for women. While the prevalence for women showed no statistically significant increase during the period between the 1999-2000 survey and the 2007-2008 survey (prevalence 35.5%), the prevalence for men increased further during the first years, but showed no significant changes in the period between the 2003-2004 survey and the 2007-2008 survey (prevalence 32.2%). Although the prevalence of obesity in the United States is still on a very high level, these recent findings suggest that it will not further increase at the same rate as in the past decades [15].

Even though the obesity epidemic is generally regarded as a problem concerning solely developed nations with high economic status, more and more developing countries (e.g. in Africa, Central and South America) are also experiencing an increase in obesity prevalence, mainly in urban areas [3, 12]. Urbanization and the associated changes in lifestyle (e.g. energy dense diet, lack of physical activity, socioeconomic factors) are considered to be the main causes of obesity in these nations, and hence may lead to a coexistence of over- and undernutrition in the same country, as the rural population is often still struggling for sufficient food supply [3, 12].

2.1.2 Associations between Obesity and Cardiovascular Disease

Obesity is associated with a number of health conditions which are known to promote cardiovascular disease and are considered as classical cardiovascular risk factors, including diabetes mellitus, dyslipidemia and hypertension [16]. The concomitant presence of excess visceral fat, insulin resistance, dyslipidemia and hypertension is summarized under the term metabolic syndrome and regarded as an important risk factor for cardiovascular disease [7].
The relationship between obesity and cardiovascular disease is well established and has been reviewed in detail by Poirier et al. and by Lavie et al. [17, 18]. Obesity is associated with an increased risk of cardiovascular disease such as cardiomyopathy and congestive heart failure, hypertension, coronary artery disease and stroke [17, 18]. The risk of hypertension has been shown to be up to five times higher among obese people compared to a normal-weight population [19]. A raise in body weight of 10 kg corresponds with an increase in the systolic and diastolic blood pressure of 3.0 mmHg and 2.3 mmHg respectively [20]. As a clinical consequence of this raise in blood pressure, these increases correspond to an heightened risk for coronary heart disease by 12% and for stroke by 24% [20]. A study with asymptomatic probands showed, that the BMI was significantly associated with an increased progression of coronary artery calcification [21].

Increasing evidence supports the existence of an obesity-related cardiomyopathy, independent of accompanying conditions such as hypertension, coronary artery disease and diabetes mellitus [22, 23]. The risk of heart failure is heightened proportionally to the grade of obesity [24] and the duration of obesity seems to be a major risk factor for heart failure [25]. After 20 years of obesity the risk of developing heart failure is 66%, after 25 years of obesity 93% [26].

Several possible mechanisms may explain the underlying connection between obesity and the emergence of cardiovascular disease. For instance, obesity is accompanied by a state of systemic low-grade inflammation [4, 7, 27] and increased levels of oxidative stress [5, 9], both of which are known to contribute to the development of cardiovascular disease [6, 8, 28]. Adipose tissue is able to secrete a variety of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP) which contribute to the development of cardiovascular disease [4, 6, 7]. In addition, increased oxidative stress may
contribute to elevated cardiovascular risk in obese patients. Oxidative stress is characterized by an imbalance between reactive free radicals and antioxidants in the tissue, a state which can be observed in obese individuals [5, 9]. Accumulating evidence points to an important role for free radicals in the progression of cardiovascular disease [8, 28, 29]. Free radicals and their implications on cardiovascular disease will be discussed in chapter 2.3 in further detail.

Various stimulating mechanisms have been proposed to up-regulate oxidative stress in obesity. Besides direct up-regulation by systemic low-grade inflammation, free radical accumulation is stimulated by hyperglycemia, elevated tissue lipid levels and hyperleptinemia [9]. In addition, decreased antioxidant defense mechanisms may play a role in oxidative stress in obesity. This may partly be due to the fact, that obese patients consume lower amounts of antioxidant agents, which are contained in fruits, vegetables, whole grains, wine, olive oil and nuts [9].

The reason for the development of obesity-related cardiomyopathy is most likely to be

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**Fig. 1.** Pathophysiological mechanisms contributing to the development of obesity-related cardiomyopathy. Adapted from Wong et al. [30].
multifactorial, as no singular primary cause has yet been identified [22, 23]. The postulated pathogenetic mechanisms include ventricular and myocardial remodeling due to hemodynamic changes in obese individuals (discussed in chapter 2.2.3); harmful influences of metabolic factors like insulin resistance; direct lipotoxicity of excess fatty acid accumulation; systemic low-grade inflammation; up-regulation of the renin-angiotensin-aldosterone system (RAAS) (with angiotensin II – being a growth factor for cardiac myocytes – leading to cell hypertrophy, apoptosis and myocardial dysfunction [31]); sympathetic nervous system activation; microvascular disease and endothelial dysfunction in the vasculature of the heart (Fig. 1) [22, 23]. It is very well conceivable, that oxidative stress also contributes to the progression of obesity-related cardiomyopathy – either via direct obesity-induced production of free radicals or via stimulation by cytokines. The role of oxidative stress in cardiac hypertrophy and failure is addressed in chapter 2.3.5.

2.1.3 Rodent Models of Obesity

The development and examination of animal models of obesity has contributed substantially to the investigation of obesity and its sequelae over the last decades. But also today, animal models are still of great significance for current obesity research [32]. Rodent models are the most commonly used animal models for obesity due to their similar genetic structure to humans and the availability of genetic manipulation techniques, their convenient size and the relatively inexpensive cost for breeding and housing of the animals [33]. Several different methods have been developed in order to evoke obesity in animal models. On one hand, animals were genetically altered – either by spontaneous genetic mutations or by deliberate deletion or overexpression of a gene – on the other hand selective breeding or high caloric dietary treatment has been applied to induce obesity [32]. Examples for genetically manipulated animals are the leptin-deficient ob/ob-mouse and the leptin-receptor-deficient
db/db-mouse [32]. A wild-type mouse strain which is known to be highly prone to obesity when fed a high caloric diet is the C57BL/6J mouse [32, 34]. In response to a high-fat diet it develops obesity and diabetes mellitus [34]. For these reasons, the C57BL/6J mouse is a suitable animal model to study the impact of a high-fat diet on the organism.
2.2 The Heart

2.2.1 Anatomy and Function of the Heart

The heart is a hollow, rhythmically contracting muscle, which primary function is to provide the necessary force to ensure a sufficient, constant blood circulation through the body. It consists of four chambers: the right and left atria and the right and left ventricles. Venous blood, coming from the peripheral areas of the body, flows via the venae cavae superior and inferior to the right atrium and subsequently to the right ventricle. By muscular contraction, the blood is pumped via the pulmonary artery into the lungs and through the pulmonary capillaries, where the exchange of carbodioxide and oxygen takes place. The freshly oxygenated blood then flows back to the heart through the pulmonary veins and via the left atrium into the left ventricle. From there, the blood is transported by contraction via the aorta into every arterial vessel in the body and reaches the internal organs and peripheral areas. Due to the high force and energy needed to pump the blood through the arterial system, the left ventricle muscular walls are physiologically of greater width than the muscular walls of the right ventricle. The right atrium is separated from the right ventricle by the tricuspid valve,
the left atrium is separated from the left ventricle by the mitral valve. In the aorta and pulmonary artery, the aortic and pulmonary semilunar valves prevent the reflux of the expelled blood (Fig. 2).

2.2.2 Histology of the Heart

The wall of the heart can histologically be divided in three different layers – the endocardium, the myocardium and the epicardium.

The endocardium is the innermost layer of the heart wall. The heart chambers are lined by an endothelium of polygonal, squamous cells, which is continuous with the endothelium of the vessels entering and leaving the heart. Underlying the endothelium, is a thin layer of collagenous and elastic fibers, followed by a layer of denser connective tissue, rich in elastic fibers and containing smooth muscle cells. The myocardium is separated from the endocardium, by a layer of loose connective tissue, which contains blood vessels and bundles of fibers of the electrical conduction system of the heart.

The major constituent of the myocardium are cardiac muscle fibers, which enable the heart to perform the rhythmic contraction. Certain muscle cells are specialized on the initiation of these contractions by spontaneous depolarization in a regular rate. These pacemaker cells are located in the sinoatrial node at the junction of the superior vena cava with the right atrium.

The epicardium is built of an inner layer, consisting of fibroelastic connective tissue, and an outer layer of squamous mesothelial cells. The coronary blood vessels, which provide the myocardium with blood, are embedded in the connective tissue of the epicardium, as well as varying amounts of adipose tissue.
2.2.3 Effects of Obesity on Heart Structure and Function

Obesity has a significant impact on blood circulation, heart structure and function. Obesity has been shown to increase left and right ventricular wall thickness and mass and cause ventricular dilatation [36-40]. Autopsy studies showed increased heart weight, left and right ventricular hypertrophy and – on the histological level – diffuse myocyte hypertrophy in the obese population [37, 41]. Moreover, in both animal models and obese humans myocardial fibrosis was found [37, 42].

As schematically represented in Fig. 3 [43] hemodynamic changes occur in the circulatory system of obese individuals. In a first step, the presence of excessive adipose tissue leads to

![Diagram of the effects of obesity on heart structure and function](image)

**Fig. 3. Schematic Representation of the Pathophysiology of Obesity Cardiomyopathy.** Adapted from Alpert [43].
an increase in blood volume and a consecutive augmentation in cardiac output and stroke volume, while the heart rate remains unchanged. In order to cope with the increased cardiac output, systemic vascular resistance decreases. The augmented cardiac output then leads to left ventricular dilatation and thereby to elevated left ventricular wall stress, which in turn may cause left ventricular hypertrophy. If hypertrophy proves to be inadequate to compensate for a given ventricular dilatation, however, systolic dysfunction results and left ventricular heart failure develops [43].

In combination with further factors discussed in chapter 2.1.2, these changes in heart function may lead to the clinical manifestation of obesity-related cardiomyopathy. Chronic heart failure ensues, with symptoms such as progressive dyspnea on exertion, paroxysmal nocturnal dyspnea, orthopnea and lower extremity edema [22, 43].

2.3 Physiological Roles of Free Radicals and Antioxidants and Impact on Cardiovascular Disease

2.3.1 Free Radicals

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), together referred to as free radicals, can be described as molecules capable of independent existence, containing one or more unpaired electrons in their molecular orbitals [44]. Due to this property, free radicals are very reactive molecules and play an important role in a variety of both physiological and pathophysiological processes [28, 45]. In healthy conditions, ROS and RNS are generated by tightly regulated enzymes and neutralized by a number of antioxidative enzymes and molecules [28, 46]. If either an overproduction of free radicals or an underproduction of antioxidants occurs, the physiological equilibrium is disturbed and a state of oxidative stress results [28, 45]. An overbalance of free radicals may result in severe damage to cell
structures, including lipids and membranes, proteins and DNA [28, 29, 45]. Increased oxidative stress is known to play a pivotal role in the pathogenesis of cardiovascular disease [8, 29, 47]. Available in moderate concentrations, however, free radicals fulfill a variety of physiological functions [28, 45]. Therefore, organisms need to regulate the redox state in living cells and maintain redox homeostasis in order to keep the balance between beneficial and harmful effects of free radicals and prevent oxidative damage [45].

2.3.1.1 Reactive Oxygen Species (ROS)

When an oxygen molecule acts as an electron acceptor, different reactive oxygen species are formed depending on the number of electrons which are accepted [44]. The ROS formed include the superoxide anion radical (O$_2^-$) (one electron-reduced oxygen molecule), hydrogen peroxide (H$_2$O$_2$) (two electron-reduced oxygen molecule) and the hydroxyl radical (OH) (three electron-reduced oxygen molecule) [44]. The major sources of O$_2^-$ in the cells are nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, xanthine oxidase, cytochrome P-450 systems, the mitochondrial electron transport chain and arachidonate metabolism [46]. The O$_2^-$ molecule is rather unstable with a half life of a few seconds in aqueous solution and because of its poor cell membrane permeability it is usually restricted to the cell compartment where production takes place [29]. If O$_2^-$ is existent in moderate concentration, it is rapidly dismutated to H$_2$O$_2$, a reaction which is catalyzed by superoxide dismutase (SOD) [29]. However, the O$_2^-$ molecule may also react with nitric oxide (NO) to form the highly reactive peroxynitrite anion (ONOO$^-$), a molecule which is able to directly damage proteins, lipids and DNA [44]. The hydroxyl radical (OH), a further oxygen-derived free radical, is a reactive molecule with a very short half life of approximately $10^{-9}$s [48]. Another group of ROS are the lipid hydroperoxides (LOOH), which are formed through
oxidation of polyunsaturated fatty acids, contained as phospholipids in the cell membrane [44].

2.3.1.2 Reactive Nitrogen Species (RNS)

Nitric oxide (NO), containing one unpaired electron and therefore being a free radical, belongs to the group of reactive nitrogen species (RNS) [46]. In aqueous environment NO has a half life of only a few seconds [28]. The half life is prolonged (>15 seconds), however, in an environment with low oxygen concentration [28]. Due to its both hydrophilic and lipophilic solubility, NO is able to permeate through cytoplasm and cell membranes [28]. In biological tissues NO is produced by nitric oxide synthases (NOSs) and NO functions as an important signaling molecule in several physiological processes and is of great importance in the regulation of vascular tone [49]. When NO reacts with O₂⁻, however, highly reactive ONOO⁻ is formed, which is a powerful oxidizing agent and causes direct damage to proteins, lipids and DNA [44].

2.3.2 Antioxidants

To attenuate the exposure of healthy body tissue to free radicals, a variety of defense mechanisms have been developed [46]. The enzymatic antioxidant defense is represented by superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase [28, 46]. Examples for non-enzymatic antioxidants are ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), coenzyme Q, glutathione (GSH), carotenoids and flavonoids [28, 46]. Vitamins act as strong antioxidants \textit{in vitro} and \textit{in vivo} and scavenge a variety of free radicals [46]. Thereby, ascorbic acid acts as antioxidant in hydrophilic environment, whereas α-tocopherol scavenges radicals in hydrophobic environment [46]. The enzymes superoxide dismutase and glutathione peroxidase will be discussed in further detail in chapter 2.3.3.3 and chapter 2.3.3.4 respectively.
2.3.3 Selected Enzymes involved in Regulation of Redox Homeostasis

2.3.3.1 NAD(P)H Oxidase

NAD(P)H oxidases are of particular importance for redox signaling and are associated with a multitude of physiological as well as pathophysiological functions [29]. The enzyme catalyzes the one-electron reduction of an oxygen molecule (O$_2$) using reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor [29, 50]. The prototype of the NAD(P)H oxidase family is contained in phagocytes of the immune system, where it contributes to phagocytosis and degradation of pathogens by releasing large amounts of O$_2^-$, a process which is referred to as the oxidative burst [29]. Phagocytic NAD(P)H oxidase is composed of several subunits (Fig. 4) [50].

![Diagram of Phagocytic NAD(P)H Oxidase](image)

**Fig. 4. Phagocytic NAD(P)H Oxidase.** Cytosolic regulatory subunits p47phox, p40phox, p67phox and the G-protein Rac attach to the subunits Nox2 and p22phox upon activation, thus forming a functioning enzyme (A). The NAD(P)H oxidase then catalyzes the reduction of oxygen molecules to superoxide radicals (O$_2^-$), using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor (B). Adapted from Cave et al. [29].
subunit p22phox and the subunit gp91phox – also known as Nox2 – together form a membrane-associated flavocytochrome-complex, which already contains the entire catalytic machinery necessary to transfer an electron from the donor NAD(P)H to oxygen (O_2) in order to produce superoxide (O_2^-) [50]. Nevertheless, the cytosolic regulatory subunits p47phox, p40phox, p67phox and the G-Protein Rac1 or Rac2 are needed to associate with p22phox and Nox2 to activate the enzyme [29, 50]. Expression of NAD(P)H oxidase isoforms was found in a wide range of different cells and tissues [29, 50]. In the cardiovascular system NAD(P)H-dependent oxidases were detected in vascular smooth muscle cells, endothelial cells, adventitial and cardiac fibroblasts and cardiomyocytes [29]. A common property of nonphagocytic NAD(P)H oxidases seems to be the intensity of their O_2^- release. Rather than releasing large amounts of ROS as a reaction on a specific stimulus, O_2^- is generated continuously on a steady and low level without any stimulation needed but may also be activated by specific stimulation [29]. A number of homologs of the subunit Nox2 have been discovered and were termed Nox 1, Nox 3, Nox 4, Nox 5, Duox 1 and Duox 2 [29, 50]. All of these isoforms have specific expression patterns and thus are limited to specific tissues [29]. In cells of the cardiovascular system the subunit p22phox and the cytosolic components of the oxidase are expressed ubiquitarily [29]. In contrast, the expression of the catalytic Nox-subunit varies between different cell types. Examination of cardiomyocytes showed significant expression of Nox 2 and Nox 4 [29].

In addition to basal ROS production, the activity of NAD(P)H oxidases in cardiovascular cells may be up-regulated in different ways. Potential stimuli include: Oscillatory shear stress, angiotensin II, cytokines, and metabolic factors such as elevated glucose, insulin, free fatty acids and hypercholesterolemia [29, 47, 51].
2.3.3.2 Nitric Oxide Synthase (NOS)

Nitric oxide synthase catalyses a reaction using L-arginine, \( \beta \)-nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen as substrates, leading to the production of citrulline, NADP and the free radical NO [52]. In order for the reaction to occur, NOS needs tetrahydrobiopterin, heme, flavin adenine dinucleotide, flavin mononucleotid and calmodulin as cofactors [52]. Up to now, three different main isoforms of the enzyme have been described [52, 53]. NOS1 – also known as neuronal NOS – is found predominantly in neuronal tissue. NOS2 – also known as inducible NOS – is inducible in many different kinds of cells and tissues, including macrophages, smooth muscle cells, heart and liver tissue. NOS3 – also known as endothelial NOS – is the isoform, which is mainly found in vascular endothelial cells [52]. Since NO is synthesized in a wide range of different tissues and fulfills a variety of functions, the regulation of NOS is a complex matter. By transcriptional regulation the cell and tissue specific localization of the NOS isoforms is controlled [54] and NOS activity is also regulated by posttranslational modification [52].

In pathophysiological conditions, however, NOS may cease NO production and contribute to the accumulation of free radicals [55, 56]. If the the essential NOS cofactor tetrahydrobiopterin is oxidized by \( O_2^- \) or \( ONOO^- \), NOS loses the ability to produce NO and generates \( O_2^- \) instead [55]. This process is called uncoupling of the nitric oxide synthase.

2.3.3.3 Superoxide Dismutase (SOD) and Antioxidant-1 (Atox1)

Superoxide dismutases (SOD) are metalloenzymes, which are found in cells of various species [57]. These enzymes catalyze the chemical reaction from \( O_2^- \) to \( H_2O_2 \) and thus play a major role in scavenging of reactive oxygen species [57]. Three main isoforms of the enzyme are known. The cytoplasmic SOD (SOD1), the mitochondrial SOD (SOD2) and the extracellular SOD (SOD3) [58]. SOD1 and SOD3 both contain copper and zinc in their
catalytic centre, SOD2 uses manganese as a cofactor [58]. The conversion of O$_2^-$ to H$_2$O$_2$ is conducted in two steps. During the first step, the O$_2^-$ anion is bound to the reduced form of the enzyme and reacts with the prosthetic group of SOD, which acquires a proton and releases molecular oxygen. During the second step, the reduced form of the enzyme binds another O$_2^-$ anion and proton, liberates H$_2$O$_2$ and returns to its oxidized form. H$_2$O$_2$ then is further disproportionated by the enzymes catalase or glutathione peroxidase [59]. All the SOD isoforms are tightly regulated on transcriptional level by mechanical, chemical and biological messengers [58]. In vascular tissue, SOD3 is the most abundant isoform, where it influences blood pressure and endothelial function by regulating the concentration of O$_2^-$ in the vascular cells [60, 61]. The expression of SOD3 is up-regulated by a variety of factors, including angiotensin II, nitric oxide, exercise training and in pathological states, such as atherosclerosis and hypertension [62]. Since SOD1 and SOD3 both contain copper, the presence of a soluble, cytosolic copper-carrier – a copper chaperone – is needed for the proper function of the enzymes. Copper chaperone for SOD1 (CCS) and Antioxidant-1 (Atox1) – the chaperone for SOD3 – carry out this task [59, 60]. In addition to its role as copper-carrier, Atox1 stimulates the expression of SOD3 at transcriptional level [60].

2.3.3.4 Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) is a selenoprotein which contains a selenocystein residue in its active site [46]. Glutathione peroxidases catalyze the reduction of hydrogen peroxide (H$_2$O$_2$) or organic hydroperoxides to water or corresponding alcohols, using glutathione as a coenzyme [46]. Thereby, reduced glutathione (GSH) acts as an electron donor (H$_2$O$_2$ + 2GSH $\rightarrow$ GSSG + 2H$_2$O) [46]. Five different types of glutathione peroxidase have been found in mammals [46, 63]. Cytosolic GPx (GPx-1), gastrointestinal GPx (GPx-2), plasma GPx (GPx-3), phospholipid hydroperoxide GPx (GPx-4) and a GPx located in the human olfactory
system (GPx-6) [46, 63]. The five isozymes show a distinct expression pattern in different tissues [64, 65]. Although glutathione peroxidase family has long been regarded solely as antioxidant enzymes, this is only true to a certain extent. Over the last years the image of H$_2$O$_2$ has changed from a mere toxic oxidant, to a molecule involved in physiological functions, such as cellular signaling, programmed cell death, and proliferation and differentiation of cells [65].

### 2.3.4 Free Radicals in Physiological Functions

Free radicals are involved in numerous physiological functions and, beyond that, play a major role in intracellular signaling and regulation [45]. Physiological functions which are controlled by redox-responsive signaling pathways include ROS production by phagocytic NAD(P)H oxidase, redox regulation of immune responses, ROS production as a sensor of oxygen concentration, ROS-induced apoptosis and the regulation of vascular tone [28].

When neutrophils and macrophages are activated by a pathogenic agent, large quantities of O$_2^-$ radicals are produced by the phagocytic isoform of NAD(P)H oxidase (oxidative burst) in order to neutralize the pathogen [28, 29]. In addition, O$_2^-$ radicals and H$_2$O$_2$ are able to induce T-lymphocyte functions [28]. A role for ROS was implicated in the oxygen homeostasis of the blood, as changes in oxygen concentration are sensed by ROS-producing proteins and a changing rate of mitochondrial ROS may be associated with the function of the oxygen sensor in the carotid bodies [28]. Apoptosis, the programmed cell death, is needed both for normal development and constant renewal of the body. Even though cell death can be induced by a variety of stimuli, production of ROS is a commonly observed phenomenon in apoptotic processes [45]. NO regulates vascular smooth muscle tone and inhibits platelet adhesion, using cyclic guanosine monophosphate as a second messenger [28, 45]. NO plays a major role
in the regulation of blood pressure and the prevention of cardiovascular disease, as it has anti-atherogenic, anti-proliferative and anti-thrombotic properties [49].

### 2.3.5 Free Radicals in Cardiac Disease

Oxidative stress has been implicated in a number of cardiovascular diseases, including hypertension, atherosclerosis and cardiac hypertrophy [28, 29]. However, the mechanisms of how ROS contribute to the pathogenesis of these diseases are incompletely understood and will be subject to future research.

Various cell types within the heart tissue are able to produce ROS [66]. In cardiomyocytes, the most commonly found ROS are $\text{O}_2^-$, $\text{H}_2\text{O}_2$, $\text{ONOO}^-$ and $\text{OH}$ [67]. The main sources of these free radicals include NAD(P)H oxidase, the mitochondrial respiratory chain, xanthine oxidase and uncoupled nitric oxide synthase [66, 67]. The production of ROS by one source may also activate ROS production by another source. ROS generation by uncoupled nitric oxide synthase, for example, may be triggered by NAD(P)H oxidase $\text{O}_2^-$ production [68].

NAD(P)H oxidase is probably the best examined source of ROS in cardiac disease. In pressure-overload cardiac hypertrophy in guinea pigs, activity of NAD(P)H has been shown to be increased, along with augmented expression of Nox2 oxidase in cardiomyocytes and endothelial cells [69]. But also in failing human hearts elevated NAD(P)H activity could be detected [70, 71]. In a further experiment, wild-type and Nox2 deficient mice were treated with angiotensin II, which induced cardiac hypertrophy in wild-type but not in Nox2 deficient mice [72, 73]. However, in an examination of cardiac hypertrophic response to constriction of the aorta, no difference was found between wild-type and Nox2-knockout mice [73]. Nevertheless, these findings confirm a crucial role of NAD(P)H oxidase in the development of cardiac hypertrophy. Cardiac NAD(P)H oxidases have also been suggested to promote
cardiomyocyte apoptosis, interstitial fibrosis and contractile dysfunction of the heart, thus contributing to the pathogenesis of cardiac failure [66].

Nitric oxide synthase isoforms are also involved in the prevention and genesis of cardiac disease [74]. The production of NO by NOS3 is generally attributed a beneficial effect on the heart tissue [74]. Induction of cardiac pressure overload by constriction of the thoracic aorta has been demonstrated to lead to a higher degree of left ventricular hypertrophy and dysfunction in NOS3-deficient mice, compared to wild-type mice [75]. However, it has also been proposed that uncoupling of NOS3 and subsequent ROS production by NOS3 may contribute to cardiac dysfunction in cardiac pressure overload [76]. NOS2 – whose expression is induced by pro-inflammatory cytokines – has been implicated in the emergence of cardiac hypertrophy and failure [74]. In cultured cardiomyocytes of neonatal rats, stimulation with cytokines caused an increase in NOS2 expression, nitrite production and subsequent cardiomyocyte apoptosis [77]. Furthermore, it has been shown that in NOS2-deficient mice, induction of cardiac pressure overload by constriction of the thoracic aorta induced less cardiac hypertrophy and dysfunction than in wild-type mice [78].
2.4 Aim of the Study

The objective of this study was to examine the impact of different dietary treatments varying in fat content and the impact of Nox2-deficiency on cardiac expression levels of enzymes involved in the regulation of the redox homeostasis in male C57BL/6 mice. A low fat control diet (LFD), a high fat diet (HFD) and a very high fat diet (VHFD) were compared in this study. In Nox2-deficient mice, the influence of a low fat control diet and a high fat diet was studied. The following questions were addressed:

- Is messenger RNA (mRNA) of the genes Nox2, Nox4, p22phox, NOS2, NOS3, SOD1, SOD3, Atox1 and GPx-1 detectable in the heart tissue of mice fed either a low fat control diet, a high fat diet or a very high fat diet as well as in Nox2-knockout mice fed a low fat or a high fat diet?
- How do 15 weeks of dietary treatment with either a high fat diet or a very high fat diet affect cardiac mRNA expression levels of redox genes, compared to mRNA expression levels in mice fed a low fat diet?
- Has Nox2-deficiency in the presence or absence of a high fat diet an effect on cardiac mRNA expression levels of redox genes?
3 Methods and Materials

3.1 Animals and Dietary Treatments

Healthy male C57BL/6 mice (obtained from Charles River; Sulzfeld, Germany) and C57BL/6 Nox2<sup>-/-</sup> mice (obtained from Charles River; Sulzfeld, Germany) were housed in the institutional animal facilities on a 12h:12h light-dark cycle. The facilities, as well as the experimental protocols, were approved by the local authorities for animal research (Kommission für Tierversuche des Kantons Zürich, Switzerland) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health [79]. Throughout the phase of dietary treatment, the animals were given free access to water and food ad libitum. At four weeks of age, the C57BL/6 mice were assigned randomly to one of the following three 15-weeks-during diets: low fat control diet (LFD: 12% of total kcal from fat, 57% of total kcal from carbohydrate, 31% of total kcal from protein; Kliba Nafag 3430; Kaiseraugst, Switzerland), high fat diet (HFD: 41% of total kcal from fat, 43% of total kcal from carbohydrate, 17% of total kcal from protein; Research Diets D12079B; New Brunswick, New Jersey, USA) or very high fat diet (VHFD: 58% of total kcal from fat, 25.5% of total kcal from carbohydrate, 16.4% of total kcal from protein; Research Diets D12331; New Brunswick, New Jersey, USA). The Nox2-deficient mice were assigned either to the control diet (LFD-N) or the high fat diet (HFD-N).

The specific macronutrient dietary composition of each diet is listed in chapter 8.1 in the appendix. Group size was 8 animals for all diets, except for the high fat diet (HFD) where group size was 7 animals.
3.2 Tissue Sampling and Treatment

The mice were anesthetized (xylazine 100mg/kg body weight, ketamine 23mg/kg body weight, acepromazine 3mg/kg body weight) and exsanguinated by cardiac puncture. Subsequently, the hearts were removed, flash-frozen in liquid nitrogen and stored at -80°C until further analysis.

3.3 Isolation of RNA from Heart Tissue

3.3.1 Precautions

In order to prevent damage to the heart tissue and spontaneous degradation of the RNA contained within it, a number of precautions were taken. Whenever the heart tissue was handled, benches were cleaned beforehand with ethanol 70% and a bench guard was used. Latex Gloves were worn at all times and all instruments, used to gain appropriately sized portions of tissue, were cleaned thoroughly with ethanol 70%. Furthermore, sample tubes were kept closed whenever possible to avoid contamination. During the isolation process, the heart tissue as well as the sample tubes containing lysed tissue were stored on ice or kept in liquid nitrogen to prevent thawing of the tissue samples.

3.3.2 Different Steps of RNA Isolation

The RNA isolation process was performed using the Qiagen RNeasy Mini Kit® and according to the Qiagen RNeasy Mini protocol (Qiagen; Hilden, Germany). A heart tissue sample of 30 mg weight was excised and in a second step disrupted and homogenized in 600 µl Buffer RLT® (Qiagen), using a rotor-stator homogenizer. Afterwards, the tissue lysate was centrifuged at 10'000 rounds per minute (rpm) for 3 minutes. The supernatant was transferred to a new tube, the pellet was discarded. 600 µl of 70% ethanol were added to the cleared lysate and immediately mixed by pipetting. The sample was transferred to an RNeasy mini
column® (Qiagen) in a 2 ml collection tube, in aliquots of up to 700 µl, and the tubes were centrifuged for 15 seconds at 10'000 rpm after addition of each aliquot. In the next step, the flow-through was discarded, 700 µl of Buffer RW1® (Qiagen) were added to the RNeasy column and the tubes were again placed in the centrifuge for 15 seconds at 10'000 rpm to wash the column. Subsequently, the RNeasy column was transferred into a new 2 ml collection tube and 500 µl of Buffer RPE® (Qiagen) were added to the column, followed by centrifugation for 15 seconds at 10'000 rpm. The addition of 500 µl Buffer RPE was repeated and the tubes were first centrifuged for 2 minutes at 10'000 rpm and, after exchange of the collection tube, centrifuged again for 1 minute at 10'000 rpm, in order to dry the silica-gel membrane of the RNeasy column. For the elution of the RNA, the column was placed in a new 1.5 ml collection tube and 30 µl of RNase-free water were pipetted onto the silica-gel membrane, followed by centrifugation for 1 minute at 10'000 rpm.

3.3.3 Determination of isolated RNA

In order to determine the concentration and the grade of purity of the extracted RNA, a spectrophotometry was performed after the completion of the isolation process. As an absorbance of 1 unit at 260 nm (A_{260}) corresponds with an RNA concentration of 40 µg/ml, the amount of RNA could be calculated by using the formula: RNA-concentration = 40 x A_{260} x dilution factor. RNA samples were only used for further analysis if a ratio of the readings at 260 nm and 280 nm between 1.9 and 2.1 was reached.

To rule out the presence of genomic DNA, able to interfere in the polymerase chain reaction, a reverse transcriptase-negative control was conducted for each sample.
3.4 Reverse Transcription

The reverse transcription was performed by using the Qiagen QuantiTect Reverse Transcription Kit® and according to the Qiagen QuantiTect Reverse Transcription protocol (Qiagen). In order to eliminate genomic DNA present in the RNA samples, the following DNA elimination step was performed: 2 µl of genomic DNA Wipeout Buffer® (Qiagen) and a variable amount of RNase-free water was added to 1 µg template RNA, so that a total reaction volume of 14 µl was reached. The tube was incubated for 2 minutes at 42º C and then placed on ice immediately. A reverse-transcription master mix, containing all the required components for first-strand complementary DNA (cDNA) synthesis except template RNA, was prepared on ice as follows: 1 µl Quantiscript Reverse Transcriptase® (Qiagen), 4 µl Quantiscript RT Buffer® (Qiagen) and 1 µl RT Primer Mix® (Qiagen). In a last step, the 14 µl template RNA were added to 6 µl reverse-transcription master mix, the tube was incubated for 15 minutes at 42º C and subsequently incubated for 3 minutes at 95º C, in order to inactivate the reverse transcriptase.

3.5 Real-time Quantitative Polymerase Chain Reaction

3.5.1 Introduction

The real-time polymerase chain reaction (PCR) is a technique in molecular biology to amplify specific sequences of DNA in order to allow relative quantification or further analysis. The underlying principle of this method is thermal cycling. In a thermal cycler, the DNA samples are repeatedly heated up and cooled down to enable DNA melting and enzymatic replication by the means of a DNA polymerase. Thermus aquaticus (Taq) DNA polymerase is a commonly used polymerase for PCR, due to its high thermostability. The specific amplification of a designated DNA region is accomplished by the use of specific 3’- and 5’-
primers, containing a complimentary sequence to the target region. The primers bind to the 3’- and the 5’-end of the selected DNA region and represent the starting point of the replication process. Apart from DNA template, DNA polymerase and specific primers, a PCR buffer solution as well as deoxynucleoside triphosphates (dNTP) are needed for a functioning PCR reaction. The former, to promote primer annealing and to provide an advantageous environment for the polymerase, the latter as building blocks for the synthesis of DNA.

During the different cycles of the reaction, the following actions take place: 1) Initialization step: The reaction is heated up to 95°C for 3 minutes in order to activate the DNA polymerase. 2) Denaturation step: The reaction remains at 95°C for 15 seconds, the double-stranded DNA melts due to the high temperature and single-stranded DNA is formed. 3) Annealing / elongation step: The temperature is lowered to approximately 60°C for 1 minute, both primers bind to the respective complementary sequences of the gene of interest and the Taq polymerase initiates DNA elongation, producing a new piece of double-stranded DNA of the selected sequence. Steps 2 and 3 are repeated up to 45 times, doubling the amount of DNA product with every repeat.

During the PCR process three main phases can be observed, as the number of cycles and the amount of product increases. In the first phase, the PCR product accumulates exponentially, as the polymerase has ideal working conditions and the dNTPs are present in abundance, thus not limiting the progression of the reaction. In this phase, the efficiency of the reaction is closest to 100% (100% efficiency of a PCR reaction corresponds to doubling the amount of product with every cycle). During the second phase a stabilization of the reaction begins, as the product continues to accumulate but reaction efficiency starts to decrease. The product now accumulates linearly. In the final or plateau phase of the PCR reaction, dNTPs and other reagents are running out and the polymerase activity decreases. The DNA amplification process slowly comes to a halt.
For quantification of the PCR product, real-time quantitative PCR has become the standard method. The amount of PCR product is measured in “real-time” after each PCR cycle with the aid of a fluorescent detector molecule, such as SYBR Green. SYBR Green is an intercalating dye that fluoresces upon binding to double-stranded DNA. After the primer-mediated replication of the target sequence, SYBR Green binds to the newly synthesized double-stranded DNA and emits a fluorescent signal, which is detected and recorded by the thermal cycler. The quantification of the PCR product is expressed in crossing threshold \( (C_T) \) values, \( C_T \) being the number of cycles that it takes each reaction to reach an arbitrary amount of fluorescence [80, 81].

### 3.5.2 Real-time PCR Analysis

The real-time PCR has been carried out using the Qiagen QuantiTect SYBR Green PCR Kit\(^\text{®} \) (Qiagen). As a precautionary measure, the work surface and instruments were treated with ultraviolet light during 40 minutes to minimize the risk of DNA contamination. A master mix with all the necessary components for the polymerase chain reaction was prepared in the following ratio: 12.5 µl SYBR-Green master mix\(^\text{®} \) (Qiagen) (containing HotStarTaq DNA Polymerase\(^\text{®} \), QuantiTect SYBR Green PCR Buffer\(^\text{®} \), dNTP mix, SYBR Green I dye, ROX dye), 9.5 µl RNase-free water, 0.5 µl forward (3’) primer 20 µM, 0.5 µl reverse (5’) primer 20 µM (Microsynth AG; Balgach, Switzerland). The master mix was thoroughly mixed and centrifuged. The synthesized cDNA was diluted with RNase-free water in a ratio of 1/10. 2 µl of the cDNA sample and 23 µl PCR-master mix were transferred on a 96-well PCR plate (Bio-Rad; Hercules, California, USA), adding up to a total volume of 25 µl per well. Each sample was measured in duplicate or triplicate and for each target gene a no-template negative control was conducted, in order to monitor possible contaminations of the PCR reaction. The plate was then covered with protective foil (Bio-Rad) – to prevent evaporation during the
PCR analysis – and centrifuged. The PCR was run on the iCycler IQ (Bio-Rad). The protocol for the amplification process consisted of cycle 1 heating up to 95°C for 3 minutes in order to activate the hot start Taq polymerase, cycle 2 with 15 seconds at 95°C to accomplish DNA melting, followed by 1 minute at 60°C, allowing annealing of the primers to the single stranded DNA and DNA synthesis by the polymerase. Cycle 2 was repeated 45 times. Cycle 3 heating up to 95°C for 1 minute, cycle 4 cooling down to 55°C, cycle 5 increasing the temperature by 0.5°C every 10 seconds (repeated 80 times) in order to measure the melt curve of the samples and the final cycle 6 at 20°C for short time storage of the PCR plate.

3.6 Primer Design and Testing

All primers used in the experiment were designed with the Beacon designer® 2.06 software (Premier Biosoft; Palo Alto, California, USA). The primer sequences are listed in chapter 8.4 in the appendix.

Efficiency of each pair of primers was tested by performing a real-time PCR reaction, using a cDNA dilution series with the dilution factor two (1:10, 1:20, 1:40, 1:80, 1:160, 1:320) as target for the reaction. The standard curve thus obtained was used for calculation of efficiency (E) for each gene, using the formula E=10^{-1/s} (where s is the slope of the standard curve). The PCR reaction was optimized, until its efficiency reached a value between 0.99 and 1 (corresponding to a slope value of 3.30 to 3.39). The PCR product was verified by measurement of the melting curve and comparison to the specific melting point of the target DNA, by agarose gel electrophoresis and by sequencing, so as to exclude artifact influence or amplification of an unspecific DNA product.
3.7 Calculations and Statistical Analyses

For analysis of the relative changes in gene expression, the target gene expression was compared to two internal controls, housekeeping genes murine β-actin and protein tyrosine kinase 3 (TYRO3). All measurements were performed for both housekeeping genes and were only accepted, if the C_T value of the housekeeping gene lay within ±2 of the overall mean C_T value of the housekeeping gene measured in this study. However, the housekeeping gene TYRO3 was expressed more consistently in the different sample tissues examined than β-actin, so that it was selected as the internal control for the relative quantification of target gene expression. The latter was accomplished by using the $2^{-\Delta C_T}$ method. The amount of target gene expression equals $(1+E)^{\Delta C_T}$, where $\Delta C_T$ represents the difference between the average crossing threshold of the housekeeping gene and the target gene ($C_T, Target - C_T, Housekeeper$) and $E$ is the efficiency of the PCR reaction, which is 0.99 (see above) [82]. The resulting amount of target gene expression is expressed in arbitrary units.

As group sizes were too small to test for normal distribution, normal distribution could not be assumed and the non-parametric Kruskal-Wallis test was used to test for variances between the groups. For comparison between two groups, the non-parametric Mann-Whitney-U test was applied whenever necessary. All data are expressed as mean±standard error of the mean (SEM). A $P$-value ≤0.05 was considered statistically significant. All calculations and statistical analyses were conducted using StatView® 5.0.1 software (SAS Institute Inc; Cary, North Carolina, USA).
4 Results

4.1 Metabolic Parameters

Metabolic parameters of animals examined in this study were assessed and published by Mundy et al. in 2007 [83]. After 15 weeks of dietary treatment, mice showed increasing weight gain with incremental dietary fat content. In addition, glucose intolerance was observed in both the high fat diet (HFD) and the very high fat diet (VHFD) groups [83]. Detailed information about metabolic parameters is given in chapter 8.2 in the appendix.
4.2 Overview of Steady State Redox Gene mRNA Expression Levels in LFD-mice

Steady state mRNA expression of SOD1, SOD3, Atox1, GPx-1, Nox2, Nox4, p22phox, NOS2 and NOS3 was found in heart tissue of lean control mice (Fig. 5). SOD1, SOD3, Atox1 and GPx-1 showed higher average mRNA expression levels than the NAD(P)H oxidase subunits Nox2, Nox4 and p22phox as well as NOS2 and NOS3 (Fig. 5, panel A-B). SOD1 showed the highest mRNA expression levels of all genes investigated, followed by GPx-1 which was approximately 15-fold lower expressed than SOD1 (Fig. 5, panel A). Messenger RNA expression levels of NAD(P)H oxidase subunit p22phox were approximately two-fold higher than expression levels of Nox2. Nox4 mRNA expression levels were even lower (Fig. 5, panel B).

Fig. 5 Cardiac SOD1, SOD3, Atox1 and GPx-1 mRNA expression (panel A) and cardiac Nox2, Nox4, p22phox, NOS2 and NOS3 mRNA expression (panel B) in LFD-mice. Data are shown as means±SEM and are expressed in arbitrary units=ΔCT of gene of interest and housekeeping gene. n=4-8 per group. Note different scale y-axis panel A-B.
4.3 Effects of Dietary Fat Intake on Cardiac Steady State Redox Gene mRNA Expression Levels in C57BL/6 Wild-type Mice

4.3.1 Nox2, Nox4 and p22phox

Steady state expression levels of Nox2 mRNA showed a trend to decrease with increasing concentrations of fat in the diet but did not reach significance ($P=0.06$ VHFD vs. LFD; Fig. 6, panel A). Likewise, no significant differences occurred in Nox4 mRNA expression, although expression levels tended to be higher in the high fat diet group (HFD) than in the control group ($P=0.12$ vs. LFD; Fig. 6, panel B). Expression of p22phox mRNA was lower in the very high fat diet group (VHFD) compared to both other groups ($P=0.02$ vs. LFD, $P=0.03$ vs. HFD; Fig. 6, panel C).
Fig. 6 Effects of a low fat control diet (LFD), high fat diet (HFD) and very high fat diet (VHFD) on relative cardiac expression of Nox2 mRNA (panel A), cardiac expression of Nox4 mRNA (panel B) and cardiac expression of p22phox mRNA (panel C). Data are shown as means±SEM and are expressed in arbitrary units=ΔCT of gene of interest and housekeeping gene. n=5-8 per group. *P=0.02 vs. LFD, P=0.03 vs. HFD (panel C). Note different scale y-axis panel A-C.
### 4.3.2 NOS2 and NOS3

Steady state mRNA expression levels of NOS2 were at a similar level in LFD- and HFD-mice (Fig. 7, panel A), while being lower in VHFD-mice. However, this difference in expression levels was not significant ($P=0.18$ vs. LFD; Fig. 7, panel A). Moreover, no significant differences in NOS3 expression levels were detected among the three groups. (Fig. 7, panel B).

**Fig. 7** Effects of a low fat control diet (LFD), high fat diet (HFD) and very high fat diet (VHFD) on relative cardiac expression of NOS2 mRNA (panel A) and cardiac expression of NOS3 mRNA (panel B). Data are shown as means±SEM and are expressed in arbitrary units=$\Delta$CT of gene of interest and housekeeping gene. $n=4-8$ per group.
4.3.3 SOD1, SOD3 and Atox1

Steady state mRNA expression levels of SOD1 were approximately 2.5-fold lower in mice treated with the VHFD in comparison to both the LFD and the HFD diet ($P=0.01$ vs. LFD, $P=0.01$ vs. HFD; Fig. 8, panel A). Expression levels of SOD3 were not significantly altered by the different dietary treatments (Fig 8. panel B). Atox1 expression levels were approximately 3.5-fold decreased in the VHFD group compared to the LFD and the HFD group ($P=0.004$ vs. LFD, $P=0.007$ vs. HFD; Fig. 8, panel C).

![Graph A](image1)

**A**

![Graph B](image2)

**B**

![Graph C](image3)

**C**

**Fig. 8** Effects of a low fat control diet (LFD), high fat diet (HFD) and very high fat diet (VHFD) on relative cardiac expression of SOD1 mRNA (panel A), cardiac expression of SOD2 mRNA (panel B) and cardiac expression of Atox1 mRNA (panel C). Data are shown as means±SEM and are expressed in arbitrary units=$\Delta$CT of gene of interest and housekeeping gene. n=4-6 per group. *$P=0.01$ vs. LFD, $P=0.01$ vs. HFD (panel A); † $P=0.004$ vs. LFD, $P=0.007$ vs. HFD (panel C). Note different scale y-axis panel A-C.
4.3.4 GPx-1

No differences in GPx-1 steady state mRNA expression levels were detected between the three dietary groups examined (Fig 9).

Fig. 9  Effects of a low fat control diet (LFD), high fat diet (HFD) and very high fat diet (VHFD) on relative cardiac expression of GPx-1 mRNA. Data are shown as means±SEM and are expressed in arbitrary units=ΔCT of gene of interest and housekeeping gene. n=6-7 per group.
4.4 Cardiac Steady State Redox Gene mRNA Expression Levels in C57BL/6 Nox2\(^{-}\) mice

4.4.1 Nox2, Nox4 and p22phox

Steady state expression of Nox4 and p22phox mRNA was detected in the heart tissue of both C57BL/6 Nox2-deficient mice fed a low fat control diet (LFD-N) and Nox2-deficient mice fed a high fat diet (HFD-N) (Fig. 10). As expected, no expression of Nox2 was found in the heart tissue of Nox2-deficient animals (data not shown). Neither Nox2-deficiency alone nor in combination with high fat dietary treatment affected Nox4 mRNA expression among the groups (Fig. 10, panel A). Expression levels of p22phox mRNA were lower in the HFD-N group compared to the LFD-N group \((P=0.05 \text{ vs. LFD-N; Fig. 10, panel B})\), while not reaching significance in comparison to the LFD or HFD group \((P=0.07 \text{ vs. LFD, } P=0.39 \text{ vs. HFD; Fig. 10, panel B})\).

![Fig. 10](image)

**Fig. 10** Differences between wild-type mice fed a low fat control diet (LFD) or a high fat diet (HFD), Nox2-deficient mice fed a low fat control diet (LFD-N) and Nox2-deficient mice fed a high fat diet (HFD-N) in relative cardiac expression of Nox4 mRNA and relative cardiac expression of p22phox mRNA. Data are shown as means±SEM and are expressed in arbitrary units=\(\Delta CT\) of gene of interest and housekeeping gene. \(n=5-8\) per group.

\(*P=0.05\text{ vs. LFD-N, } P=0.07\text{ vs. LFD, } P=0.39\text{ vs. HFD (panel B). Note different scale y-axis panel A-B.}\)
4.4.2 NOS2 and NOS3

NOS2 and NOS3 mRNA expression was found in the heart of LFD-N-mice and HFD-N-mice (Fig 11). No significant difference in expression levels of NOS2 mRNA was found among the LFD, LFD-N, HFD and HFD-N group (Fig. 11, panel A). Likewise, the expression levels of NOS3 mRNA did not differ significantly among the four groups examined (Fig 11, panel B).

Fig. 11 Differences between wild-type mice fed a low fat control diet (LFD) or a high fat diet (HFD), Nox2-deficient mice fed a low fat control diet (LFD-N) and Nox2-deficient mice fed a high fat diet (HFD-N) in relative cardiac expression of NOS2 mRNA and relative cardiac expression of NOS3 mRNA. Data are shown as means±SEM and are expressed in arbitrary units=ΔCT of gene of interest and housekeeping gene. n=4-8 per group.
4.4.3 SOD1, SOD3 and Atox1

Messenger RNA of SOD1, SOD3 and Atox1 was shown to be expressed in the heart tissue of LFD-N and HFD-N mice (Fig. 12). Nox2-deficiency and Nox2-deficiency in combination with a high fat diet did not affect expression levels of SOD1 and Atox1 mRNA (Fig. 12, Panel A, C). Expression levels of SOD3 mRNA were reduced in HFD-N mice compared with HFD mice ($P=0.03$ vs. HFD; Fig. 12, panel B), however no significant difference was reached in comparison to LFD or LFD-N mice ($P=0.44$ vs. LFD, $P=0.66$ vs. LFD-N; Fig 12, panel B).

![SOD1 mRNA expression](image)

**Fig. 12** Differences between wild-type mice fed a low fat control diet (LFD) or a high fat diet (HFD), Nox2-deficient mice fed a low fat control diet (LFD-N) and Nox2-deficient mice fed a high fat diet (HFD-N) in relative cardiac expression of SOD1 mRNA, relative cardiac expression of SOD3 mRNA and relative cardiac expression of Atox1 mRNA. Data are shown as means±SEM and are expressed in arbitrary units=$\Delta$CT of gene of interest and housekeeping gene. n=4-8 per group. *$P=0.03$ vs. HFD, $P=0.44$ vs. LFD, $P=0.66$ vs. LFD-N (panel B). Note different scale y-axis panel A-C.
4.4.4 GPx-1

Steady state mRNA expression levels of GPx-1 remained unaffected by high fat dietary treatment and Nox2-deficiency (Fig. 13).

**Fig. 13** Differences between wild-type mice fed a low fat control diet (LFD) or a high fat diet (HFD), Nox2-deficient mice fed a low fat control diet (LFD-N) and Nox2-deficient mice fed a high fat diet (HFD-N) in relative cardiac expression of GPx-1 mRNA. Data are shown as means±SEM and are expressed in arbitrary units=ΔCT of gene of interest and housekeeping gene. n=6-7 per group.
5 Discussion

In the present study, the impact of dietary treatment and deletion of Nox2 on steady state mRNA expression levels of enzymes involved in redox-state regulation in the heart of C57BL/6 mice was investigated. The data showed that both the high fat diet (HFD) and the very high fat diet (VHFD) had little influence on mRNA expression levels of most pro- and antioxidant enzymes investigated after a feeding period of 15 weeks. Interestingly, however, cardiac mRNA expression levels of the redox genes SOD1, Atox1 and p22phox were down-regulated after treatment with a VHFD. In HFD-fed Nox2-deficient mice (HFD-N), cardiac expression levels of p22phox mRNA were lower compared to Nox2-deficient mice fed a low fat control diet (LFD-N) and cardiac expression levels of SOD3 mRNA were reduced compared to wild-type mice fed a high fat diet (HFD). Although these data indicate that neither dietary interventions nor Nox2-deficiency has completely evident effects on the expression patterns of the majority of enzymes involved in the regulation of cardiac redox homeostasis in the conditions investigated, it is of notice that steady state mRNA expression levels of SOD1 – the highest expressed gene in mouse cardiac tissue – were reduced by approximately 65% after treatment with a VHFD. This decrease in SOD1 expression may lead to relevant impairment of antioxidant defense mechanisms in the heart and subsequently to redox state imbalance, which in turn may promote the progression of cardiac disease.

5.1 Steady State Redox Gene mRNA Expression Levels in LFD-Mice

The examination of steady state mRNA expression levels in cardiac tissue of lean control mice showed that mRNA expression levels of the antioxidant genes SOD1, SOD3, Atox1 and GPx-1 were higher than mRNA expression levels of NAD(P)H oxidase subunits Nox2, Nox4 and p22phox, as well as NOS2 and NOS3. Moreover, SOD1 was found to be the highest
expressed redox gene in mouse cardiac tissue, followed by the approximately 15-fold lower expressed GPx-1. The abundance in which SOD1 is found in cardiac cells, may suggest a substantial role for this enzyme in cardiac antioxidant defense. However, studies investigating the impact of SOD isoforms on the prevention of ROS-mediated cardiac reperfusion injury after ischemia produced contradictory results about the cardioprotective properties of SOD1 [84-87]. Yoshida et al. reported increased ischemic reperfusion injury in the heart of SOD1-knockout mice in relation to wild-type mice and therefore postulated an important role of SOD1 in cardiac antioxidant defense [84]. Furthermore, Wang et al. found attenuated superoxide generation, reduced infarct size and increased recovery of contractile function in the hearts of transgenic mice with overexpression of human SOD1 compared to nontransgenic controls, following 30 min of global ischemia [87]. In contrast, Asimakis et al. and Jones et al. did not find relevant changes in cardiac injury after ischemia and reperfusion due to SOD1 overexpression and partial or complete SOD1-deficiency [85, 86]. The presence of SOD2, on the other hand, attenuated cardiac injury in these studies [85, 86]. Moreover, in SOD2-deficient mice high neonatal mortality and severe growth retardation has been demonstrated [88], whereas SOD1-deficient mice grow normally and reach adulthood [89]. Although the role of SOD1 in cardiac redox state regulation in physiological conditions and cardiovascular disease is not yet entirely elucidated, it seems well conceivable - due to its high cardiac expression levels - that SOD1 is substantially involved in these processes.

Whereas NOS3 is generally attributed beneficial properties for cardiac health, NOS2 has been proposed to be only expressed in pathological cardiac conditions and has been implicated in the development of cardiac hypertrophy and failure [74]. However, NOS2 and NOS3 mRNA expression levels were found to be within the same range in lean control mice. The levels of steady state mRNA expression should, however, not be over-interpreted, as the amount of
redox gene mRNA expression may not necessarily correspond to the amount of protein expression or to the production and bioavailability of free radicals. For instance, small amounts of expressed prooxidant enzymes may already lead to high concentrations of free radicals and therefore necessitate high amounts of antioxidant enzymes.

Interestingly, NAD(P)H oxidase subunit p22phox is expressed in higher levels than subunits Nox2 and Nox4. This may be due to the fact, that p22phox forms the catalytic membrane-associated complex together with Nox2, Nox4 and all other Nox isoforms. Therefore p22phox is needed in much higher amounts than the respective Nox isoforms.

5.2 The Impact of Dietary Fat Intake on Cardiac Steady State Redox Gene mRNA Expression

5.2.1 Obesity and Oxidative Stress

Several studies have shown that obesity enhances markers of oxidative stress in the plasma, serum and urine of obese individuals throughout all age groups [5, 9]. In addition, the concomitant presence of metabolic dysregulation such as diabetes mellitus further increases oxidative stress in obese patients [9]. The reasons for the accumulation of excess free radicals in obesity are not entirely understood, but several possible trigger factors have been identified so far. On the one hand, obesity is associated with a state of systemic low-grade inflammation, which is known to contribute to increased oxidative stress and cardiovascular disease [4, 6, 7]. On the other hand, the production of free radicals in obesity is augmented directly by factors such as hyperglycemia, elevated levels of tissue lipids, excessive angiotensin-II production by the renin-angiotensin-aldosterone system (RAAS) and hyperleptinemia [9]. Moreover, impaired antioxidant defense due to obesity and its associated changes in metabolism may also contribute to the impaired redox balance in obese individuals [9].
Adipose tissue is no longer regarded as a passive lipid storage, but more and more recognized as an active endocrine and paracrine organ with multiple influences on the homeostasis of the human body. Both adipocytes and macrophages – which are contained abundantly in adipose tissue – are able to secrete a variety of cytokines and hormone-like factors, including the cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP), the adipokines leptin and adiponectin and the hormone angiotensinogen, which is subsequently converted to angiotensin-II [4, 7]. Moreover, adipocytes and macrophages communicate through cytokine secretion and create positive feedback loops. For instance, TNF-α stimulates IL-6 production and IL-6 leads to an increased CRP release by the liver [7]. Levels of these protein markers of systemic inflammation are positively correlated with the amount of excess visceral adipose tissue and the severity of the symptoms of the metabolic syndrome [7]. But even more importantly, increased CRP concentration is an independent predictor of cardiovascular risk [7], thus confirming that systemic inflammation is substantially involved in the pathogenesis of cardiovascular disease. Although the effects of low-grade systemic inflammation on the cardiovascular system are not entirely elucidated yet, pro-inflammatory cytokines have been shown to contribute to endothelial dysfunction and subsequent progression of cardiovascular disease [6, 90]. These cytokines play a major role in leukocyte cell migration and activation, enhance the expression of various cell surface molecules on endothelial cells, smooth muscle cells and macrophages and stimulate cell proliferation [6, 90]. The adipokine leptin – which is an important neuroendocrine regulator of food intake – is attributed with pro-inflammatory properties, whereas adiponectin acts as an anti-inflammatory agent [7, 27]. The inflammatory state in obesity enhances leukocyte tissue infiltration and activation along with the production of free radicals, since cytokines are known to up-regulate the activity of redox enzymes, including NAD(P)H oxidase and NOS2.
Moreover, angiotensin-II is an important stimulus for NAD(P)H oxidase activation [51]. Whereas increased oxidative stress in obesity has been demonstrated by the means of determination of markers of oxidative stress in the plasma, serum and urine of obese individuals [5, 9], relatively little is known about the impact of obesity on ROS and RNS production in the heart and only few studies have yet attempted to elucidate the potential underlying mechanisms [92-96]. Although some studies have shown elevated levels of free radicals or markers of oxidative stress in the heart of animal models for obesity and diabetes mellitus [92-96], these studies were very heterogeneous in nature with respect to the selection of animal models and the selection of methods for measuring oxidative stress levels. Due to these differences in study protocols, a direct comparison between results of the existing studies and the present study is difficult and should only be made with caution. More research has yet to be done to completely understand the role and importance of obesity-induced oxidative stress in the emergence of cardiac disease.

5.2.2 Steady State mRNA Expression Levels of Nox2 and Nox4

In the present study, the levels of Nox2 and Nox4 mRNA expression did not differ among the dietary treatments. These findings are in line with a study conducted by Serpillon et al. in 2009 [93]. In this study, the redox state of the heart was examined in Zucker fa/fa rats – a rodent model of type 2 diabetes characterized by genetic obesity, insulin resistance and diabetes mellitus – and lean Zucker rats [93]. The hearts of the animals were removed at 9-11 weeks of age and superoxide (O$_2^-$) levels, hydrogen peroxide (H$_2$O$_2$) levels, protein expression levels of NAD(P)H oxidase subunits Nox2, Nox4, p47phox and p67phox as well as enzymatic activity of SOD and GPx were determined. Interestingly, protein expression levels of NAD(P)H oxidase subunits Nox2, Nox4, p47phox and p67phox showed no
difference between hearts of Zucker fa/fa and lean Zucker rats, whereas the level of $O_2^-$ in the heart of Zucker fa/fa rats was approximately two-fold higher than in lean Zucker rats. In addition, Nox-enzymes and uncoupling of the mitochondrial respiratory chain were identified as major sources of $O_2^-$ in lean Zucker and Zucker fa/fa rat hearts, as treatment with a Nox-enzyme inhibitor and mitochondrial respiratory chain complex inhibitors lowered $O_2^-$ production in both groups. H$_2$O$_2$ levels did not differ between lean Zucker and Zucker fa/fa rats [93]. In summary, these results demonstrate that although $O_2^-$ production by Nox enzymes and uncoupling of the mitochondrial respiratory chain is elevated in the hearts of Zucker fa/fa rats in comparison to lean Zucker rats, protein expression levels of NAD(P)H oxidase subunits are not increased. Augmented cardiac $O_2^-$ production by NAD(P)H oxidase in Zucker fa/fa rats may therefore be caused by up-regulation of NAD(P)H oxidase enzymatic activity, rather than enhancement of NAD(P)H oxidase expression. In the activation process of Nox2 NAD(P)H oxidase, phosphorylation of the cytoplasmic subunit p47phox by protein kinases is a crucial step [97, 98]. Phosphorylation leads to a conformational change of p47phox and subsequent translocation of the cytoplasmic subunit-complex – consisting of p47phox, p67phox, p40phox and Rac – to the cell membrane, where it attaches to the membrane-associated catalytic complex constituted by subunits Nox2 and p22phox [97, 98]. Thus, a functioning, $O_2^-$ producing NAD(P)H oxidase is formed [97, 98]. Serpillon et al. demonstrated, that pharmacological inhibition of different kinases contributing to the phosphorylation of p47phox, led to decreased $O_2^-$ production in the hearts of Zucker fa/fa rats, but not in the hearts of lean Zucker rats [93]. Hence, NAD(P)H oxidase activation by phosphorylation of p47phox may be a major mechanism of increased cardiac oxidative stress in Zucker fa/fa rats. What remains unanswered, however, is which stimulus or stimuli may lead to phosphorylation and subsequent translocation of the p47phox subunit in obese Zucker fa/fa rats. Potential stimuli might include angiotensin-II, TNF-α or direct obesity-related
factors such as hyperglycemia and elevated tissue lipids. For instance, in TNF-α-mediated activation of NAD(P)H oxidase in isolated coronary microvascular endothelial cells from mice, it was demonstrated that the subunit p47phox plays a pivotal role [99]. O$_2^-$ production by NAD(P)H oxidase could be induced by TNF-α in endothelial cells from wild-type mice, but not in in endothelial cells from p47phox$^{-/-}$-mice [99]. Touyz et al. examined the impact of treatment with angiotensin-II on cultured human vascular smooth muscle cells [100]. Within minutes of angiotensin-II stimulation an increased rate of subunit p47phox phosphorylation was found, and p47phox translocation from cytoplasm to the cell membrane was higher upon angiotensin-II stimulation. O$_2^-$ production was increased by the vascular smooth muscle cells and reached maximal activity after 10-15 minutes of Angiotensin-II treatment. Interestingly, when exposed to angiotensin-II for a longer time period of 6-24 hours, protein expression levels of subunits Nox2, p22phox, p47phox and p67phox were elevated in the vascular smooth muscle cells examined [100]. These data propose a two phase effect of angiotensin-II in cultured cells, namely a short-term effect causing NAD(P)H oxidase activation followed by a long-term-effect causing de novo synthesis of NAD(P)H oxidase subunits.

Although the findings of Serpillon et al. and the in vitro findings of Li et al. and Touyz et al. may not necessarily be transferable to other animal models of obesity, let alone the human organism, one might hypothesize that in obesity similar short- and long-term effects on cardiac redox balance by angiotensin-II and potentially other prooxidative agents may occur. In early stages of obesity, O$_2^-$ production by NAD(P)H oxidases may predominantly be increased due to enzymatic activation, while expressional up-regulation of Nox subunits may only emerge in later stages of obesity. This hypothesis would be in line with the fact that no alterations in expression levels of subunit Nox2 and Nox4 mRNA were found in the relatively young animals used in the present study after 15 weeks of high fat dietary treatment.
However, more research has to be done to elucidate the exact mechanism of activation and transcriptional up-regulation of NAD(P)H oxidases by its different stimuli in obesity, before conclusive conclusions can be drawn. Moreover, the role of other sources of ROS in obesity – such as the uncoupling of the mitochondrial respiratory chain – has likewise to be examined in further detail.

Another possibility to consider is that the heart – at least in early stages of obesity – may be less susceptible than other organs to the harmful low-grade inflammatory state and metabolic changes that are associated with the presence of excessive adipose tissue. In fact, elevated Nox2 protein expression in the kidneys and aorta of rats after 28 weeks of dietary treatment with a diet high in fat and refined sugar has been reported [101]. For this study, Roberts et al. used two month old female Fischer 334 rats and assigned the animals to either a control diet low in saturated fat and containing mostly complex carbohydrates or to a high fat / high refined sugar diet, which was high in saturated and monounsaturated fat from lard and to a lesser amount from corn oil and high in sucrose. After dietary treatment for 28 weeks, lipoperoxides were measured in plasma samples to assess the levels of oxidative stress in both animal groups and protein expression levels of Nox2, SOD1, SOD2, GPx and catalase were determined. Levels of lipoperoxides were significantly higher in the serum of animals fed a high fat / high refined sugar diet compared to control animals. In addition, animals fed a high fat / high refined sugar diet showed increased protein expression levels of Nox2 in the kidneys and the aorta and decreased expression levels of SOD1, SOD2 and GPx in the kidneys. Expression levels of SOD1 and GPx were also lowered in the aorta of these animals, whereas no difference in expression levels of catalase was found between the two groups [101]. These findings show that in Fischer 334 rats, a diet high in fat and refined sugar induces up-regulation of Nox2 expression in the vasculature and in the kidney, whereas – as
shown in the present study – a high fat diet does not affect Nox2 expression levels in the heart of C57BL/6 mice. One has to consider that in the study by Roberts et al. dietary treatment was maintained during 28 weeks, versus only 15 weeks in the present study. The duration of a harmful diet may possibly be an important influencing factor in the emergence of oxidative stress. Nevertheless, one might hypothesize that the kidneys and the vasculature are affected earlier than the heart by the adverse effects of obesity and thus oxidative stress in kidney and vascular tissue develops prior to oxidative stress in heart tissue.

In summary, several lines of argumentation may explain the unaltered mRNA expression levels of Nox2 and Nox4 in mice fed a high fat diet. Firstly, 15 weeks of dietary treatment may be too short a period to fully observe the adverse effects of high fat dietary treatment. Secondly, the heart may be less susceptible to the effects of a high fat diet than other organs. If at all, alterations in NAD(P)H oxidase subunit expression might therefore be observed only in later stages of obesity. Thirdly, NAD(P)H oxidase $O_2^-$ production in obesity may mainly be increased by posttranscriptional activation of the enzyme and not by up-regulation of NAD(P)H oxidase subunit expression.

In this context, it is interesting that also in human heart failure augmented NAD(P)H oxidase activity without a concomitant increase in subunit expression levels has been reported [70, 71], thus confirming that activation and transcriptional up-regulation of the enzyme do not necessarily have to be associated in pathological cardiac conditions. The results of these studies are discussed in the following chapter in further detail.

5.2.2.1 NAD(P)H Oxidase Activity and Expression in Cardiac Failure

Nediani et al. examined $O_2^-$ production as well as Nox2 and p47phox protein expression levels in explanted end-stage failing hearts from patients undergoing heart transplantation and in non-failing donor hearts, which were unsuitable for transplantation for technical reasons [71].
In the presence of the substrate NADPH, increased $\text{O}_2^-$ production was found in both right and left ventricles of failing hearts compared to non-failing hearts. Whereas subunit p47phox was overexpressed in failing hearts, no difference in Nox2 protein expression was found between failing and non-failing hearts [71]. Heymes et al. measured $\text{O}_2^-$ production as well as mRNA and protein expression levels of NAD(P)H oxidase subunits Nox2, p22phox, p47phox and p67phox in explanted end-stage failing hearts from patients undergoing cardiac transplantation and non-failing donor hearts, which were unsuitable for transplantation for technical reasons [70]. While the activity of NAD(P)H oxidase was significantly higher in failing hearts compared to non-failing hearts, no differences were found in both mRNA and protein expression levels of NAD(P)H oxidase subunits between failing and non-failing hearts. However, translocation of subunit p47phox to the myocyte membrane was increased in failing hearts [70]. These findings suggest that NAD(P)H oxidase activity but not expression is up-regulated in cardiac failure. However, Li et al. reported increased $\text{O}_2^-$ production accompanied by augmented protein expression levels of Nox2, p22phox, p47phox and p67phox in guinea pigs with left ventricular hypertrophy, which was experimentally induced by constriction of the abdominal aorta [69]. Nevertheless, it is conceivable that in obese individuals increased cardiac NAD(P)H oxidase activity – and thus $\text{O}_2^-$ production – may contribute to obesity-related cardiomyopathy and resulting cardiac failure, without a concomitant increase in NAD(P)H oxidase subunit expression.

5.2.3 Steady State mRNA Expression Levels of p22phox

Surprisingly, in the present study p22phox mRNA expression levels were lowered by 67% by treatment with the VHFD in comparison to the LFD group and by 72% in comparison to the HFD group. The subunit p22phox is essential to the NAD(P)H oxidase in order to produce $\text{O}_2^-$ [50]. Therefore, a decrease in p22phox expression would indicate that the total number of
NAD(P)H oxidase enzymes capable of O$_2^-$ production is diminished. However, as discussed above, NAD(P)H oxidase activity may be up-regulated in obesity, so that total O$_2^-$ production could still be increased. Following this line of argument, one could also hypothesize that p22phox down-regulation may not primarily occur due to increased dietary fat content, but as a compensatory response to increased levels of oxidative stress in obesity. It was beyond the scope of this study, however, to clarify these speculations. As no total amounts of ROS and RNS were measured, it was impossible to draw conclusions about the actual level of oxidative stress in the cardiac tissue of the animals examined. Another explanation for down-regulation of p22phox may lie within the composition of the fat portion of the diets. This will be addressed in further detail in the discussion of the limitations of the study in chapter 5.4.

5.2.4 Steady State mRNA Expression Levels of NOS2 and NOS3

The levels of NOS2 and NOS3 mRNA expression were not altered by dietary treatment. To my knowledge, the only previous study to investigate the effects of obesity on cardiac NOS expression was conducted by Saraiva et al. [96]. This group studied 2-6 month old ob/ob mice – a mouse strain which is leptin deficient due to a genetic defect and therefore shows massive obesity – and age-matched C57BL/6 wild-type mice. In both groups, cardiac mRNA and protein expression levels of NOS1 and NOS3 were determined. In addition, the amount of NO metabolites (nitrites and nitrates) was measured in the cardiac tissue of both groups and the level of oxidative stress was estimated by measurement of the GSH/GSSG ratio. While NOS3 mRNA and protein expression levels were unchanged in ob/ob mice in relation to wild-type mice, both NOS1 mRNA and protein expression levels were decreased by 47% and by 52%, respectively. The amount of NO metabolites and thus NO production was 47% lower in ob/ob mice compared to wild-type mice. Oxidative stress – demonstrated by a decreased GSH/GSSG ratio – was higher in ob/ob mice than in control mice [96]. In line with the
present study, these results show that expression of NOS3 is not affected by obesity. NOS3 is generally attributed a beneficial role in cardiac function, as it has been implicated in the prevention of cardiac hypertrophy and failure [74]. NOS1 has been suggested to have regulatory functions in the control of cardiac contractility [74]. Its reduced expression in obese ob/ob mice [96] may therefore have detrimental effects on cardiac function. NOS2 – whose mRNA expression levels were not affected by dietary treatment in the present study – has been proposed to have deleterious effects on cardiac health, as NOS2-derived NO has been implicated in cardiac hypertrophy and failure [74]. However, high fat dietary treatment seems to have minor impact on mRNA expression levels of both NOS2 and NOS3, irrespective of these enzymes harmful or beneficial effects on cardiac function.

5.2.5 Steady State mRNA Expression Levels of SOD1, SOD3, Atox1 and GPx1

In the present study, decreased SOD1 and Atox1 mRNA expression levels were found in mice fed a VHFD. Expression levels of SOD1 mRNA were reduced by 62% in relation to the LFD group and by 65% in relation to the HFD group. Given the high cardiac mRNA expression levels of SOD1 in relation to other redox enzymes, the observed mRNA reduction due to high dietary fat intake may result in substantial impairment of cardiac antioxidant defense, although contradictory data about the antioxidant and cardioprotective potency of SOD1 has been published [84-86]. Since SOD1 antioxidant activity and the ROS load in the cardiac tissue of HFD- and VHFD-animals were not determined in the present study, no definite conclusions about the impact of dietary treatment on SOD1 function can be drawn from these results. Considering the high expression levels of SOD1 mRNA in cardiac tissue, however, it seems highly probable that a reduction of SOD1 expression by 65% causes a relevant decrease in SOD1 antioxidant activity and in the general antioxidant capacity of cardiac cells.
A high fat diet may thus lead to impaired antioxidant defense by SOD1 and hence contribute to the development and progression of cardiovascular disease.

Atox1 mRNA expression levels were lowered by 73% in comparison to the LFD group and by 66% compared with the HFD group. In contrast, no difference in SOD3 and GPx-1 expression was observed between the groups.

These findings, however, did not correspond to the results of a previous study on the subject. Fujita et al. examined mRNA expression levels of SOD1 and GPx-1 in the heart of a mouse model of obese insulin-resistant diabetes, similar to diabetes type 2 in humans [95]. For this purpose, 11 weeks old KKAy mice were used, which are characterized by genetic obesity, hyperinsulinemia and hyperglycemia [102]. Animals were only included in the study, if fasting blood glucose was over 8.3 mmol/l and age-matched non-diabetic C57BL/6J mice were used as control animals. GPx-1 mRNA expression levels were found to be elevated in KKAy mice in relation to the control mice, while no significant differences in SOD1 mRNA expression levels were detected [95]. Whereas this study shows transcriptional up-regulation of GPx-1 in a state of obesity and hyperglycemia, the data of the present study suggests that in response to a high fat diet mRNA expression levels of antioxidant enzymes remain unchanged or – as in the case of SOD1 – are even reduced. As the mRNA expression levels of Atox1 – the copper chaperone for SOD3 – were decreased in the VHFD group, one might even speculate that the function of SOD3 may be impaired as well, in spite of its steady expression levels throughout all groups. Furthermore – as Atox1 is known to stimulate SOD3 expression [60] – low levels of Atox1 may result in transcriptional SOD3 down-regulation in the course of time. It is conceivable, however, that the disparate findings of the study by Fujita et al. and the present study may originate from the differences between the type of animal models used, as the implications of diet-induced obesity may differ remarkably from the effects of genetic
Discussion

Obesity. However, obesity may not only lead to changes in expression levels of SOD1, SOD3, Atox1 and GPx-1, but also to alterations in enzymatic activity. Both Serpillon et al. and Vincent et al. investigated antioxidant activity in the hearts of animal models of obesity [92, 93]. In the study discussed previously, Serpillon et al. examined the antioxidant activity of SOD and GPx in the hearts of genetically obese Zucker fa/fa rats, compared to lean Zucker rats [93]. Interestingly, both enzymes showed lower cardiac activity levels in obese Zucker fa/fa rats [93]. Vincent et al. compared the enzymatic activity of SOD1, SOD2 and GPx in the left ventricle of 12 months old obese male Zucker fa/fa rats compared to lean Zucker rats. Total SOD activity and activity of GPx did not differ between the hearts of lean and obese Zucker rats. However, when examined individually, SOD2 activity was higher in the hearts of obese rats, whereas the cardiac activity of SOD1 did not differ between lean and obese rats [92].

In summary, there is only little evidence in the literature that obesity may lead to up-regulation of SOD and GPx expression and activity in the heart. It is more probable, however, that obesity does not affect levels of SOD and GPx expression and activity or may even lead to down-regulation of the antioxidant defense performed by these enzymes, thus contributing to the formation of a prooxidant state in cardiac cells. The accumulating free radicals in the hearts of obese individuals may hence remain unscavenged and promote cardiac disease. Inadequate antioxidant defense may therefore play an important role in the emergence of oxidative stress and subsequent cardiovascular disease in obesity.
5.3 The Impact of Nox2-Deficiency on Cardiac Steady State Redox Gene mRNA Expression

5.3.1 Nox2 in Cardiovascular Health and Disease

NAD(P)H oxidase is one of the most important sources of ROS in the human body and is attributed an important role in cardiovascular health and disease [29]. But NAD(P)H oxidase is also a regulator of oxidative stress, as NAD(P)H oxidase-derived ROS interact with other redox enzymes. Superoxide originating from NAD(P)H oxidase, may for instance interact with NO produced by NOS3, which results in the production of highly reactive ONOO⁻, able to directly damage proteins, lipids and DNA [44]. The generated ONOO⁻ may, however, also oxidize the essential NOS3 cofactor tetrahydrobiopterin. Thereby, the reaction step of O₂ reduction by NOS3 is uncoupled from the formation of NO and NOS3 starts to directly produce O₂⁻ [55]. Thus, NOS3 loses its ability to produce NO and contributes to the harmful accumulation of O₂⁻.

In order to better understand the regulating effects of Nox2-containing NAD(P)H oxidase on redox homeostasis, in the present study mRNA expression levels of enzymes involved in cardiac redox balance were examined in C57BL/6 Nox2-knockout mice. Previous studies with Nox2-knockout mice have demonstrated inhibited neovascularization in response to treatment with vascular endothelial growth factor and in response to hindlimb ischemia in Nox2-deficient mice, compared to wild-type mice [103, 104]. Hence, a critical role for Nox2-derived ROS in angiogenesis has been suggested [103, 104]. Furthermore, Nox2-deficiency prevented angiotensin-II-induced cardiac hypertrophy in mice, but did not affect development of cardiac hypertrophy in response to constriction of the aorta [72, 73]. A role of Nox2 in the mediation of the adverse effects of angiotensin-II on the heart therefore appears probable.
5.3.2 Steady State mRNA Expression Levels of Nox4, p22phox, NOS2, NOS3, SOD1, SOD3, Atox1 and GPx-1

Whereas the mRNA expression levels of Nox4 did not significantly differ between wild-type and Nox2-deficient mice, mRNA expression levels of p22phox were decreased by 54% in Nox2-deficient mice fed a high fat diet (HFD-N), compared to Nox2-deficient mice fed a low fat control diet (LFD-N). The difference to wild-type mice fed a low fat control diet (LFD) did not reach statistical significance. Thus, in Nox2-deficient mice, a diet with 41% of total kcal from fat (HFD) did already reduce p22phox mRNA expression levels, whereas in wild-type mice only a diet with 58% of total kcal from fat (VHFD) led to decreased expression levels of p22phox mRNA. Nox2-deficiency may therefore augment the effects of a high fat diet on the expression of NAD(P)H oxidase subunit p22phox. It remains unclear, however, why this effect is limited to the subunit p22phox and what underlying mechanisms might contribute to a higher susceptibility of Nox2-deficient animals to a high fat diet.

Neither for NOS2 nor for NOS3 a significant difference in mRNA expression levels was detected among the wild-type groups and the Nox2-deficient mice groups. In addition, SOD1 and GPx-1 mRNA was expressed without significant differences in wild-type and Nox2-deficient mice. SOD3 mRNA expression was reduced by 40% in Nox2-deficient mice fed a high fat diet (HFD-N) compared to wild-type mice fed a high fat diet (HFD). This finding suggests that Nox2-deficiency may adversely affect antioxidant defense by SOD3.

However, since the effects of Nox2-deficiency are limited to the mRNA expression levels of SOD3 and p22phox and very moderate in quantity, it can be concluded that in the conditions examined, absence of Nox2 has no major regulatory effect on the mRNA expression levels of the genes investigated in the present study.
5.4 Limitations of the Study

This study had some limitations worth mentioning. Firstly, group sizes were relatively small to reach a statistically significant conclusion. By examination of larger groups of animals and further investigation of cardiac gene expression in obesity, more precise conclusions could be drawn about the connections between obesity and cardiac oxidative stress. Secondly, as previously mentioned, 15 weeks of high fat dietary treatment may be a too short of a period for the increase in oxidative stress to fully develop. Little is known about the duration of the pathogenesis process of increased oxidative stress, up- or down-regulation of redox enzymes and consequent development of cardiac disease. However, considering the long lifespan of humans compared to that of animal models such as obese C57BL/6 mice, a long duration pathogenesis of increased oxidative stress and related heart disease is very well conceivable and changes in oxidative stress levels might not necessarily be detectable after 15 weeks already. The fact that the duration of obesity is a major risk factor for development of heart failure [25] indicates that the harmful effects on the heart grow with increasing duration of obesity. To entirely understand the implications of high dietary fat intake and the presence of excessive adipose tissue for the development of cardiac disease, expensive and time-consuming long-term studies would be necessary. Thirdly, in this experiment only steady state mRNA expression levels of redox genes were determined. However, determination of mRNA expression levels alone only provide very limited information about the actual redox state of the heart. Both the fractions of inactivated and activated enzymes as well as the total ROS and RNS load remain unknown. According to the data in the literature, rather than regulating gene transcription, obesity and / or Nox2-deficiency could have an impact on activation, activity or deactivation of redox enzymes by posttranscriptional regulation and thereby lead to changes in ROS and RNS production. In order to examine changes in the
activity of redox enzymes due to dietary influences and to fully assess the level of oxidative stress in cardiac tissue of obese animals, direct measurements of the actual ROS and RNS load would be necessary. Fourthly, in contrast to the fat fraction, carbohydrate and protein fractions of the diets used in this study were not standardized and differed in relative amount and composition. Moreover, and possibly more importantly, the fat portion consisted of fat from different sources. The high fat diet was mainly based on animal-fat (milk fat and corn oil), whereas the very high fat diet was based on plant-fat entirely (coconut and soybean oil). Therefore, observed differences in mRNA expression levels may not only be due to the different amount of fat content but also caused by the different constitution of the dietary fat portion. Varying fractions of different types of fatty acids as well as varying amounts of phenolic phytochemicals may have disparate effects on cardiac redox gene expression. For instance, polyunsaturated fatty acids have been attributed with anti-inflammatory and anti-atherogenic properties and are known to lower cardiovascular risk when consumed in appropriate amount [105]. On the other hand saturated fatty acids and trans fatty acids are known to increase cardiovascular risk [106]. However, beneficial effects on cardiovascular health have been reported for medium-chain fatty acids, a group of saturated fatty acids that are for example contained in coconut oil [107]. As the very high fat diet (VHFD) used in the present study contained large amounts of coconut oil, it is conceivable that the abundance of medium-chain fatty acids in the diet affected redox balance in the hearts of the animals examined. In terms of contribution to cardiovascular disease, dietary fat quality – or in other words fatty acid composition – may be more important than dietary fat quantity [106].

Coconut and soybean oil – which are both contained in the VHFD of the present study – are known to contain phenolic phytochemicals [108, 109]. Phenolic phytochemicals are chemical compounds contained in plants, which are characterized by their antioxidant activity [110]
and therefore may have a positive effect on diseases caused by increased oxidative stress [108]. Thus, the beneficial antioxidant effects of coconut and soybean oil may outweigh the negative effects of a high caloric diet with a large fat portion and hence lead to a decrease in oxidative stress. According to this hypothesis, the lower expression levels of p22phox mRNA in the VHFD-group in the present study, may also be caused by the beneficial effects of phenolic phytochemicals. Décordé et al. examined the impact of phenol substitution in the form of grape seed extract on superoxide production and protein expression of p22phox in the left cardiac ventricle of Syrian golden hamsters fed a high fat diet in order to evoke obesity [94]. Compared to a control group, which was fed a high fat diet without any additional treatment, superoxide production was decreased by 74% and protein expression of p22phox was reduced by 30% in animals supplemented with phenolic phytochemicals [94]. The fat portion of the high fat diet (HFD) used in the present study contained both milk fat and – although in 20-fold smaller concentration – corn oil. Like coconut and soybean oil, corn oil contains phenolic phytochemicals and therefore possesses antioxidant activity [111]. Therefore, changes in expression levels in the groups fed a high fat diet may also partially be affected by the antioxidant activity of corn oil. Of course, this argumentation remains speculative and many further questions arise. On the one hand, it remains unclear why the supposedly beneficial effects were limited to p22phox expression and could not be demonstrated for the other NAD(P)H oxidase subunits. On the other hand, the high fat diet (HFD) lowered p22phox expression only in Nox2-deficient but not in wild-type mice, which would then indicate a heightened sensitivity for the beneficial effects of phytochemicals in the absence of Nox2. In spite of this ambiguity, these findings may suggest a possible role of alimentary phenolic phytochemicals in the redox balance of cardiac cells. To further examine the individual impact of dietary fat content and dietary fat composition on cardiac redox gene expression, studies employing strictly standardized experimental diets would be needed.
5.5 Clinical Implications

Obesity is associated with a state of systemic low-grade inflammation and increased oxidative stress and both of these pathological conditions are implicated in the development of cardiovascular disease [4-9]. Therefore, several prophylactic measures and therapeutic interventions have been proposed to prevent the adverse effects of increased oxidative stress on cardiovascular health in obesity. The possibly most important measure would be to lower the prevalence of obesity by dietary and lifestyle recommendations. But also in existing obesity, adaptation of diet, increased physical exercise and subsequent weight loss may lead to a decrease of biomarkers of oxidative stress in the blood [112]. The American Heart Association Diet and Lifestyle Recommendations for Cardiovascular Risk Reduction currently suggest a diet rich in vegetables and fruits, wholegrain, fiber and fish – which contains a high amount of polyunsaturated fatty acids – and limitation of saturated fat intake to <7% and trans fat intake to <1% of total energy intake. In addition, regular physical exercise, moderate alcohol consumption and avoidance of tobacco products is recommended [113]. Therefore, in prevention and therapy of obesity, a healthy lifestyle should always be the foundation for any additional intervention. As promising results of dietary and lifestyle interventions in the prevention of cardiovascular disease are, poor patient adherence to diet and lifestyle adaptations constitutes a major problem of this therapeutic approach [114]. Extensive instruction by health care professionals, the setting of clearly defined and attainable goals, social support and regular contact with the medical attendant may help patients to maintain healthy diet and lifestyle [114]. However, all these measures are very expensive and time-consuming and therefore rarely ever applied to an adequate extent. Therefore, drugs or food supplements counteracting the emergence of oxidative stress in obesity and metabolic
syndrome would be a desirable addition to the therapy options for these conditions. In the following section, current approaches in this field are discussed.

Both antioxidant dietary supplements and antioxidant foods have been proposed in the treatment of increased oxidative stress. The best studied antioxidant vitamins for this purpose are vitamin E ($\alpha$-tocopherol), vitamin C (ascorbic acid) and $\beta$-carotene. These vitamins have antioxidant activity \textit{in vitro} and \textit{in vivo} [46] and observational studies showed a positive correlation between intake or tissue levels of antioxidant vitamins and low risk of cardiovascular disease [115]. The positive results from these observational studies led to randomized trials, which, however, could not confirm the beneficial role of antioxidant vitamins in the prevention of cardiovascular disease. Vivekananthan et al. showed in a meta-analysis of fifteen randomized trials that neither $\alpha$-tocopherol nor $\beta$-carotene have beneficial effects on all-cause mortality and the risk of cardiovascular death [116]. In fact, patients with supplementation of $\beta$-carotene even had a slightly increased all-cause and cardiovascular mortality, suggesting a potential harmful effect of $\beta$-carotene substitution [116]. Similarly, positive effects of ascorbic acid were only reported sporadically in clinical trials [117]. Considering these results, supplementation of vitamins cannot be recommended at present time for the prevention of cardiovascular disease. The questions remain, however, why results of observational studies indicate a positive effect of increased vitamin intake and why the demonstrated antioxidant effects of vitamin \textit{in vitro} do not seem to have an equally positive effect on cardiovascular risk in clinical trials. The answer to the first question may lie within the design of the observational studies examining the correlation between vitamin intake and cardiovascular risk. Probands in this study were divided into different groups according to their serum vitamin concentration or daily intake of vitamins contained either in natural dietary ingredients or in vitamin supplementation [116]. But additional factors such as
composition of their habitual diet, frequency and intensity of physical exercise, nicotine abuse and possibly existing concomitant diseases were not taken into account [116]. Therefore, with utmost probability these studies are confounded by additional characteristics of the different groups examined. For example, a proband who has a high dietary vitamin intake or consumes a vitamin supplement on a daily basis, may also set value on a healthy diet altogether and therefore consume higher doses of vegetables, fibers, polyunsaturated fatty acids and phenolic phytochemicals – all of which may contribute independently to a lower cardiovascular risk [113]. In addition, it is very likely that these probands also maintain a healthier lifestyle – with regular exercise and avoidance of tobacco products – than control probands. Thus, the group of probands with high vitamin consumption may have a lower risk for cardiovascular disease, independent of the actual influence of vitamin intake. The second question – as why observations of vitamin antioxidant activity in vitro and in animal models are not necessarily transferable to clinical medicine – is more difficult to answer satisfyingly. On the one hand molecular mechanisms involved in maintaining redox homeostasis and scavenging free radicals in the human body are still incompletely understood, on the other hand clinical trial studies examining antioxidant vitamins only examined effects on cardiovascular events or mortality and did not assess the actual effect on oxidative stress levels. In fact, neither antioxidant status or oxidative stress was evaluated before or after treatment in these studies [118]. Therefore, it has been proposed that the level of oxidative stress may vary between individuals with the same clinical symptoms and that antioxidant status may be an important factor to identify patients who could benefit from a antioxidant therapy [118]. Future vitamin trial studies might possibly produce more successful results, if participating patients were selected and assessed according to their actual antioxidant status before and after treatment. Although these considerations are entirely hypothetical in nature today, adapted study designs are necessary to clarify the relations between obesity and metabolic syndrome, levels of
oxidative stress and the effect of antioxidant vitamin treatment. At present time, however, supplementation of vitamins in order to prevent cardiovascular disease in asymptomatic or obese patients cannot be recommended. The American Heart Association currently suggests to consume food sources of antioxidant agents such as fruit, vegetables, whole grains and vegetable oils but does not recommend vitamin supplementation [113, 117].

Another antioxidative nutrition supplement is selenium, which acts as a cofactor of GPx [46]. Although selenium supplementation increases antioxidant activity, studies examining its beneficial impact on cardiovascular health remain inconclusive [119]. Therefore - without additional investigation - selenium supplementation cannot be recommended either.

Medicamentous treatment is a further therapeutic option to decrease oxidative stress in obesity. Klein et al. reported inhibition of TNF-α-induced – and ROS-mediated – cardiomyocyte apoptosis by metallothionein in cultured neonatal cardiomyocytes [120]. Metallothionein is a thiol-rich protein with antioxidant properties that – according to Klein et al. – could pharmacologically be up-regulated in the heart tissue [120]. However, these promising experimental results may not be easily transferable to clinical medicine, as experience with vitamins with antioxidant properties has shown. Nevertheless, up-regulation of antioxidant agents directly in the cardiac tissue may be a more efficient approach than mere oral antioxidant supplementation.

But also established drugs are employed to reduce oxidative stress and subsequent disease. Interruption of the renin-angiotensin-aldosterone system (RAAS) may be a promising approach in order to reach this goal. Inhibition of the RAAS can be achieved with angiotensin-converting-enzyme-inhibitors (ACEI) or angiotensin-receptor blocking drugs. ACEI inhibit angiotensin-converting-enzyme and thereby the conversion of angiotensin-I to angiotensin-II. Currently, ACEI are already widely used in the therapy of hypertension and
chronic cardiac failure and have been shown to reduce cardiovascular events such as myocardial infarction or stroke and to reduce cardiovascular mortality [121]. Ceconi et al. reported that treatment with the ACEI perindopril decreased plasma levels of angiotensin-II and TNF-α in humans [122]. Moreover, SOD3 activity was increased in cultivated human umbilical vein epithelial cells after incubation with serum from patients treated with perindopril [122]. Inhibition of the RAAS may therefore be an important step in order to lower systemic inflammation, maintain redox balance and consequently prevent cardiovascular disease. Given that angiotensin-II stimulates NAD(P)H oxidase activity [51], decreased NAD(P)H oxidase O$_2^-$ production may also be achieved by RAAS inhibition. NAD(P)H oxidase activity is also lowered by statins, a group of drugs which inhibit cholesterol synthesis [123]. In addition, it was demonstrated by Bagi et al. that rosiglitazone – a drug applied in the treatment of diabetes mellitus – reduced O$_2^-$ production by NAD(P)H oxidase and increased catalase activity in the aorta of db/db mice – a mouse strain which shows massive obesity due to a genetic leptin-receptor deficiency [32] – while not affecting SOD activity [124].

As our present knowledge about the effectivity of different approaches in the prevention and therapy of obesity-induced oxidative stress and cardiovascular disease is still very limited, further research is needed to identify the most efficient methods. Until then, emphasis should be placed on a balanced diet and regular exercise in order to lower the incidence of cardiovascular disease in the obese population and – possibly most importantly – to prevent the development of obesity in children and adults. In addition, commonly used drugs in the treatment of the metabolic syndrome like ACEI, statins and antidiabetic drugs may have an anti-inflammatory and antioxidative component and should therefore be continued to be applied in these patients. However, the development of new drugs targeting the redox system
5 Discussion

specifically is desirable and might be an important step in the prevention and therapy of obesity-related cardiovascular disease.

5.6 Conclusion

High dietary fat intake and obesity are associated with various changes in body homeostasis, which eventually lead to the development of cardiovascular disease [17, 18] Systemic low-grade inflammation and increased oxidative stress are known to play a major role in the emergence of obesity sequelae, although the underlying mechanism are not yet fully understood [4-9]. Both the pathways of up-regulation of oxidative stress in obesity and the mechanisms of how free radicals exactly contribute to the progression of cardiac and cardiovascular disease need to be elucidated by future research, to broaden our understanding of the correlations between obesity and cardiovascular disease. The present study has shown, that in mice a diet with a very high fat content (VHFD) had little influence on mRNA expression levels of most pro- and antioxidant enzymes investigated. Only cardiac mRNA expression levels of SOD1, Atox1 and p22phox were reduced, whereas expression levels of Nox2, Nox4, NOS2, NOS3, SOD3 and GPx-1 remained unchanged. However, according to these results, high dietary fat intake may lower SOD1 expression levels. As SOD1 was shown to be the most abundantly expressed antioxidant enzyme in cardiac tissue, a reduction of its expression level may result in significant impairment of antioxidant defense and thus contribute to a state of oxidative stress in the heart. Obesity may also lead to up-regulation of cardiac prooxidant enzymes by enzymatic activation, rather than increased expression. Since increased cardiac oxidative stress and \( \text{O}_2^- \) production in obesity has been reported in different studies [92-96], it is conceivable that free radicals play a role in the connection between obesity and cardiac disease, even though transcriptional up-regulation of prooxidant enzymes may not be necessarily involved in the emergence of obesity-related cardiac disease.
Nox2-deficiency showed to be of little importance for steady state mRNA expression levels of redox enzymes in the heart. Only expression levels of p22phox were decreased in Nox2-deficient mice fed a high fat diet (HFD-N) compared to Nox2-deficient mice fed a low fat control diet (LFD-N) and SOD3 mRNA expression was decreased in Nox2-deficient mice fed a high fat diet (HFD-N) compared to wild-type mice fed a high fat diet (HFD). It can therefore be concluded that the presence of the NAD(P)H oxidase subunit Nox2 is of minor importance for the transcriptional regulation of the cardiac redox enzymes investigated.

Different therapeutic approaches have been proposed in order to lower levels of oxidative stress in obesity and thus attenuate the development of cardiovascular disease, including the inhibition of the RAAS and supplementation of antioxidant substances. However, these therapeutic interventions only partially show the beneficial effect desired, so that prevention of obesity and adaptation of nutritional and lifestyle factors in obese patients should be paramount in order to diminish obesity-related cardiovascular disease. As the worldwide prevalence of obesity is increasing in both developed and developing countries [1-3], obesity-related diseases will remain to be a major global health problem in the next decades. Therefore, the molecular connections of obesity and oxidative stress, its implications for cardiovascular disease and potential therapeutic interventions will remain an important area for future investigation in the coming years.
6 References


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8 Appendix

8.1 Composition of the Diets

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<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>175</td>
<td>700</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>25</td>
<td>225</td>
</tr>
<tr>
<td>Coconut Oil, Hydrogenated</td>
<td>333.5</td>
<td>3001.5</td>
</tr>
<tr>
<td>Mineral Mix S10001</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>10.5</td>
<td>0</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Potassium Citrate, 1 H20</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin Mix V10001</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>FD&amp;C Red Dye #40</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1000.1</strong></td>
<td><strong>5558.5</strong></td>
</tr>
</tbody>
</table>
8.2 Metabolic Parameters

Metabolic Parameters were assessed and published by Mundy et al. in 2007 [83]. After 15 weeks of dietary treatment, metabolic examinations were conducted prior to organ removal. Following an overnight fasting period of 14 hours, the body weight of the mice was determined and baseline glucose (0 minutes) was measured. In addition to that, glucose tolerance was tested by intraperitoneal injection of D-glucose 2mg/g body weight and subsequent glucose measurements in the venous blood at 5, 10, 15, 30, 45, 60, 90 and 120 minutes. For all measurements, venous blood was obtained from the tail vein of the animals. Glucose levels were determined using an AccuChek Advantage glucose meter (Roche Diagnostics; Rotkreuz, Switzerland).

After 15 weeks of dietary treatment mice fed a low fat control diet (LFD) had gained 11±1g of body weight, mice fed a high fat diet (HFD) had gained 17±1g and mice fed a very high fat diet (VHFD) had gained 21±1g of body weight (P<0.004 LFD vs. HFD, P<0.001 LFD vs. VHFD, P<0.04 HFD vs. VHFD) [83].

While the amount of fat intake and weight gain did not affect baseline fasting glucose levels (in mmol/l, LFD=5.5±0.2, HFD=5.2±0.4, VHFD=5.9±0.3), a significant impairment of glucose tolerance could be observed in both the high fat diet (HFD) and the very high fat diet (VHFD) groups (P<0.04 vs. LFD control group) [83].
8.3 Reverse Transcription

<table>
<thead>
<tr>
<th>Reverse Transcription Mastermix:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µl Quantiscript Reverse Transcriptase&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Qiagen; Hilden, Germany</td>
</tr>
<tr>
<td></td>
<td>Containing RNase inhibitor</td>
</tr>
<tr>
<td>4 µl Quantiscript RT Buffer&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Qiagen; Hilden, Germany</td>
</tr>
<tr>
<td></td>
<td>Containing dNTPs</td>
</tr>
<tr>
<td>1 µl RT Primer Mix&lt;sup&gt;®&lt;/sup&gt;</td>
<td>Qiagen; Hilden, Germany</td>
</tr>
<tr>
<td></td>
<td>Containing oligodeoxythymidylic acid primer and random primers</td>
</tr>
</tbody>
</table>
8.4 Polymerase Chain Reaction

The following cDNA primers (Microsynth AG; Balgach, Switzerland) were used in the polymerase chain reaction experiments:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Basepairs</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse TYRO3</td>
<td>forward 5'-CGA GCA GGC AGA GCG ATA TG-3'</td>
<td>149</td>
<td>347</td>
</tr>
<tr>
<td>(NM_011740)</td>
<td>reverse 5'-AGA CGA CCC TCC ACG ATG AC-3'</td>
<td>476</td>
<td></td>
</tr>
<tr>
<td>Mouse Nox2</td>
<td>forward 5'-AAC TCC TTG GGT CAG CAC TG-3'</td>
<td>130</td>
<td>131</td>
</tr>
<tr>
<td>(MMU43384)</td>
<td>reverse 5'-GAG CAA CAC GCA CTG GAA-3'</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>Mouse Nox4</td>
<td>forward 5'-GTG AAG ATT TGC CTG GAA GAA C-3'</td>
<td>148</td>
<td>851</td>
</tr>
<tr>
<td>(NM_015760)</td>
<td>reverse 5'-TGA TGA CTG AGA TGA TGG TGA C-3'</td>
<td>998</td>
<td></td>
</tr>
<tr>
<td>Mouse p22phox</td>
<td>forward 5'-GTG GAC TCC CAT TGA GCC TA-3'</td>
<td>130</td>
<td>451</td>
</tr>
<tr>
<td>(NM_007806)</td>
<td>reverse 5'-CTC CTC TTC ACC CTC ACT CG-3'</td>
<td>580</td>
<td></td>
</tr>
<tr>
<td>Mouse NOS2</td>
<td>forward 5'-CAC CTT GGA AGA GGA GCA AC-3'</td>
<td>145</td>
<td>1900</td>
</tr>
<tr>
<td>(NM_010927)</td>
<td>reverse 5'-AAG GCC AAA CAC AGC ATA CC-3'</td>
<td>2044</td>
<td></td>
</tr>
<tr>
<td>Mouse NOS3</td>
<td>forward 5'-GGA GAG GAG CAA GGG TGA AC-3'</td>
<td>148</td>
<td>1397</td>
</tr>
<tr>
<td>(NM_008713)</td>
<td>reverse 5'-GGT GGG TTG TCT GCT AGA GG-3'</td>
<td>1544</td>
<td></td>
</tr>
<tr>
<td>Mouse SOD1</td>
<td>forward 5'-TGG GTT CCA CGT CCA TCA GTA-3'</td>
<td>151</td>
<td>136</td>
</tr>
<tr>
<td>(NM_05725)</td>
<td>reverse 5'-ACC GTC CTT TCC AGC AGT CA-3'</td>
<td>286</td>
<td></td>
</tr>
<tr>
<td>Mouse SOD3</td>
<td>forward 5'-TTG TTC TAC GGC TTG CTA CTG-3'</td>
<td>141</td>
<td>282</td>
</tr>
<tr>
<td>(NM_011435)</td>
<td>reverse 5'-CGT GTC GCC TAT CTT CTC AAC-3'</td>
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<td></td>
</tr>
<tr>
<td>Mouse Atox1</td>
<td>forward 5'-GCT CTT CTC CAC AAT GCT AAC C-3'</td>
<td>135</td>
<td>299</td>
</tr>
<tr>
<td>(NM_009726)</td>
<td>reverse 5'-CTT AAC ACC AGT CAC ACC CTT G-3'</td>
<td>433</td>
<td></td>
</tr>
<tr>
<td>Mouse GPx-1</td>
<td>forward 5'-TCA GTT CGG ACA CCA GGA GAA-3'</td>
<td>124</td>
<td>269</td>
</tr>
<tr>
<td>(NM_008160)</td>
<td>reverse 5'-CTC ACC ATT CAC TTC GCA CTT C-3'</td>
<td>392</td>
<td></td>
</tr>
</tbody>
</table>

**PCR Mastermix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5 µl SYBR-Green master mix®</td>
<td>Qiagen; Hilden, Germany</td>
<td>Containing HotStarTaq DNA Polymerase®, QuantiTect SYBR Green PCR Buffer®, dNTP mix, SYBR Green I dye, ROX dye</td>
</tr>
<tr>
<td>9.5 µl RNase free water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µl Primer forward</td>
<td>Microsynth AG; Balgach, Switzerland</td>
<td></td>
</tr>
<tr>
<td>0.5 µl Primer reverse</td>
<td>Microsynth AG; Balgach, Switzerland</td>
<td></td>
</tr>
<tr>
<td>2 µl template cDNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
9 Curriculum Vitae

Hannes Samuel Rich von Bassersdorf ZH / Neuhausen am Rheinfall SH

14.12.1983 Geboren in Zürich
1990-1996 Primarschule in Bassersdorf ZH
1996-1998 Sekundarschule in Bassersdorf ZH
1998-2002 Gymnasium Unterstrass, Zürich (Matura Typ M)
2003-2009 Medizinstudium an der Universität Zürich
10 / 2009 Staatsexamen an der Universität Zürich