RELEVANCE OF VASCULAR NADPH OXIDASES IN THE FRENCH PARADOX

Inaugural-Dissertation
zur Erlangung des Grades eines Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von
Anna Bertram
aus Limburg a.d. Lahn

Gießen 2008

Aus dem Rudolf-Buchheim-Institut für Pharmakologie
des Fachbereichs Medizin der Justus-Liebig-Universität Gießen
Gf. Direktor: Prof. Dr. Michael Kracht

Gutachter: Prof. Dr. H. Schmidt

Gutachter: Prof. Dr. N. Weissmann

Tag der Disputation: 06.02.2009

Table of Contents

1 INTRODUCTION 1

1.1 Overview 1

1.2 Clinical Implications of Reactive Oxygen Species 2

1.3 French Paradox 3

1.4 The Family of NADPH Oxidases 11

2 AIM OF THE THESIS 16

3 MATERIALS AND METHODS 18

3.1 Ethical Approvals 18

3.2 Materials 18

3.3 Cell Culture 22

3.4 Animals 23

3.5 Experimental Design of the *in vivo* Study 23

3.6 Feeding Protocol 24

3.7 Organ Preparation 25

3.8 Protein Analysis 26

3.9 Detection of Superoxide and Nox Activity 31

3.10 Isometric Tension Measurements 31

3.11 Statistical Analysis 33

4 RESULTS 37

4.1 Effects of Purple Grape Products *in vitro* 37

4.2 Effects of Purple Grape Products in Living Animals 41

5 DISCUSSION 48

5.1 The *in vitro* Role of NADPH Oxidases in the French Paradox 48

5.2 Discrepancy between *in vitro* and *in vivo* Findings 51

5.3 Organ Bath Studies on Aortic Vasofunction 55

5.4 Relevance of NADPH Oxidases in the French Paradox 59

6 SUMMARY 63

7 ZUSAMMENFASSUNG 65

8 REFERENCES 67

9 ABBREVIATIONS 81

10 DANKSAGUNG 83

11 CURRICULUM VITAE 84

ERKLÄRUNG 85

1 INTRODUCTION

1.1 Overview

Cardiovascular diseases are a major source of mortality in the industrialized world. In the years 2004 to 2006, 41% of the cases of death of the 65- to 84-years-olds in the European Union were caused by cardio- or cerebrovascular events. Among these, the most frequent disease was ischemia of the heart with 17% (Niederlaender, 2006). There have been many attempts to reduce cardiovascular morbidity and mortality, either by primary prophylaxis (minimizing risk factors), or by treatment of already diseased patients. Primary prophylaxis, however, is the more reasonable way from an economic point of view, too. Acute cardiovascular events like myocardial infarction or stroke frequently result from atherosclerosis (Ross, 1999). Thus, prevention of atherosclerosis is one of the first steps in primary prophylaxis of cardiovascular morbidity. Apart from age, obesity, cigarette smoking, hypertension and diabetes mellitus, high serum low density lipoprotein-cholesterol (LDL-C) has been identified as a main risk factor for atherosclerosis (Ames et al., 1993; Ross, 1999; Law and Wald, 2002; Singh and Jialal, 2006). High serum LDL-C results mostly from dietary fat intake and is positively correlated to cardiovascular mortality (Schaefer, 2002).

Interestingly, there is a lower cardiovascular mortality in Southern Europe and especially France than in many other developed countries like Germany and Great Britain, despite the fact of a similar high consumption of dietary fat and serum cholesterol in all these populations. This paradoxical situation (the so-called “French paradox”) was attributed to the high consumption of red wine in France (Renaud and de Lorgeril, 1992). Various studies on the impact of red wine on different cardiovascular parameters have shown that beneficial red wine effects could not be attributed to its alcoholic content, but rather to its polyphenolic components (e.g. Fitzpatrick et al., 1993; Wollny et al., 1999). Some of the examined parameters that were improved by polyphenolic agents are linked to the oxidative homeostasis of the body. Oxidative stress seems to be crucial in the pathogenesis of hypertension (Lassègue and Clempus, 2003) and atherosclerosis (Singh and Jialal, 2006), which often underlie cardiovascular diseases. It was proposed that a modulation of the oxidative state is responsible for the beneficial effect of polyphenolic agents present

in red wine and other beverages (Freedman et al., 2001; Al-Awwadi et al., 2004). NADPH oxidases, xanthine oxidase and uncoupled endothelial nitric oxide synthase (eNOS) are contributors to the generation of reactive oxygen species (ROS) causing oxidative stress in vasculature. As a consequence, changes in the expression or activity of these enzymes have immense effects on vascular homeostasis. In the last years, especially the role of NADPH oxidases in cardiovascular diseases has been highlighted. For this reason, NADPH oxidases are also relevant targets of investigations on the French paradox. The aim of this study was to evaluate the effects of polyphenolic beverages like red wine and purple grape juice on the expression and activity of NADPH oxidases in the aorta of the rat.

1.2 Clinical Implications of Reactive Oxygen Species

Hypertension is an important risk factor for the development and progression of atherosclerosis, which can result in acute cardiovascular events like ischemia of the heart, brain or extremities and succeeding infarction (Ross, 1999). Oxidative stress due to excessive ROS production leads to nitric oxide (NO) breakdown, which results in reduced vasorelaxation and, therefore, hypertension. ROS directly influence the vascular contractility, too. Furthermore, hypertensive damage of vessels is a consequence of exceeding ROS formation (Touyz, 2004). The destructive processes involved in hypertensive vascular damage include deposition of oxidized LDL-C and extracellular matrix in the vessel wall, proliferation of vascular smooth muscle cells, and monocyte invasion across the endothelial barrier. Finally, atheromata develop as a sign of atherosclerosis. Clinical manifestations like myocardial infarctions originate from sudden disruption of these plaques, which can be induced by mechanical forces of the blood flow with hypertensive blood pressure (Stokes et al., 2001).

As described above, oxidative stress seems to be crucial in the development of cardiovascular diseases based on hypertension. Nevertheless, clinical trials on a possible beneficial effect of dietary antioxidants (vitamin E, vitamin C, β-carotene)

were disappointing (e.g. Rapola et al., 1997; Yusuf et al., 2000)[1]. Therefore, research has focussed on a decrease of ROS generation as a possible way of reducing cardiovascular mortality.

1.3 French Paradox

1.3.1 Background

High dietary intake of saturated fat and elevated serum cholesterol are associated with a high incidence of cardiovascular diseases. Nevertheless, epidemiological investigations showed that there is a lower cardiovascular mortality in France than, for example, in Germany or Great Britain, despite a similar high consumption of dietary fat in these populations. The World Health statistics from 1989 noticed a yearly coronary heart disease mortality of 78 per 100000 men in Toulouse, France, and of 348 per 100000 men in Belfast, UK. Mean serum cholesterol was 230 mg/dl for Toulouse and 232 mg/dl for Belfast (World Health Organisation, 1989). That surprising situation, commonly termed as the "French paradox", was attributed to the high red wine consumption in France (Renaud and de Lorgeril, 1992). Newer data from the European statistical system Eurostat show similar regional differences for causes of death in Europe. From 2004 to 2006, the highest rate of fatal ischemic heart disease in 65- to 84-years-old residents was found in Estonia with 2305 male and 1318 female per 100000. In France, only 402 male and 169 female per 100000 died from this disease. That was the lowest rate in the European Union. All in all, Northern and Eastern Europe (Baltic states, Czech Republic, Slovakia) had a much higher incidence of fatal ischemic heart disease than Southern Europe (France, Portugal, Spain, Italy). In countries like Germany (874 male, 524 female per 100000) and Great Britain (1100 male, 602 female per 100000), deaths from ischemic heart disease were a little more frequent than in the European average (788 male, 451 female per 100000). The complete data can be obtained from Eurostat, Luxembourg, and was summarized by Niederlaender (2006).

---

[1] A great variety of clinical trials on these dietary antioxidants were reviewed by Kritharides and Stocker (2002).

The reciprocal correlation between red wine intake and cardiovascular mortality was easily detected, but it is not as easy to prove the supposed causal relationship between high red wine intake and reduced morbidity. Several *in vitro* and *in vivo* studies have tried to illuminate this appealing field of research.

1.3.2 Ethanol or Red Wine Polyphenols?

The first arising question is which red wine component might be responsible for its beneficial effect on the cardiovascular system. In healthy volunteers, alcohol was able to increase serum concentrations of high density lipoprotein-cholesterol (HDL-C)[2] that is known to be antiatherogenic by transporting cholesterol from peripheral tissues to the liver (Rimm et al., 1999). In apolipoprotein E-deficient mice, which serve as a disease model for atherosclerosis, both red wine and ethanol elevated serum HDL-C levels, whereas dealcoholized red wine did not (Bentzon et al., 2001). Alcohol also seems to inhibit platelet aggregation (Renaud and de Lorgeril, 1992). This is consistent with the observation that alcoholics die rarely from ischemic diseases in comparison to non-alcoholics, but more often from hemorrhagic strokes (Díaz et al., 2003; Iso et al., 2004). Many epidemiological case control studies only assessed alcohol consumption but not its distribution to different beverages. They showed that moderate alcohol consumption reduced incidence and recurrence of myocardial infarctions (Gaziano et al., 1999; Hines et al., 2001; de Lorgeril et al., 2002). Mukamal et al. (2003) examined the correlation between consumption of different alcoholic beverages (beer, red and white wine, liquor) and found a reduction of nonfatal myocardial infarctions in all groups.

---

[2] In the blood, cholesterol and triglycerides are transported in the core of lipoproteins, which are made water-soluble by an outer shell of phospholipids. Lipoproteins are classified by their density. LDL (low density lipoprotein) provides the periphery of the body with cholesterol, whereas HDL (high density lipoprotein) transports cholesterol from peripheral organs back to the liver. The LDL-C serum level correlates with the risk for atherosclerosis. Conversely, high HDL-C serum levels correlate with a low risk for atherosclerosis. For further information, see one of the plenty of textbooks of clinical pathology.

However, in a couple of experimental studies trying to explain the mechanism of the French paradox, alcohol alone failed to exhibit the same beneficial effects as red wine (e.g. Fitzpatrick et al., 1993; Wollny et al., 1999). These findings led to the hypothesis that these effects are caused by another large group of red wine components, the polyphenols. These compounds are found in many plants including grapes. The most common polyphenols in red wine are resveratrol, quercetin, catechin, curcumin, rutin, kaempferol, tannins, anthocyanins, caffeic acid and gallic acid (Tapiero et al., 2002). The beneficial effect of red wine is mainly ascribed to these antioxidant compounds. Therefore, research does not only focus on red wine, but also on certain polyphenols alone or on other polyphenolic beverages like purple grape juice or green and black tea.

1.3.3 Role of Lipoproteins

Considering the pathogenesis of atherosclerosis, which mostly underlies cardiovascular events, there are different parameters that could play important roles in the French paradox mechanism. HDL-C has antiatherogenic properties, whereas LDL-C, especially in its oxidized form, acts proatherogenic (Stocker and Keaney, 2004). As described above, serum HDL-C levels seem to be elevated by ethanol but not by polyphenols. In hamsters with atherosclerosis induced by a high-fat diet, serum LDL-C levels were decreased, and the oxidation of LDL-C was decelerated by *in vivo* application of purple grape juice and red wine with and without alcohol (Vinson et al., 2001). The oxidation of LDL-C was reduced by red wine but not by dealcoholized red wine in apolipoprotein E-deficient mice, a disease model for atherosclerosis (Bentzon et al., 2001). This was confirmed for dealcoholized red wine in Sprague Dawley rats, in which quercetin and catechin did not change LDL-C oxidation, too (Benito et al., 2004). However, a mixture of these two red wine polyphenols was able to diminish LDL-C oxidation in Wistar rats fed with a high-fat diet (Fremont et al., 1998). In contrast to the findings of Bentzon et al. (2001), Stocker and O'Halloran (2004) reported a reduction of LDL-C oxidation after administration of dealcoholized red wine to apolipoprotein E-deficient mice. Nevertheless, red wine did not affect LDL-C oxidation products in humans (Ziegler et al., 2005).

These partially controversial results might be due to different study designs, but they also prompt to investigate other vascular parameters to elucidate the mechanism of the French paradox.

1.3.4 Reactive Oxygen Species in the French Paradox

Reactive oxygen species (ROS) play a physiological role not only in host defence (phagocytic respiratory burst), but also in normal cell metabolism (cell growth, cell aging and apoptosis, cell migration) (Stocker and Keaney, 2004). There are ROS producing enzymes (NADPH oxidases, xanthine oxidase, uncoupled nitric oxide synthase), and enzymes that eliminate ROS (superoxide dismutase, catalase, peroxidases). ROS strongly react with biomolecules like DNA, proteins, lipids and carbohydrates. If exceeding ROS production and / or diminished endogenous antioxidants disturb the physiological balance, potentially harmful oxidative stress will occur (Sies, 1991). The latter plays an important role in the pathomechanism of cardiovascular dysfunction.

In the group of ROS, there are free radicals and nonradical oxidants. Free radicals, e.g. the superoxide anion (O_2^-), the hydroxyl radical (OH) and nitric oxide (NO), contain one or more unpaired electrons. When two radicals react with each other, they link their unpaired electrons to a covalent bond and form a nonradical molecule. That is the case in the breakdown of the radical NO by the radical superoxide, which results in the nonradical, but highly reactive oxidant peroxynitrite. A free radical may also react with a nonradical molecule to form a new radical, frequently followed by a chain reaction. An example relevant for the vasculature is the oxidation of lipids that often leads to a chain reaction with production of more and more oxidized lipids. Examples of nonradical oxidants are hydrogen peroxide (H_2O_2), hypochlorite (^-OCl), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) (Stocker and Keaney, 2004).

NADPH oxidases, xanthine oxidase and uncoupled eNOS are the major sources of ROS in the vasculature. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid with hydrogen peroxide as a by-product. There is evidence in literature that xanthine oxidase is involved in endothelial dysfunction (Guthikonda et al, 2003). Nevertheless, its role in the French paradox has not been

analyzed closely yet. Endothelial NO synthase (eNOS) generates NO from the substrate L-arginine, but it can also produce superoxide and hydrogen peroxide instead of NO, a process that is called uncoupling of eNOS (Mayer et al., 1991; Pritchard et al., 1995) and that has been associated with hypertension (Landmesser et al., 2003). Many research groups investigated the role of eNOS in the French paradox, but a real correlation could never be proven (cf. chapter **1.3.5**). As an endogenous antioxidant defence mechanism, superoxide dismutase (SOD) metabolizes superoxide to hydrogen peroxide, which is decomposed to water and oxygen by catalase or used to oxidize other substrates. Dietary-derived antioxidants include vitamin C (ascorbate), vitamin E (α-tocopherol) and a number of polyphenols (Stocker and Keaney, 2004).

It was shown that exceeding vascular ROS production and oxidative stress are implicated in the pathogenesis of atherosclerosis, hypertension and diabetic vascular dysfunction (Taniyama and Griendling, 2003). ROS, especially superoxide, lead to the breakdown of endothelium-derived NO (Afanas'ev, 2004). Thus, they play a key role in vascular pathophysiology and may be an important factor in the decryption of the French paradox. In Sprague Dawley rats with insulin resistance, which is associated with enhanced superoxide production in the thoracic aorta, ingestion of red wine polyphenols with and without ethanol decreased superoxide production in comparison to controls; ethanol alone did not affect superoxide production in aorta (Al-Awwadi et al., 2004). In humans, purple grape juice diminished the formation of superoxide both after *in vitro* application on platelets and *in vivo* uptake for 14 d (Freedman et al., 2001). Not only grape-derived beverages but also green and black tea, which are known to contain a great variety of polyphenols, exhibited this effect *in vitro* (Ying et al., 2003).

ROS also play an important role in tumorgenesis (McCord, 2000). This leads to the hypothesis that polyphenolic beverages might exhibit a similar protective effect on morbidity caused by malignancy as on cardiovascular morbidity. Indeed, epidemiological studies have revealed a preventive effect of red wine but not other alcoholic beverages on lung cancer (Ruano-Ravina et al., 2004) and prostate cancer (Schoonen et al., 2005). In a follow-up study including 24523 persons in Denmark, Grønbaek et al. (2000) found a lower relative risk for dying from cancer in wine

drinkers, but not in persons drinking beer or spirits regularly. In addition, the polyphenolic antioxidant resveratrol seems to be protective against various cancer types (Aziz et al., 2003).

Since ROS are a main link between pathogeneses of cardiovascular and malignant diseases (McCord, 2000), the hypothesis that oxidative stress plays a key role in the French paradox is convincing. Oxidative stress can be due to increased oxidant generation, declined endogenous antioxidant defence, or both. The investigation of endogenous antioxidants is complicated by the fact that defence mechanisms are induced by oxidative stress. Thus, an enhancement of antioxidant enzymes may indicate either a better protection or a greater need of these enzymes (Beckman and Ames, 1998).

1.3.5 Nitric Oxide and Endothelial Dysfunction

Endothelium-derived nitric oxide (NO), formerly known as endothelium-derived relaxing factor (EDRF)[3], plays a crucial role in maintaining normal vascular function and structure. It acts as a vasodilator and inhibits platelet aggregation, monocyte adhesion, and proliferation and migration of vascular smooth muscle cells (VSMC). NO breakdown due to ROS and concomitant formation of peroxynitrite results in endothelial dysfunction with an enhanced risk of cardiovascular diseases. The term endothelial dysfunction includes a state of impaired vascular tone, monocyte adhesion to the vascular wall and platelet aggregation. It is one of the earliest events in atherogenesis (Ross, 1999).

NO is generated from the substrate L-arginine by NO synthases (NOS). NO interacts with its receptor, soluble guanylyl cyclase, and generates cGMP which mediates many effects of NO. In absence of their substrate L-arginine or their activating cofactor tetrahydrobiopterin (e.g. because it was oxidated by ROS), NO synthases can become uncoupled, i.e., they transfer electrons to molecular oxygen instead of L-

[3] Robert F. Furchgott described the discovery of EDRF and its identification as NO in his Nobel lecture for receiving the Nobel Prize in Physiology or Medicine 1998 together with Ferid Murad and Louis J. Ignarro (Furchgott, 1998).

arginine, and thereby they generate superoxide instead of NO (Mayer et al., 1991; Pritchard et al., 1995). Under certain conditions, there can be a partial uncoupling, and NO synthases produce superoxide and NO concurrently. As described above, these two molecules combine to peroxynitrite. Some cardiovascular risk factors like hypercholesteremia, diabetes and hypertension seem to be involved in the uncoupling of NOS (Griffith and Stuehr, 1995; Hemmens and Mayer, 1998; Davis et al., 2001; Stocker and Keaney, 2004). *Figure 1* summarizes the impact of oxidative stress on endothelial function.

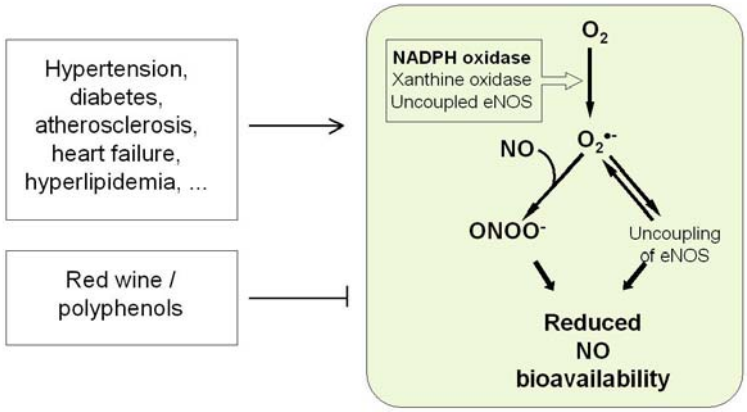


Figure 1: Oxidative stress and endothelial dysfunction – a simplified scheme. Pathological states are associated with Nox activation, oxidative stress and succeeding reduced NO-bioavailability, i.e. endothelial dysfunction.

In summary, NO is essential for endothelial function. An initial event in atherogenesis seems to be endothelial dysfunction, which is attributed to a lack of NO (Beckman et al., 1990). NO may either be generated to a lesser extent by NO synthase (NOS), or be depleted by interaction with superoxide and subsequent formation of peroxynitrite (Hemmens and Mayer, 1998; Afanas'ev, 2004).

Wallerath et al. (2003) reported that application of red wine on human endothelial cells from the umbilical vein enhanced the expression of the endothelial NOS isoform (eNOS), both on mRNA and protein level. Certain polyphenols like resveratrol and caffeic acid also increased eNOS protein expression, whereas others like quercetin

and catechin did not (Wallerath et al., 2005). In addition, red wine and resveratrol were able to elevate NOS activity (Wallerath et al., 2002; 2003). Leikert et al. (2002) could not show an effect of resveratrol on NOS activity in the same cells, but dealcoholized red wine enhanced both NOS activity and eNOS protein expression. *In vivo* experiments with Wistar rats revealed that dealcoholized red wine increased NOS activity (Bernátová et al., 2002), but in a similar study eNOS protein expression was not affected (Ralay Ranaivo et al., 2004).

NO acts as a vasodilator and inhibits platelet aggregation, monocyte adhesion, and VSMC proliferation and migration. In the literature, the impact of red wine on vasofunction and platelets was assessed with many experimental designs (e.g. Fitzpatrick et al., 1993; Freedman et al., 2001). The insufficient inhibition of monocyte adhesion and VSMC proliferation and migration by NO was examined by measuring the extent of atherosclerotic plaques (Vinson et al., 2001).

Common experimental setups for endothelial function are vessel studies in organ bath chambers. Fitzpatrick et al. (1993) applied the substances of interest directly on rat aortic rings. Both red wine and purple grape juice improved endothelium-dependent vasorelaxation in response to acetylcholine, whereas ethanol and white wine had no effect. Interestingly, an extract from grape skins but not from grape pulp could copy the red wine effect. Additionally, various polyphenols were applied on aortic rings. Quercetin and tannic acids improved vasorelaxation, whereas resveratrol and malvidin did not. In rings from rat mesenteric and femoral artery, dealcoholized red wine enhanced relaxation (Zenebe et al., 2003; Duarte et al., 2004). The latter substance had a similar relaxing effect when administered to Wistar rats 7 d before the organbath experiments were performed (Diebolt et al., 2001). In contrast to pure ethanol and white wine, feeding of red wine with and without alcohol enhanced bleeding time and decreased platelet adhesion to fibrillar collagen in Sprague Dawley rats (Wollny et al., 1999). Osman et al. (1998) fed purple grape, orange and grapefruit juice to monkeys and dogs; only the first one inhibited platelet aggregation in both species. The *in vitro* aggregation of human platelets was also reduced by application of purple grape juice. This could be confirmed *in vivo* by demonstrating that after 14 d of regular grape juice intake platelet aggregation was inhibited (Freedman et al., 2001).

Results concerning the influence of red wine on atherosclerotic plaque formation are rare and controversial. Vinson et al. (2001) reported that in hamsters with preexisting atherosclerosis, the plaque size could be decreased by a 10 weeks feeding period with red wine with and without alcohol, purple grape juice, and ethanol alone. These findings could be confirmed in apolipoprotein E-deficient mice which were fed with dealcoholized red wine (Stocker and O'Halloran, 2004). However, Bentzon et al. (2001) found no effect at all on the plaque size in the same animal model when feeding red wine, dealcoholized red wine or ethanol, although a positive effect of red wine and ethanol on HDL-C levels and on LDL-C oxidation could be demonstrated.

1.3.6 Role of NADPH Oxidases

The ROS producing NADPH oxidases represent a relatively young field of research. Thus, there were only few attempts to elucidate the role of these enzymes in the French paradox, although the hitherto existing results are intriguing.

NADPH oxidases (with their calatytic subunits that are called Nox) are the major ROS producing enzymes in the vasculature (Griendling et al., 2000), and they are the only ROS source studied in this work. It has been shown that resveratrol was able to inhibit *in vitro* Nox activity in rat aortae (Orallo et al., 2002) and in inflammatory macrophages from Wistar rats (Leiro et al., 2004). Apart from resveratrol, the polyphenols curcumin and rutin decreased Nox activity in human monocytes infected with *Chlamydia pneumoniae*, which seems to promote atherosclerotic lesions (Deby-Dupont et al., 2005). In endothelial cells from bovine carotid artery, polyphenolic extracts from green and black tea diminished the protein expression of the Nox subunits $p22^{phox}$ and $p67^{phox}$ (Ying et al., 2003).

1.4 The Family of NADPH Oxidases

1.4.1 The Phagocytic NADPH Oxidase

The first known nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) was the phagocytic one which is responsible for the microbicidal oxidative

burst in neutrophils, monocytes and macrophages – a major mechanism in host defence. The active phagocytic oxidase consists of six different subunits. The catalytic subunit Nox2 (also called gp91^{phox}) and p22^{phox} form the membrane-bound cytochrome b558, which is activated by assembly of the regulatory cytosolic subunits p47^{phox}, p67^{phox}, p40^{phox}, and rac1 or rac2. The active enzyme, illustrated in *Figure 2A*, rapidly produces great amounts of superoxide by electron transfer from NADPH to molecular oxygen (Babior, 2002) according to the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+.$$

Nox2 has a size of approximately 65 kDa (Royer-Pokora et al., 1986) and is associated to the membrane of phagosomes by six transmembrane helices. It contains two hemes, one FAD and one NADPH binding site. The cytochrome b558 complex alone is inactive. The regulatory subunits p47^{phox}, p67^{phox} and p40^{phox} are complexed in the cytosol. In activated phagocytes, an autoinhibitory domain in p47^{phox} needs to be phosphorylated by proteinkinase C to make the binding to p22^{phox} possible. Thereby, the whole cytosolic complex is recruited to the membrane (Groemping et al., 2003). The subunit p67^{phox} activates the electron transfer from NADPH to FAD (Nisimoto et al., 1999), and is the binding partner for rac, which is also necessary for oxidase activation (Lapouge et al., 2000). The last subunit, p40^{phox}, is not required for Nox activity, but it facilitates the translocation of p47^{phox} and p67^{phox} to the membrane and enhances superoxide generation (Kuribayashi et al., 2002).

Apart from phagocytes, the catalytic subunit Nox2 was also detected in different vascular cells, i.e. endothelial cells, VSMC and fibroblasts, and in the three layers of the vessel wall intima, media and adventitia (summarized by Lassègue and Clempus, 2003). Some experiments indicate a role of Nox2 in vascular diseases: Firstly, its mRNA expression was enhanced in diabetes (Kusaka et al., 2004) and by oscillatory stress in endothelial cells (Hwang et al., 2003). Secondly, angiotensin II-induced left ventricular hypertrophy was not elicited in Nox2-deficient mice in comparison to wild type animals (Byrne et al., 2003). Finally, Nox2 (and p22^{phox}) mRNA levels were correlated with the severity of atherosclerosis in human coronary arteries (Sorescu et al., 2002).

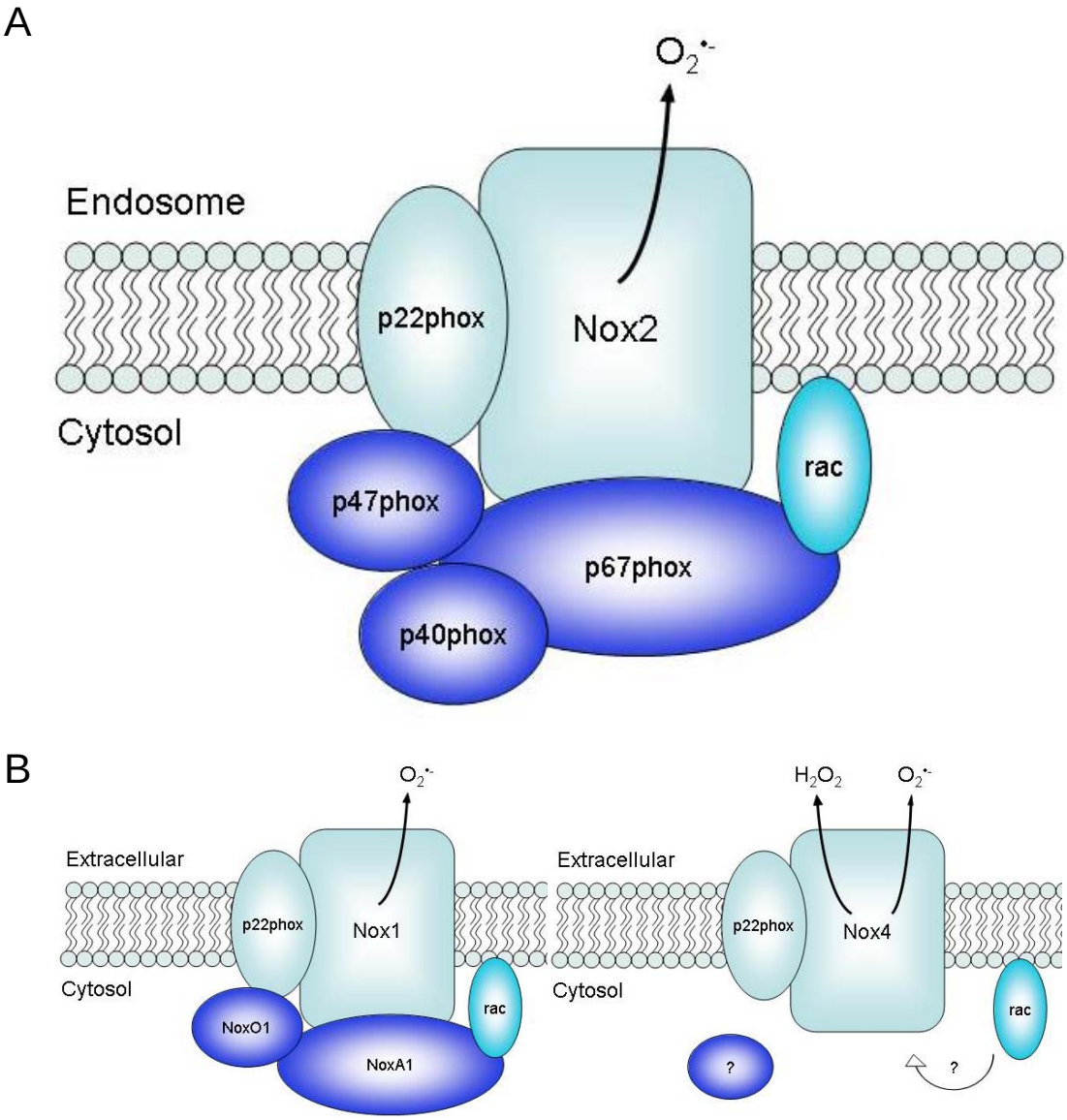


Figure 2: Vascular NADPH oxidases. A. Together with $p22^{phox}$, the catalytic Nox2 ($gp91^{phox}$) forms the membrane-bound cytochrome b558. Activation of the phagocytic NADPH oxidase requires the assembly with the cytosolic subunits $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, which are complexed in the cytosol, and rac. **B.** Active Nox1 and Nox4. NoxO1 and NoxA1 are homologues of $p47^{phox}$ and $p67^{phox}$, respectively. Apart from $p22^{phox}$, there is no evidence for Nox4-regulating subunits until now (modified from Opitz et al., 2007).

1.4.2 Discovery of New NADPH Oxidase Isoforms

In the late 1990's, Suh et al. (1999) discovered Nox1, the first homologue of the catalytic Nox2. Since this time, five other catalytic subunits – Nox3, Nox4, Nox5, Duox1 and Duox2 – have been described. Nox1, Nox3 and Nox4 have approximately

the same size as Nox2 and a similar structure with six transmembrane α-helices, two hemes, the FAD and the NADPH binding site (Lambeth, 2004). Nox1 is mainly expressed in the colon (Suh et al., 1999), but also in endothelial cells, VSMC and adventitial fibroblasts (Sorescu et al., 2002). Nox3 was firstly cloned in fetal kidney (Cheng et al., 2001). Recently, Bánfi et al. (2004) found very high Nox3 mRNA levels in the inner ear. Geiszt et al. (2000) discovered Nox4 in renal tubule cells. Nox4 mRNA is also expressed in vascular endothelial cells, VSMC and adventitial fibroblasts (Sorescu et al., 2002). Additionally to the Nox2 homologous region, Nox5 possesses a calcium-binding site by which it is regulated (Bánfi et al., 2001). Nox5 mRNA was detected in spleen, sperm and fetal tissues (Cheng et al., 2001). There are two Nox2 homologues cloned from thyroid gland that also contain a calcium-binding site, one additional transmembrane α-helix and a peroxidase-like domain. Because of these two oxidase domains (NADPH oxidase plus peroxidase), they were called Duox1 and Duox2 (dual oxidase) (Dupuy et al., 1999).

1.4.3 Vascular NADPH Oxidases

NADPH oxidases represent a major source of ROS in the vasculature (Griendling et al., 2000). In contrast to phagocytes, which show an inducible Nox activity, vascular cells (endothelial cells, VSMC, fibroblasts) seem to have constitutively active oxidases, because they exhibit a basal superoxide generation. However, vascular Nox isoforms can also be activated, for example by angiotensin II (Lassègue et al., 2001; Rueckschloss et al., 2002). Apart from Nox2, the isoforms Nox1 and Nox4 were detected in non-phagocytic cells, too (Lassègue and Clempus, 2003). Newer data also showed mRNA expression of Nox3 (Zhang et al., 2006) and Nox5 in endothelial cells (Bánfi et al., 2001), but these isoforms were not investigated in this study.

Nox1 is present in all vessel wall layers including endothelial cells, VSMC and fibroblasts (Sorescu et al., 2002). Like Nox2, it is localized at membranes together with $p22^{phox}$, which is necessary for its function (Ambasta et al., 2004). Nox1 possesses its own regulatory subunits. NoxO1 (Nox organizer 1) is almost identical to $p47^{phox}$, but lacks the autoinhibitory region that prevents binding to $p22^{phox}$ in the inactive state (Takeya et al., 2003). Thus, it is prelocalized at the membrane together

with Nox1 and $p22^{phox}$, even without cell activation (Cheng and Lambeth, 2004). NoxA1 (Nox activator 1) is a homologue of $p67^{phox}$ and activates Nox1 (Takeya et al., 2003). A scheme of the active Nox1 enzyme complex is demonstrated in *Figure 2B*.

Nox4 seems to be constitutively active. Furthermore, this enzyme seems to generate hydrogen peroxide in addition to superoxide. The common cytosolic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, rac, NoxO1 and NoxA1 are not required for the production of hydrogen peroxide by Nox4, and they are not able to increase its activity (Martyn et al., 2006). The only necessary subunit appears to be $p22^{phox}$ (Ambasta et al., 2004), but one or more Nox4-specific regulatory proteins might still be discovered in the future. Like Nox1, Nox4 is present in all vessel wall layers including endothelial cells, VSMC and fibroblasts (Sorescu et al., 2002). The enzyme complex of Nox4 is illustrated in *Figure 2B*.

Vascular Nox isoforms are implicated in different cardiovascular diseases like hypertension, atherosclerosis, diabetic vascular diseases and heart failure (Rajagopalan et al., 1996; Azumi et al., 1999; Sorescu et al., 2002; Tojo et al., 2002; Sonta et al., 2004; Takayama et al., 2004). Nox-derived ROS are enhanced in genetic hypertension in spontaneously hypertensive rats (SHR) and in hypertension caused by angiotensin II (Rajagopalan et al., 1996; Zalba et al., 2000). In SHR, $p22^{phox}$ mRNA levels and media thickness are enhanced, and vasorelaxation is impaired in comparison to healthy controls. Treatment with angiotensin II-receptor antagonists improves vasofunction, and decreases media thickness, $p22^{phox}$ mRNA levels and Nox activity (Zalba et al., 2000). Angiotensin II is a major stimulus of Nox activity in the vasculature (Griendling et al., 1994). Its effects include VSMC hypertrophy (Geisterfer et al., 1988) and cellular inflammatory responses (Ferrario and Strawn, 2006) which play important roles in atherogenesis. In fact, Nox activity and subsequent superoxide release is increased in vessels of rabbits with experimental atherosclerosis (Warnholtz et al., 1999). Azumi et al. (1999) demonstrated that $p22^{phox}$, the binding partner of all vascular Nox isoforms, is enhanced in atherosclerotic human coronary arteries in all three layers of the vessel wall. Since atherosclerosis can result in acute vascular events, its association with vascular Nox isoforms may be of great importance for the manifestation of the French paradox.

2 AIM OF THE THESIS

The origin of the French paradox is yet not fully explained. Since the French people do not suffer from cardiovascular diseases as much as other people, and since endothelial dysfunction is one of the initial steps on the way to cardiovascular diseases, it seems reasonable to assume that the French develop less endothelial dysfunction. According to recent research, endothelial dysfunction and its sequelae, e.g. hypertension and atherosclerosis, can be considered as a state of oxidative stress. This has led to the hypothesis that red wine polyphenols somehow reduce oxidative stress, either by lessening the production of ROS, or by enhancing antioxidant defence mechanisms. NADPH oxidases are main contributors to superoxide and enhanced ROS production, and are associated to endothelial dysfunction.

The purpose of this study, therefore, was to prove, whether the administration of polyphenolic beverages like red wine and purple grape juice will result in changes in Nox expression and / or activity *in vitro* and *in vivo*, which in turn would influence the oxidative homeostasis in the cardiovascular system.

To achieve this goal, protein expression of three vascular Nox isoforms (Nox1, Nox2 and Nox4) in aortic tissue homogenates and of Nox4 in rat aortic smooth muscle cells (A7r5) following administration of red wine or purple grape juice was assessed. Furthermore, Nox activity was determined via measurement of superoxide production *in vitro* in A7r5 cells and by performing isometric tension measurements on isolated thoracic aorta segments. The *in vivo* experiments were performed in healthy Wistar unilever rats (WUR) and in spontaneously hypertensive rats (SHR) which serve as a disease model for genetic hypertension.

In more detail, the aim of this thesis was to answer the following questions:

- How does red wine influence Nox activity and Nox protein levels *in vitro*?
- Does *in vivo* administration of red wine result in a change of Nox expression and / or activity in vasculature?

- Is purple grape juice able to copy potential red wine effects? What is the role of ethanol in this context?
- Is there a difference between healthy Wistar rats and cardiovascular stressed spontaneously hypertensive rats in response to polyphenolic beverages?

3 MATERIALS AND METHODS

3.1 Ethical Approvals

The experiments were approved by the Ethical Committee for Animal Experimentation of the Regional Commission, Giessen (Regierungspräsidium Giessen, Dezernat 25.3, Staatliches Amt für Lebensmittel-Überwachung, Tierschutz und Veterinärwesen), in accordance with the European Union Guidelines[4]. The experimental part of this study was performed at the Pharmacological Institute and at the animal house ("Zentrales Tierlabor") of the Justus-Liebig-University in Giessen, Germany.

3.2 Materials

3.2.1 Antibodies

The following antibodies were used for Western Blotting: anti-Nox1, anti-Nox2, anti-Nox4, anti-β-actin and HRPO-conjugated secondary antibodies. Anti-Nox1 and anti-Nox4 polyclonal antibodies were raised in our laboratories. The anti-Nox1 antibody was raised against the Nox1 amino acids 545-561, and the anti-Nox4 antibody against the N-terminal Nox4 amino acids 84-101 (Wingler et al. 2001). For the validation of the Nox4 antibody, peptide blocking, BLAST analysis and tissue expression patterns have been used by our group. Since its specificity has not been proven by MALDI-TOF-analysis and in knockout mice yet, we describe the signals obtained with this

[4] The original study design for the *in vivo* experiments intended to treat Wistar unilever rats (WUR) and spontaneously hypertensive rats (SHR) equally with red wine, ethanol, purple grape juice and sugared water. This design was approved for WUR. The committee did not approve treating SHR with potentially harmful beverages. The decision of rejection was made, because the animals already suffer from hypertension and should not be endangered by our treatment. Hence, we only fed them with purple grape juice, which was considered by the committee to be a harmless beverage.

antibody as immunoreactive signals. Anti-Nox2, anti-β-actin and the HRPO-conjugated secondary antibodies are commercially available.

Table 1: Antibodies used in this study.

Primary Antibody	Species	Source	Dilution
Anti-Nox1	Rabbit	Raised against Nox1-aa 545-561: RYSSLDPRKVQFYC (Wingler et al., 2001)	1:25000
Anti-Nox2	Mouse	BD biosciences (Heidelberg)	1:1000
Anti-Nox4	Rabbit	Raised against Nox4-aa 84-101: RGSQKVPSRRTRRLLDKS (Wingler et al., 2001)	1:10000
Anti-β-actin	Rabbit	Oncogene (San Diego, USA)	1:20000
HRPO-conjugated anti-rabbit	Goat	DAKO (Hamburg)	1:20000
HRPO-conjugated anti-mouse	Goat	DAKO (Hamburg)	1:2000

3.2.2 Chemicals

All the chemicals that were used in this study are listed in *Table 2*.

Table 2: Chemicals and suppliers.

Chemical	Source
β-Mercaptoethanol	Carl Roth GmbH (Karlsruhe)
Acrylamide (30%) with Bisacrylamide (0.8%)	Carl Roth GmbH (Karlsruhe)
Apocynin (4-hydroxy-3-methoxy-acetophenone)	Calbiochem (Darmstadt)
APS	Merck (Darmstadt)
BSA	Sigma (Deisenhofen)
$CaCl_2$	Merck (Darmstadt)
Carbogen (95% O_2 / 5% CO_2)	Lindner (Bad Nauheim)
Carbachol	Sigma (Deisenhofen)
Château de Mornag Mandagon red wine	Langguth (Traben-Trarbach)
CO_2	Lindner (Bad Nauheim)
Complete EDTA-free protease inhibitor set	Roche Molecular Biochemicals (Mannheim)
$CuSO_4$	Merck (Darmstadt)
Dulbecco's modified Eagle's medium	Sigma (Deisenhofen)
ECL Advanced	Amersham Pharmacia Biotech (Freiburg)
EDTA	Sigma (Deisenhofen)

Chemical	Source
EGTA	Sigma (Deisenhofen)
Ethanol	Merck (Darmstadt)
Folin Ciocalteu's phenol reagent	Merck (Darmstadt)
Fructose	Sigma (Deisenhofen)
Glucose	Carl Roth GmbH (Karlsruhe)
Glutamine	Sigma (Deisenhofen)
Glycerol	Merck (Darmstadt)
Glycine	Carl Roth GmbH (Karlsruhe)
HCl	Merck (Darmstadt)
KCl	Carl Roth GmbH (Karlsruhe)
KH_2PO_4	Carl Roth GmbH (Karlsruhe)
Lucigenin	Sigma (Deisenhofen)
Methanol	Merck (Darmstadt)
$MgSO_4$	Merck (Darmstadt)
Na_2CO_3	Merck (Darmstadt)
NaCl	Merck (Darmstadt)
NADPH	Sigma (Deisenhofen)
$NaHCO_3$	Merck (Darmstadt)
NaOH	Carl Roth GmbH (Karlsruhe)
Neutral red	Sigma (Deisenhofen)
Nitrogen	Lindner (Bad Nauheim)
Non-fat dry milk powder	Nestlé (Glandale, USA)
Penicillin	Sigma (Deisenhofen)
PeqGold protein marker IV prestained	PeqLab Biotechnologie (Erlangen)
Phenylephrine	Sigma (Deisenhofen)
Ponceau S	Serva (Heidelberg)
Protein extruded rodent diet No. 2019	Harlan Teklad (Borchen)
Purple grape juice	Lindavia (Lindau)
Roti-Load	Carl Roth GmbH (Karlsruhe)
SDS	Sigma (Deisenhofen)
Sodium deoxycholate	Sigma (Deisenhofen)
Sodium tartrate	Merck (Darmstadt)
Sodium pyrophosphate	Sigma (Deisenhofen)
Streptomycin	Sigma (Deisenhofen)
TEMED	Sigma (Deisenhofen)
Trichloracetic acid	Merck (Darmstadt)
Tris	Carl Roth GmbH (Karlsruhe)

Chemical	Source
Triton X 100	Serva (Heidelberg)
Trypsin	Sigma (Deisenhofen)
Tween 20	Sigma (Deisenhofen)
Vanadate	Appli Chem (Darmstadt)

3.2.3 Devices

All technical devices used are listed in *Table 3*.

Table 3: Technical devices.

Device	Source
Analytical balance AT 250	E. Mettler (Zürich, Switzerland)
Analytical balance M 5	E. Mettler (Zürich, Switzerland)
Centrifuge 5804	Eppendorf (Hamburg)
Electrophoresis system	Biorad (München)
Fluoroscan FL	Thermo labsystems (Vantaa, Finland)
Isolated organ apparatus IOA 5306	FMI Föhr Medical Instruments (Seeheim)
KL 2 shaker	E. Bühler (Bodelshausen)
Kodak EDAs 290 camera system	Kodak (New Haven, USA)
Kodak Image Station 440 CF	NEN Life Science Products, Inc. (Zaventem, Belgium)
Ohaus Explorer pro animal balance, EP 2102 C	EducaTec AG (Döttingen, Switzerland)
Power Supply SX mighty slim	Hoefer (San Francisco, USA)
Semi-dry transfer cell, Trans Blot SD	Biorad (München)
Spectra max 340 microplate reader	Molecular Devices (Sunnyvale, USA)
Thermomixer Compact	Eppendorf (Hamburg)
Varioclav Steam Sterilizer	H+P Labortechnik GmbH (Oberschleißheim)
Vortex VF 2	Janke und Kunkel IKA (Staufen)
Water bath type 1013	Gesellschaft für Labortechnik mbH (Burgwedel)

3.2.4 Software

The software used in this study and for preparing this thesis is listed in *Table 4*.

Table 4: Computer Software.

Software	Source
Adobe Photoshop 7.0	Adobe System (San José, USA)
IBJ-Amon and -Bemon vitro dat 3.4	Jaeckel (Hanau)
Kodak 1D Image Analysis	Eastman Kodak company (New Haven, USA)
Microsoft Office for Mac 2004	Microsoft Deutschland GmbH (Unterschleißheim)
Prism Graph Pad 4.0	Graph Pad Software (San Diego, USA)
Softmax Pro 1.2.0	Molecular Devices (Sunnyvale, USA)

3.3 Cell Culture

Two methods were employed in the *in vitro* part of this study, namely protein analysis and a superoxide assay. Both were performed in A7r5 cells, a rat aortic smooth muscle cell line.

The A7r5 cells were cultured in low glucose (1 g/l) Dulbecco's modified Eagle's medium, which was supplemented with 10% heat inactivated calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Confluent cells were incubated for 72 h with red wine (Château de Mornag-Mandagon, vintage 2002, produced from the grapes Carignan and Cinsault [vine variety]) in a concentration of 5% (v/v) in phosphate buffered saline (PBS), purple grape juice (Lindavia Rote Traube Direktsaft) in a concentration of 10% (v/v), an ethanol solution of 0.6% (v/v), or the vehicle PBS, respectively. These concentrations are approximately 10% of the concentrations used in the *in vivo* experiments[5]. The ethanol solution served as control for the diluted red wine, PBS was used as the appropriate control for purple grape juice. Subsequently, cells were washed three times with PBS and further treated as described in chapter **3.8.3**, **3.8.4** and **3.9**.

[5] Plasma concentrations of polyphenols reach about 10% of the ingested amount of polyphenols, depending on the chemical structure of the different polyphenols (Duthie et al., 1998).

PBS (Phosphate buffered saline), pH 7.3

KCl	2.7 mM
KH_2PO_4	1.5 mM
NaCl	137 mM
Na_2HPO_4	8 mM

3.4 Animals

Healthy Wistar unilever rats (HsdCpb:WU) and spontaneously hypertensive rats (SHR/NHsd) were obtained from Harlan Winkelmann GmbH in Borchen, Germany.

For the experiments, 12 weeks-old WUR and SHR were maintained in conventional temperature (22 °C) and humidity (60%) controlled animal facilities in a 12 h light / dark cycle. The animals were subdivided into groups of 2-3 in regular polycarbonate rodent cages (type IV). They were fed *ad libidum* with standard rodent chow (Harlan Teklad Nr. 2019) containing 20% protein, 55% carbohydrate, and 9% fat. After an adaption period of one week, the rats were weighed at day 0 and 14 of a two weeks feeding period.

3.5 Experimental Design of the *in vivo* Study

The *in vivo* study also consisted of two experimental parts, i.e. protein analysis and organ bath studies. For protein analysis in WUR, one control group and four test groups were formed. These five groups were divided into two treatment clusters, each consisting of the control group and two of the test groups. As a result, from each animal of the control group two data sets of control values were obtained by two succeeding experimental procedures. With SHR, only two parallel groups were formed, one control and one test group.

The same distribution of animals and groups was used for organ bath studies with SHR and WUR. However, in organ bath experiments with WUR, the control groups were combined to one single group to which the four test groups were compared.

With SHR, there were only two parallel groups, one control and one test group (cf. *Table 5*).

In total, 52 animals were used, 40 WUR and 12 SHR. WUR were randomly assigned to five different treatment groups, i.e., eight animals per group. Accordingly, SHR were distributed to two equally sized treatment groups.

For technical reasons, only 30 WUR were randomly assigned to the organ bath studies. Hence, in organ bath studies, each treatment group consisted of six animals.

Table 5: Treatment groups.

Animals	Treatment Cluster	Treatment / Feeding	Number of Animals	
			Protein Analysis	Organ Bath Experiments
WUR	I	Red wine dilution (1:1)	8	6
		Ethanol 6%	8	6
		Water[a]	4	3
	II	Purple grape juice	8	6
		Sugar solution	8	6
		Water[a]	4	3
SHR	-	Purple grape juice	6	6
		Water	6	6

[a] In data analysis, the water-treated animals were merged to form the control group.

3.6 Feeding Protocol

After the adaption period, the animals were randomly assigned to different treatment groups. In this study, "treatment" stands for a controlled fluid intake, i.e., all animals received only pre-defined liquids. In the first treatment cluster, WUR were treated with red wine (Château de Mornag-Mandagon) diluted 1:1 in water, or 6% ethanol in water according to the ethanol concentration of the diluted red wine. Water served as control in a third group. Red wine was diluted 1:1, because such a dilution has been described to be effective in literature (Bentzon et al., 2001; Vinson et al., 2001), and because pure red wine certainly does not meet the condition of "moderate alcohol consumption".

In the second treatment cluster, WUR were fed with purple grape juice ("Lindavia Rote Traube Direktsaft"), or a sugar mixture consisting of the main carbohydrate components of this juice, fructose (76 g/l) and glucose (77 g/l), dissolved in water (concentrations according to those in the juice). The control group received water. Purple grape juice was not diluted, because it is not thought to be harmful. Osman et al. (1998) administered pure juice to dogs and monkeys without reporting any harmful effect. Furthermore, a concentration of purple grape juice twice as high as that of red wine seemed reasonable after evaluation of the *in vitro* studies on concentration-dependency performed in this work (cf. chapter **4.1.2**). In this study, we tried to answer the question, whether purple grape juice in principle exhibits similar effects as red wine. If the answer to this question would have been "yes", further experiments about dose-dependency *in vivo* would have followed.

In statistical analyses of organ bath studies, the six water-fed animals served as control for all other treatment groups. However, within each treatment cluster, the groups fed with ethanol or sugar solution acted also as an active control. So the effect of red wine was controlled by ethanol and the effect of purple grape juice was controlled by the sugar solution.

SHR received purple grape juice or water only, because the local committee for animal protection did not approve further experiments with potentially harmful beverages in these cardiovascular stressed animals (see chapter **3.1**).

All beverages were administered *ad libidum*. The ingested amounts of chow and liquids were estimated by periodical weighing of the bottles and the chow. After a 14 d period of feeding, the animals were killed by carbon dioxide (CO_2) inhalation, and the aortae were dissected immediately.

3.7 Organ Preparation

Immediately after killing the animals by CO_2 inhalation, the thoracic aortae including the aortic arches were carefully excised and separated from the surrounding fat tissue in a Petri dish with Krebs Henseleit Buffer (KHB). Parts of the aortae were used for

organ bath experiments; the rest was frozen in liquid nitrogen and stored at –80 °C for subsequent protein determination.

KHB (Krebs Henseleit buffer), pH 7.4

NaCl	118.5 mM
KCl	4.7 mM
$MgSO_4$	1.2 mM
KH_2PO_4	1.2 mM
$NaHCO_3$	25.0 mM
$CaCl_2$	2.5 mM
Glucose	5.6 mM

3.8 Protein Analysis

3.8.1 Tissue Lysis

The frozen aortae were minced to powder in a stainless steel mortar and then stored at –80 °C. The minced organs and / or the cells from cell culture were lysed in Lysis Buffer (LB), approximately 250 µl per 30 µg, for 10 min at room temperature. The mixture was vortexed, diluted with the same amount of 2x Rotiload and heated for 15 min at 95 °C in a thermo-mixer. The emerging samples were stored at –20 °C.

LB (Lysis Buffer), pH 7.4

Tris-HCl	40 mM
NaCl	150 mM
Sodium deoxycholate	2% (w/v)
Triton X 100	2% (v/v)
SDS	0.1% (w/v)
EDTA	50 mM
EGTA	50 mM
Sodium pyrophosphate	50 mM
Vanadate	2 mM

2x Complete EDTA-free protease inhibitor set

3.8.2 Protein Determination

According to the method described by Lowry et al. (1951), 10 µl of each sample were diluted in 1 ml distilled water and incubated for 10 min with 100 µl of 0.15% sodium deoxycholate. After applying 100 µl of 72% trichloracetic acid, the samples were incubated for 15 min and then centrifuged with 16000 G for 10 min. The supernatants were removed and the pellets were dissolved in 300 µl distilled water and 300 µl Folin-I for 10 min. After incubation with 150 µl of 25% Folin Ciocalteu's phenol reagent for 30 min, the optical density of the samples was measured in a 96-well plate spectrophotometer (Spectra Max 340 microplate reader, Molecular Devices) at 595 nm. A BSA serial in different dilutions served as the standard to determine the protein concentrations of the samples. All incubation steps were carried out at room temperature on an Eppendorf shaker.

Folin-I

Na_2CO_3	224 mM
NaOH	140 mM
$CuSO_4$	1 mM
Sodiumtartrate	2.2 mM
SDS	2.5% (w/v)

3.8.3 SDS Polyacrylamide Gel Electrophoresis

Equal amounts of protein were separated at 0.4 mA/cm^2 in 9% polyacrylamide gels by SDS-PAGE according to the Laemmli method (Laemmli, 1970). The resolving gel and the stacking gel on top were generated in Biorad gel casters for gels with 36 lanes. Then, 40 µg of each sample were loaded on the gel. PeqGold protein marker (5 µl) was used for subsequent control of the approximate molecular weight. Electrophoresis was performed in a Biorad SDS-PAGE gel chamber at room temperature. The samples of each treatment cluster were applied together on one gel. To minimize the methodical error (i.e., inexact transfer at the gel margins), the sample

order was changed in each electrophoresis. Each Western Blot with one treatment cluster was performed four times for each animal.

Resolving gel (9 %), pH 8.8

AA-Bis	29.9% (v/v)
Tris-HCl	375 mM
SDS	0.1% (w/v)
Temed	0.05% (v/v)
APS	0.7% (w/v)

Stacking gel, pH 6.8

AA-Bis	16.7% (v/v)
Tris-HCl	78 mM
SDS	0.1% (w/v)
Temed	0.1% (v/v)
APS	0.5% (w/v)

Electrophoresis buffer

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)

3.8.4 Western Blot Analysis

The separated proteins were transferred from the gel to a nitrocellulose membrane (Amersham Pharmacia Biosciences, Freiburg) in a semi-dry blotting chamber with blotting buffer at 1.2 mA/cm^2 for 70 min. After completion of the protein transfer, the membrane was stained with Ponceau S, checked on transfer errors (e.g. bubbles) and decoloured with TBS-T, in the case it was error-free. Afterwards, the membrane was blocked with the blocking solution (2 h, room temperature).

Primary antibodies (see chapter **3.2.1** and *Figure 3*) were diluted in blocking solution, and the membrane was incubated over night at 4 °C. Then, the membrane was washed five times for 10 min with TBS-T, and subsequently incubated with the corresponding secondary antibody in blocking solution for 1 h at room temperature. Afterwards, it

was again washed five times for 10 min with TBS-T. To visualize the signals, the membrane was incubated with ECL Advanced for 5 min, and chemiluminescence was immediately recorded with the Kodak Image Station 440 CF.

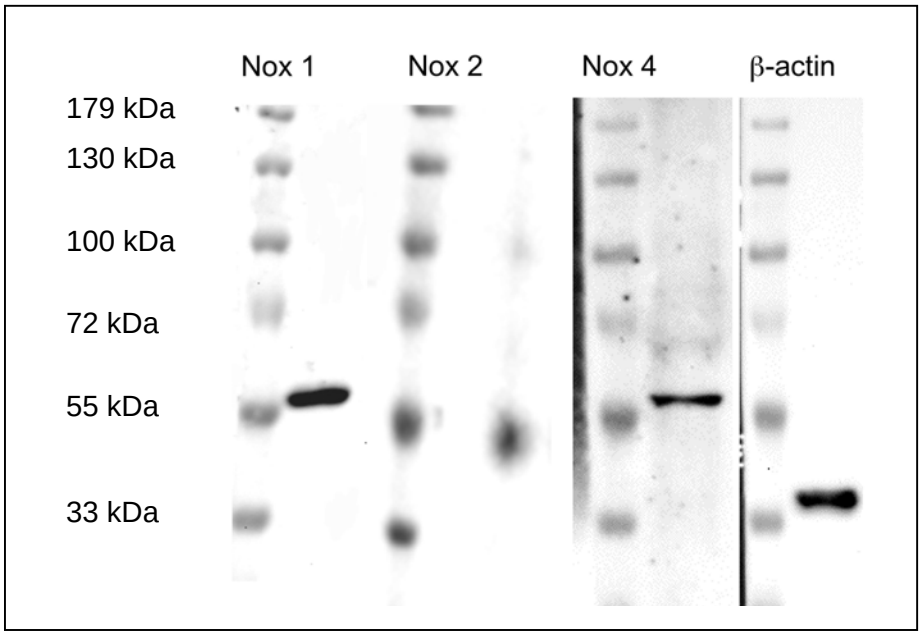


Figure 3: Original blots. From the left to the right, the immunoreactive bands obtained with the Nox1, Nox2 and Nox4 antibodies on exemplary blots are shown. 40 µg of rat aortic tissue samples were loaded on 9% polyacrylamide gels. Using the PeqGold protein marker, Nox1 and Nox4 run at a size of approximately 58 kD, while Nox2 runs at 55 kDa. The last blot on the right shows the immunoreactive band of the housekeeping protein β-actin (approximately 35 kDa) after stripping off the Nox4 antibody from the membrane.

Immediately after imaging, the membrane was incubated with stripping buffer in a water bath for 10 min at 60 °C followed by washing 4 times for 10 min with TBS-T at room temperature. Then, the membrane was blocked as mentioned above, before the overnight incubation with the next primary antibody could start[6]. The further

[6] It is possible to display the expression of different proteins on one blot by using the corresponding antibodies simultaneously, if these proteins are of different sizes, or one after another, if protein bands would interfere otherwise. Since the analyzed Nox isoforms are all of a similar size, it was necessary to strip the blots before applying the next primary antibody.

procedure was the same as described above. All incubation steps were performed on a KL 2 shaker.

Blotting Buffer, pH 10

Tris-HCl	48 mM
Glycine	39 mM
SDS	0.1% (w/v)
Methanol	20% (v/v)

TBS-Tween, pH 7.5

Tris-HCl	20 mM
NaCl	150 mM
Tween 20	0.1% (w/v)

Blocking solution

2 % (w/v) non-fat dry milk powder in TBS-T

Stripping Buffer, pH 6.8

Tris-HCl	69.2 mM
β-Mercaptoethanol	100 mM
SDS	2.5% (w/v)

3.8.5 Quantitative Analysis of Protein Immunoblots

The detected immunoreactive bands were analyzed with the Kodak 1D 3.5 software. For quantification, the bands were selected as regions of interest, and band intensity was evaluated computer-aided. To compare the protein amount of the different samples, the intensity of the bands were normalized to that of the housekeeping protein β-actin.

3.9 Detection of Superoxide and Nox Activity

Superoxide detection was performed following the protocol of Li and Shah (2001). Cells were trypsinized, washed in PBS and resuspended at 10^6/ml in HEPES buffer. Then, 5 µM lucigenin was added to the cells. This cell suspension was transferred to a 96-well plate in aliquots of 100 µl. After an incubation period of 20 min at 37 °C, the reaction was started by applying 100 µM NADPH to the wells. Chemiluminescence was recorded over a time period of 20 min using the Fluoroscan FL microplate reader. The values represent the maximum of the recorded light emission. The Nox inhibitor apocynin (1 mM) was applied at the maximum of the signal, and the amount of inhibition indicated Nox activity. For each group, each experiment was repeated at least five times.

HEPES buffer, pH 7.4

NaCl	140 mM
KCl	5 mM
$MgCl_2$	0.8 mM
$CaCl_2$	1.8 mM
Na_2HPO_4	1 mM
HEPES	25 mM
Glucose	1% (w/v)

3.10 Isometric Tension Measurements

The isometric tension measurements were in principle performed according to the protocol of Christon et al. (2005). In a Petri dish with KHB, two aortic rings of 2-3 mm length were excised from each thoracic aorta and immediately mounted in isolated organ bath chambers, which contained 5 ml KHB and were oxygenated continuously with carbogen (95% O_2 / 5% CO_2) at 37 °C. Changes in isometric tension were detected by a force transducer and recorded via a 6-channel transducer data acquisition system.

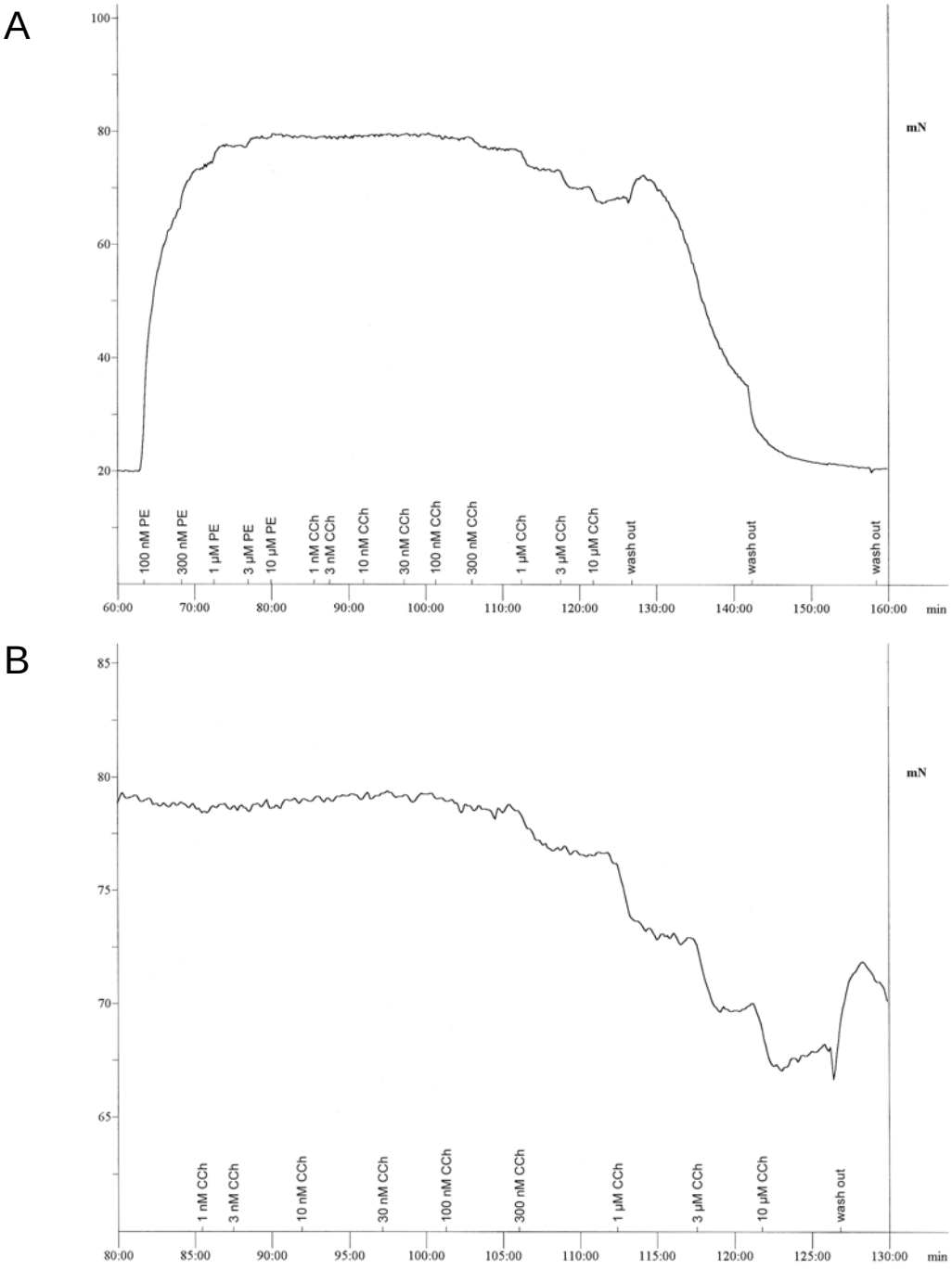


Figure 4: Isometric tension measurement in one examplary aortic ring. A. Phenylephrine (PE)-induced contraction followed by carbachol (CCh)-induced vasorelaxation and washing of the ring. **B.** The enlarged section shows the CCh-induced vasorelaxation. The doses were applied after reaching a plateau. The first effect is visible with 100 nM CCh.

The vessels were gradually stretched up to 20 mN. To determine the viability of the rings, they were challenged two times with 80 mM KCl. The KCl solution was

washed out with KHB after the contraction had reached a plateau. Thus, the rings were relaxed back to pretension (20 mN). After a recovery phase of 45 min, the rings were contracted submaximally (approximately 80%) with 10 µM of the α-adrenergic receptor agonist phenylephrine (PE). When the contraction reached a plateau, the rings were relaxed with the endothelium-dependent, parasympathomimetic carbachol (CCh) to assess a dose-response-curve (1 nM to 10 µM in semilogarithmic steps, cumulative application).

One complete PE-induced contraction followed by CCh-induced vasorelaxation, as demonstrated in *Figure 4*, took about 120 min.

Further experiments after preincubation with 100 µM of the Nox inhibitor apocynin followed by the procedure described above were performed. Thus, if any difference between groups was revealed in the organ bath experiments, the effects of purple grape products could have been specified.

The evaluation of the maximum PE-induced contractions and CCh dose-response-curves were done using the IBJ-Bemon 32 software. Therefore, the recorded curves (*Figure 5*) were compared with the chronological protocol, and event markers were set at the points of interest. Thus, each event (maximum PE-induced contraction, response to single doses of CCh) had corresponding values of force, which were calculated computer-aided.

3.11 Statistical Analysis

3.11.1 General Considerations

Data sampling, descriptive statistics (mean or median as appropriate, standard error of the mean, confidence interval, 25% - 75%-interquartil range), and modelling of the dose-response-curves were done using Graph Pad Prism 4.0 from Graph Pad Software (San Diego, USA). All experimental data presented throughout the study are mean values with SEM, unless otherwise stated. Statistical comparison of protein analyses was done with the help of Dr. R. H. Bödeker from the department of medical

statistics, Justus-Liebig-University, Giessen. A p-value of $p < 0.05$ was considered to be statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.11.2 Test Animals

Descriptive statistics were used to examine the weight gain of animals and the amount of fluid and chow ingested. Within the exploratory data analysis, significant differences between treatment groups were assessed by ANOVA analysis. In WUR, the differences between all groups within a single treatment cluster were tested by Bonferroni's post hoc test. This post-test was chosen, because Gaussian distribution was assumed, and because selective columns should be compared with each other (all columns within the single treatment clusters). In SHR, only two groups had to be compared with each other, so the student's t-test was used.

3.11.3 Immunoblots

The Western Blot data of the cell culture experiments are presented as means with SEM. Values were normalized to the values of the PBS group, which was set 100%. Statistical differences between the means were analyzed by ANOVA followed by Bonferroni's post test which is appropriate in groups with Gaussian distribution.

The statistic evaluation of the *in vivo* part of the study required the formation of treatment clusters. Each Western Blot of one treatment cluster was performed four times. Hence, each animal had four intensity values for each Nox isoform. From these values the median was assessed, because the data showed no Gaussian distribution. Values were normalized to the values of the water group, which was set to 100%. Results are given as medians with 25% - 75%-interquartil range. The groups within one treatment cluster were compared using an exact Fligner-Wolfe many-to-one test with a control of multiple range testing. This test was considered appropriate because of its robustness against potential heterogeneous distributions. In SHR, only two groups had to be compared, so the student's t-test was used.

The *null hypothesis* tested both in the *in vitro* and the *in vivo* experiments was as follows: There are no differences between the groups or between certain groups of a treatment cluster with respect to the expression of a specific Nox isoform.

The *alternative hypothesis* was: There are differences between the groups of a treatment cluster with respect to the expression of a specific Nox isoform.

The null hypothesis was to be rejected in the case $p < 0.05$.

3.11.4 Superoxide Assay

NADPH-derived superoxide production, expressed as the chemiluminescence signal, is represented as means with SEM. Statistical differences were examined by ANOVA with Bonferroni's post test. The amount of the signal that was inhibited by apocynin was normalized to the PBS control, which was set to 100%, and differences between the means were analyzed using ANOVA followed by Bonferroni's post test.

3.11.5 Vascular function

Analysis of vasofunction data was done by descriptive statistics. For analysis of isometric tension measurements, the values of the two aortic rings from each animal as obtained from the IBJ-Bemon 32 software were averaged. Statistical analysis was performed with the Graph Pad software. In WUR, maximum PE-induced contractions were compared using ANOVA analysis and Bonferroni's post test for between-group differences. In SHR, the student's t-test was used. A value of $p < 0.05$ was considered to be statistically significant.

Sigmoid dose-response-curves for CCh-induced vasorelaxation were attained with the following equation: $Y=Y_{min}+(Y_{max}-Y_{min})/(1+10^{logEC50-X})$, with $X = \log(conc)$ and $Y =$ response. This three-parameter sigmoid gave a sufficiently good fit to the experimental data in the global curve fitting procedure. Efficacy (maximum response)

and potency (EC50)[7] were compared with the Akaike's Information Criterion (AIC) test. Before attaining EC50 values by using global curve fitting, the single curves were normalized (Y_{max} = 100%), i.e., by using a global model, all curves shared the same maximum response and allowed the comparison of EC50 values. The AIC is based on information theory and is considered to be appropriate for the comparison of more than two dose-response-curves with respect to EC50 values. It does not compare a null hypothesis with an alternative hypothesis, and, therefore, does not give a p-value. Instead, two models are compared with the AIC method (e.g. model 1 "all EC50 are the same" vs. model 2 "all EC50 are different") resulting in relative probabilities w_i ("Akaike's weights") for each model. A decision between the two models can be made with the help of the evidence ratio w_i/w_j. For example, an evidence ratio of 1.6 means that one model is 1.6 times more likely to be correct than the other one. Since the AIC does not work with statistical hypothesis testing, it cannot be used to reject one model. The term "significance" is inappropriate in this context. The AIC test is comprehensibly introduced in "Fitting models to biological data using linear and nonlinear regression" (Motulsky and Christopoulos, 2003).

[7] The curve progression of dose-response-curves is mostly non-linear; in the logarithmic form, the curve has a sigmoid shape. The therapeutic range in pharmacology often lies in the intensely rising middle segment of the graph. Two values may be obtained from dose-response-curves. The efficacy (the response to a substance) is displayed on the ordinate, the potency (the dose that leads to a certain response) is displayed on the abscissa. The EC50, a measure for potency, describes the dose that exhibits 50% of the maximum effect. It is located at the inflexion point of the sigmoid graph. For further explanations see one of the current textbooks of pharmacology, e.g. „Pharmakologie und Toxikologie" (Estler and Schmidt, 2006).

4 RESULTS

4.1 Effects of Purple Grape Products *in vitro*

4.1.1 Nox Activity in A7r5 Cell Culture is Decreased by Red Wine

NADPH-derived superoxide production as a sign of oxidative stress was suppressed by purple grape products in rat aortic smooth muscle cells. As demonstrated in *Table 6*, chemiluminescence was significantly lower in A7r5 cells incubated with red wine in comparison to the active control ethanol. Red wine treated cells produced only half as much superoxide as the cells treated with ethanol ($p < 0.001$). Multiple testing of all groups revealed no significant effect of ethanol itself on superoxide production in comparison to PBS. Application of purple grape juice on A7r5 cells decreased chemiluminescence to 54% of the control PBS ($p < 0.01$).

Table 6: NADPH-derived superoxide production, expressed as chemiluminescence (CL) signal, was significantly suppressed in A7r5 cells treated with red wine compared to cells incubated with ethanol ($p < 0.001$), and in cells treated with purple grape juice in comparison to PBS ($p < 0.01$). The apocynin inhibitable part of the CL signal, a measure for Nox activity, was significantly affected by red wine in comparison to ethanol ($p < 0.05$), but not by purple grape juice in comparison to PBS. All values display means ± SEM. * displays statistical significance in comparison to ethanol and # in comparison to PBS. $N \geq 5$ experiments for each group.

Treatment	CL signal	Apocynin inhibitable part of CL signal
PBS	252.5 ± 12.2	100 ± 14.4
Ethanol	321.3 ± 16.7	131.1 ± 30.7
Red wine	159.8 ± 40.1 ***	48.3 ± 20.9 *
Purple grape juice	137.2 ± 11.6 ##	60.2 ± 13.2

Apocynin is a known inhibitor of Nox proteins, thus the apocynin inhibitable part of the chemiluminescence signal is a measure for Nox activity. *Figure 5A* shows the part of the signal that is inhibitable by apocynin. Incubation with red wine suppressed Nox

activity in A7r5 cells in comparison to ethanol to 37% ($p < 0.05$), but purple grape juice did not significantly affect Nox activity in comparison to PBS. The active control ethanol also did not alter the apocynin inhibitable part of the chemiluminescence signal compared to PBS.

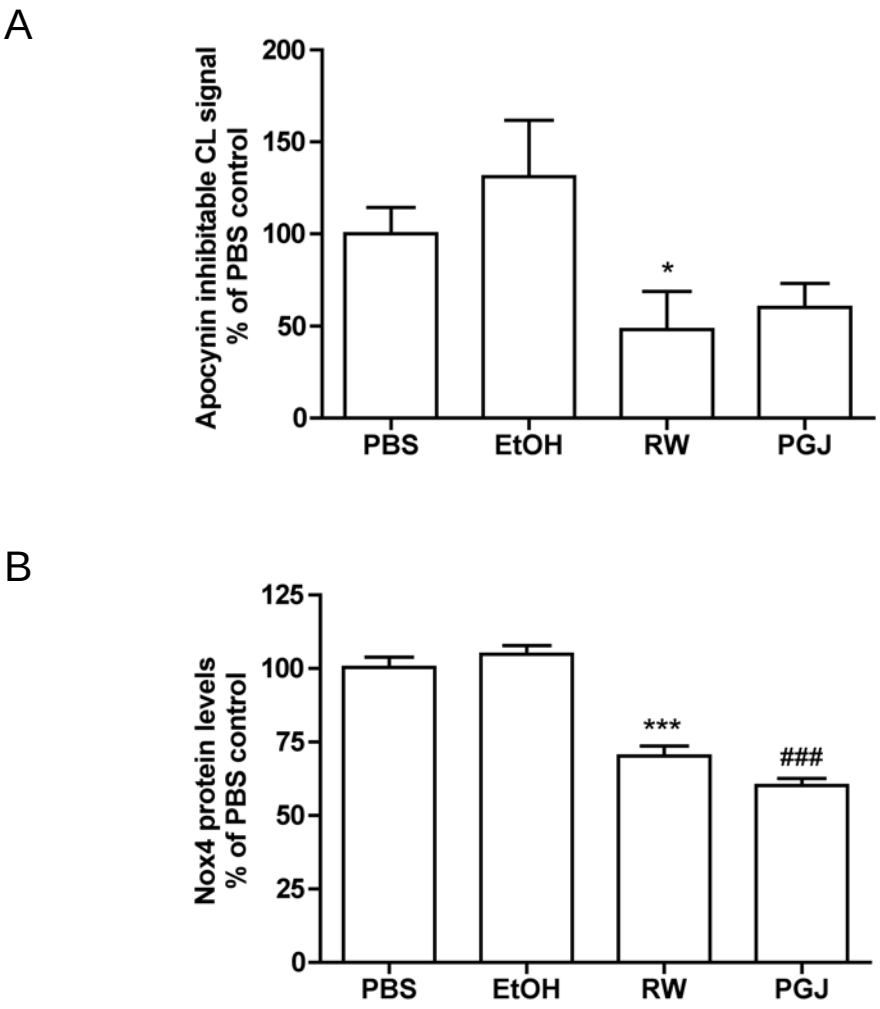


Figure 5: Red wine as well as purple grape juice significantly suppress NADPH-dependent ROS production and down-regulate Nox4 immunoreactivity in A7r5 cells. A. NADPH oxidase activity was significantly affected by red wine (RW) in A7r5 cells as shown by a significant reduction of the apocynin inhibitable part of the chemiluminescence signal as compared to PBS. **B.** Quantitative Western blot analysis revealed a significant down-regulation of Nox4 immunoreactive signals in A7r5 cells after incubation with 5% RW ($p < 0.001$ in comparison to EtOH) or 10% purple grape juice (PGJ) ($p < 0.001$ in comparison to PBS control) for 72 h. All values display means ± SEM. * displays statistical significance in comparison to EtOH and # in comparison to PBS. $N \geq 5$ experiments per group.

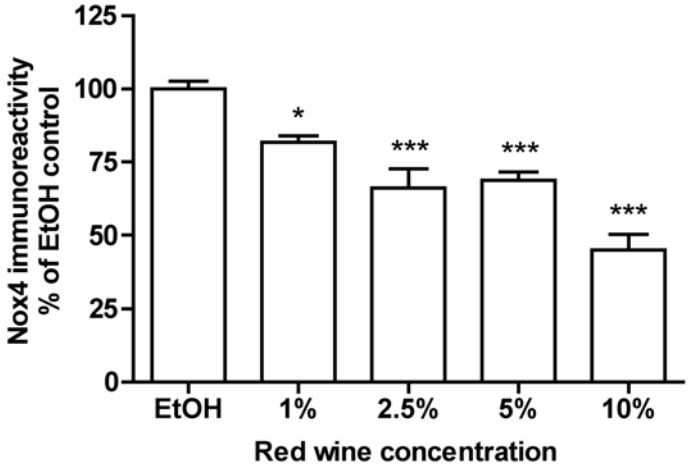
4.1.2 Nox4 Immunoreactivity in A7r5 Cells is Down-Regulated by Purple Grape Products

Nox4 is the only vascular Nox isoform that is detectable in A7r5 cells with the antibodies that are established in our laboratory. Thus, we only investigated the *in vitro* effects of purple grape products on Nox4 expression. Incubation of A7r5 cells with red wine led to a significant down-regulation of Nox4 immunoreactivity in comparison to ethanol ($p < 0.001$), which was revealed by quantitative Western Blot analysis (*Figure 5B*). Ethanol did not significantly affect protein levels in comparison to PBS. Incubation with the non-alcoholic beverage purple grape juice significantly reduced Nox4 immunoreactivity in A7r5 cells ($p < 0.001$). The red wine and purple grape juice effects on Nox4 both showed concentration-dependency, as shown in *Figure 6* and *Table7*.

Table 7: Nox4 immunoreactivity in percentage of PBS control. Red wine was compared to ethanol, and purple grape juice was compared to PBS. All values are given as means ± SEM. * displays statistical significance in comparison to ethanol and # in comparison to PBS. $N \geq 4$ experiments per group.

Treatment	Concentration (v/v)	Nox4 immunoreactivity
PBS	10%	100 ± 4
Ethanol	0.6%	104.4 ± 3
Red wine	1%	85.4 ± 2.6 *
	2.5%	66.2 ± 6.5 ***
	5%	71.2 ± 3.3 ***
	10%	46.1 ± 6.1 ***
Purple grape juice	1%	104.4 ± 6.1
	2.5%	88.6 ± 7.3
	5%	87.7 ± 8.8
	10%	60.4 ± 2.4 ###

A



B

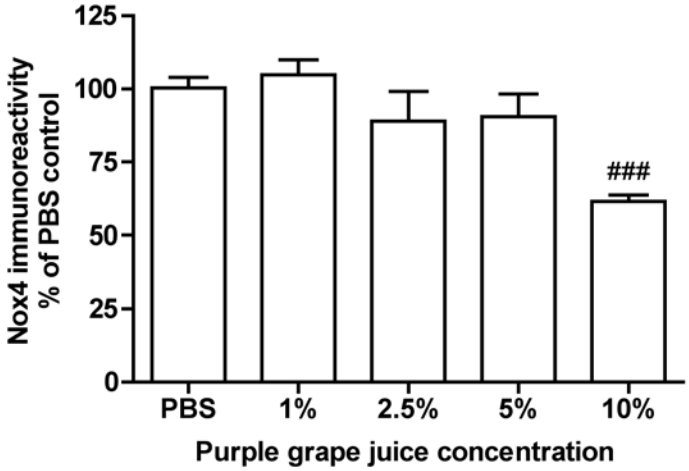


Figure 6: Concentration-dependency of Nox4 down-regulation by purple grape products in A7r5 cells. A. RW down-regulates Nox4 immunoreactivity in a concentration-dependent matter as compared to the EtOH control (0.6%). **B.** PGJ down-regulates Nox4 immunoreactivity in A7r5 cells in a concentration-dependent matter, too, but apparently with a lower potency. Just the concentration of 10% PGJ reached statistical significance in comparison to the PBS control (10%). All values display means ± SEM. * displays statistical significance in comparison to ethanol and # in comparison to PBS. N ≥ 4 experiments per group.

Red wine down-regulated Nox4 immunoreactive signals in all concentrations from 1% to 10% in comparison to a 0.6% ethanol solution which corresponds to 5% red wine. The lowest values were reached after applying 10% red wine in PBS (*Figure 6A*). This concentration led to a down-regulation to 44.2 ± 5.9% of control

($p < 0.001$). Purple grape juice reduced Nox4 immunoreactivity with a lower potency than red wine. *Figure 6B* shows Nox4 signals after applying purple grape juice in concentrations from 1% to 10% in comparison to the control PBS. While lower concentrations failed to change the signal, the 10% concentration reached a statistically significant Nox4 down-regulation to 60.4 ± 2.4% of control ($p < 0.001$). A down-regulation to such an extent was nearly reached by a red wine concentration of only 5% (68.2 ± 3.2%, $p < 0.001$).

4.2 Effects of Purple Grape Products in Living Animals

4.2.1 General features

No mortality was observed in any treatment group. 12 weeks old Wistar unilever rats (WUR) weighed approximately 330 g (334 ± 5, n = 40) before starting the treatment, with no statistically significant difference between the groups. After 14 d, WUR that were fed with purple grape products, ethanol or water had gained weight equally. The WUR receiving the sugar solution gained more weight in comparison to the water control ($p < 0.05$). The mean (± SEM) weight gains in gram were 39 ± 4 for the water and 59 ± 4 for the sugar group. SHR (12 weeks old) weighed approximately 280 g (275 ± 8, n = 12) before starting the experiments. At the end of the feeding period, SHR had gained weight equally, with no difference between treatment and control group.

WUR ingested equal amounts of water, red wine and ethanol. A remarkable greater fluid intake occurred with the two beverages with high sugar content. The animals treated with purple grape juice and sugared water drank significantly more than the other three groups ($p < 0.001$). The mean (± SEM) daily amounts in gram were 22.6 ± 0.8 for water, 34.7 ± 3.0 for purple grape juice, and 55.3 ± 2.4 for sugared water. SHR showed a similar drinking behaviour. The mean (± SEM) daily amounts in gram were 23.9 ± 1.6 in the water group, and 47.3 ± 3.7 in the purple grape juice group ($p < 0.001$).

The amount of daily-ingested chow did not differ between the four treatment groups in WUR, but three of them (red wine, ethanol, sugar) ingested a significantly higher

amount than the control group water ($p < 0.05$). WUR fed with purple grape juice did not ingest significantly more chow than the water group ($p > 0.05$). In SHR, there was no difference in daily amount of chow between treatment and control group.

All values concerning weight gain or daily amount of ingested fluid or chow are displayed in *Table 8*.

Table 8: Animal weight gain as well as daily liquid and chow intake in a 14 d feeding period. All values represent means ± SEM. Asterisks demonstrate statistical significance in comparison to the water control. N = 8 for WUR, n = 6 for SHR.

Animals	Treatment	Weight gain after 14 days [g]	Daily liquid intake [g]	Daily chow intake [g]
WUR	Water	39 ± 4	22.6 ± 0.8	7.1 ± 0.9
	Ethanol	38 ± 5	21.4 ± 0.7	14.8 ± 0.8 *
	Red wine	46 ± 7	22.3 ± 1.1	16.7 ± 0.6 *
	Purple grape juice	29 ± 4	34.7 ± 3 ***	10 ± 5.2
	Sugar solution	59 ± 4 *	55.3 ± 2.4 ***	14.2 ± 1.1 *
SHR	Water	22 ± 9	23.9 ± 1.6	25.8 ± 9.4
	Purple grape juice	33 ± 5	47.3 ± 3.7 ***	21.3 ± 3.6

4.2.2 Ethanol but not Red Wine Elevates Nox4 Immunoreactivity in Rat Aorta

The vascular Nox isoforms Nox1, Nox2 and Nox4 were detectable in the thoracic aortae of all healthy Wistar unilever rats (WUR) in all treatment groups.

Figure 7 demonstrates that feeding WUR with red wine or ethanol alone did not result in any change of Nox1 or Nox2 protein expression in relation to the control group water. In contrast, ingestion of ethanol alone led to a significant up-regulation of Nox4 immunoreactive signals to 145% of the values obtained from the group that was fed with water ($p < 0.05$, see *Table 9*). Treatment with red wine containing the same concentration of ethanol (6%) did not significantly up-regulate Nox4 signals

compared to water. Correspondingly, Nox4 immunoreactivity in the red wine group was significantly lower than in the ethanol group ($p < 0.05$). Thus, specific ingredients of the red wine seem to have prevented the Nox4 up-regulation attributed to the ethanol treatment.

WUR were also treated with purple grape juice. Grape juice ingestion did not change the expression of the vascular Nox isoforms Nox1, Nox2 and Nox4 as compared to the water treatment (*Figure 7* and *Table 9*). Additionally, the treatment group receiving the sugar solution showed no significant alteration in protein levels of any investigated Nox isoform.

Spontaneously hypertensive rats (SHR) were treated with purple grape juice only. Ingestion of this beverage did not significantly affect the expression of the investigated vascular Nox isoforms in comparison to the control water (*Table 9*).

Table 9: Immunoreactivity of vascular Nox isoforms in percentage of water control (= 100%). For this table, the values of the water control in WUR were obtained from Western Blots of the red wine treatment cluster (water – red wine – ethanol). Western Blots of the other treatment cluster (water – purple grape juice – sugar solution) led to similar values. All values are given as medians [25% - 75%-interquartil range]. * displays significance in comparison to water and # in comparison to ethanol. N = 8 for WUR, n = 6 for SHR.

Animals	Treatment	Nox1	Nox2	Nox4
WUR	Water	100 [52.2 - 357.3]	100 [43.1 - 140.2]	100 [71.5 - 146.5]
	Ethanol	150.2 [127 - 232.6]	79.8 [66.3 - 185]	144.8 [135.1 - 163.9] *
	Red wine	94.9 [77.4 - 183.9]	67.9 [43.2 - 112.4]	123.2 [114.5 - 135.3] #
	Purple grape juice	126.6 [78.6 - 165.4]	134.1 [86.9 - 211.9]	114.1 [104.1 - 158.7]
	Sugar solution	83.8 [35.1 - 153.9]	112.1 [92.6 - 157.4]	145.8 [110.5 - 162.6]
SHR	Water	100 [32.4 - 301.1]	100 [55.9 - 450.8]	100 [60.1 - 122.7]
	Purple grape juice	179 [85.1 - 679]	122 [58.4 - 328.6]	109.2 [94.7 - 128.2]

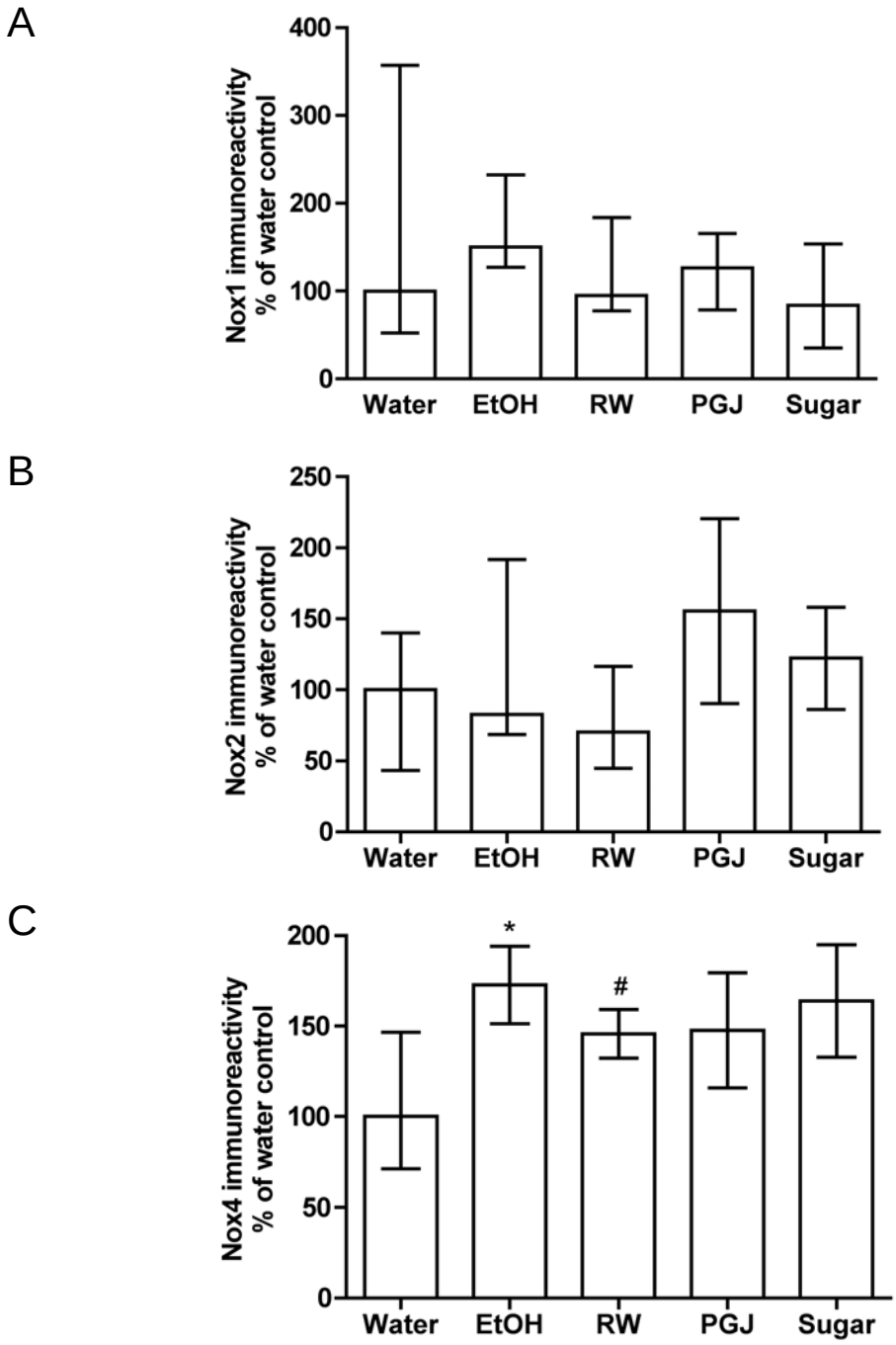


Figure 7: Purple grape products do not affect vascular Nox immunoreactivity in Wistar Unilever rat aorta *in vivo*. A. Neither EtOH and the sugar solution nor the purple grape products significantly affected Nox1 protein levels in comparison to the water control. **B.** Nox2 protein levels were not influenced by EtOH, RW, PGJ or sugar. **C.** Quantitative Western blot analysis revealed a significant up-regulation of Nox4 immunoreactivity after EtOH ingestion which did not occur in the RW group. PGJ and sugar did not significantly affect Nox4 immunoreactive signals. Values are given as medians [25% - 75%-interquartil range]. * displays statistical significance in comparison to water and # in comparison to EtOH. N = 8 animals for each group.

4.2.3 Ingestion of Purple Grape Products Does Not Influence *ex vivo* Vascular Function

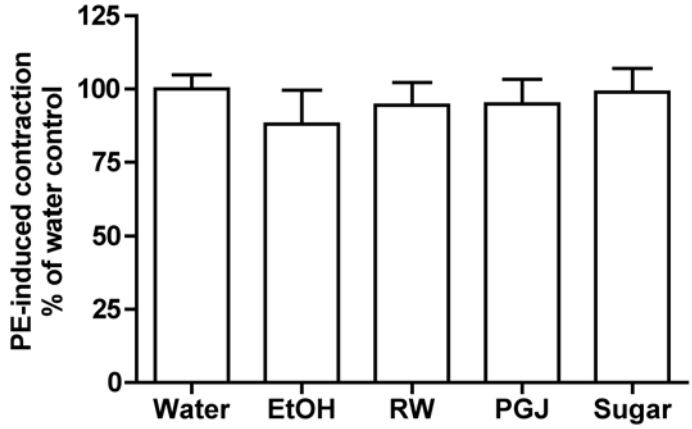
After a feeding period of 14 d, the *ex vivo* contractility of the aorta was determined by measuring phenylephrine (PE)-induced vasocontraction. Aortic rings from WUR responded similarly to the step-wise application of PE in all treatment groups and in the control group (*Figure 8A* and *Table 10*). SHR aortae displayed weaker contractility (about 20% less) than WUR aortae. However, the values of the treatment and control group in SHR did not significantly differ from each other (*Table 10).*

Endothelium-derived vasorelaxation was induced by application of the acetylcholine analogue carbachol (CCh). *Figure 8B* illustrates the CCh-induced course of vasorelaxation in percentage of the PE-induced contraction in isolated aortic rings from healthy WUR. Neither treatment with ethanol and the sugar solution nor with the purple grape products affected endothelium-dependent vasorelaxation (*Figure 8B*). *Table 10* demonstrates the EC50 values with 95% confidence interval derived from the CCh-dose-response-curves. In the ethanol group, the 95% confidence interval of the EC50 value did not overlap with that of the water group. This finding indicated a real difference between water and ethanol treatment. Nevertheless, multiple testing with the AIC led to an evidence ratio of only 1.31, i.e., the possibility that all curves are different is just 1.31 times more likely than the possibility that all curves are the same. Thus, no difference could be revealed between the groups. Furthermore, there was no remarkable difference between the groups concerning the maximum vasorelaxation (*Table 10*). In fact, the evidence ratio obtained by AIC indicated that the possibility that there is no difference between the groups is 1.2 times more probable than the alternate model with different maximum values.

In aortic rings from cardiovascular stressed SHR, CCh-induced vasorelaxation was not changed by feeding purple grape juice in comparison to water. EC50 values and maximum vasorelaxation did not differ significantly between the groups (*Table 10*).

Since no effect of purple grape products on *ex vivo* vasofunction could be revealed by these experiments, further statistical analyses of the data obtained after preincubation with the Nox inhibitor apocynin could be omitted.

A



B

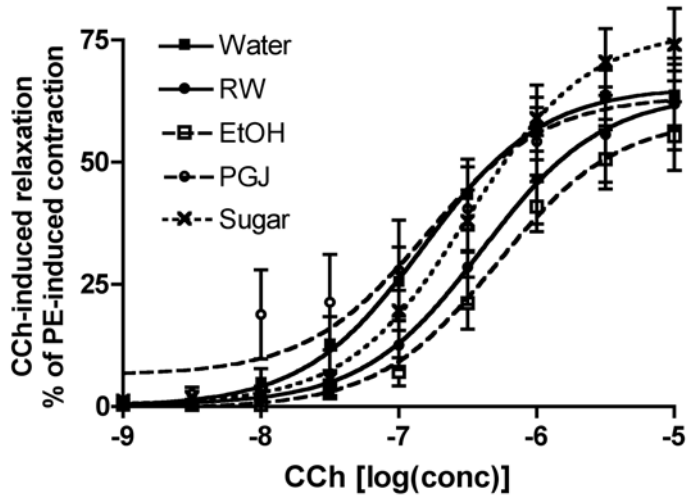


Figure 8: Vascular function is not affected by *in vivo* administration of purple grape products. A. Phenylephrine (PE)-induced contraction of Wistar Unilever rat aortic rings was not significantly affected by EtOH, RW, PGJ or the sugar solution. Values are given as means ± SEM in % of water control. **B.** Carbachol (CCh)-induced vasorelaxation was also not affected by EtOH, RW, PGJ or the sugar solution. Data points represent means ± SEM. N = 6 animals for each group.

Table 10: PE-induced vasocontraction (means ± SEM) in percentage of water control, and EC50 values (means [95% confidence interval]) as well as maximum vasorelaxation in percentage of PE-induced contraction (means [95% confidence interval]) of the CCh-dose-response-curves. N = 6 for each group.

Animals	**Treatment**	**PE-induced vasocontraction**	**CCh-induced vasorelaxation**	
			EC50 [nMol]	**Maximum relaxation**
WUR	Water	100 ± 4.9	152 [81 – 282]	65 [58 – 73]
	Ethanol	88.1 ± 11.6	521 [293 – 929]	59 [51 – 67]
	Red wine	94.4 ± 7.8	407 [185 – 891]	64 [53 – 75]
	Purple grape juice	95 ± 8.3	158 [58 – 427]	64 [53 – 74]
	Sugar solution	98.9 ± 8.1	319 [195 – 523]	77 [69 – 85]
SHR	Water	100 ± 7.9	34 [19 – 59]	67 [62 – 73]
	Purple grape juice	84.6 ± 10.6	19 [9 – 43]	67 [61 – 74]

5 DISCUSSION

5.1 The *in vitro* Role of NADPH Oxidases in the French Paradox

A reduction of oxidative stress caused by red wine polyphenols is suggested to be a key factor leading to the improved cardiovascular health in France. Oxidative stress results in NO breakdown and endothelial dysfunction, one initial event in the development of hypertension and atherosclerosis, which may result in acute events like myocardial infarction and ischemic stroke (Ross, 1999). A variety of studies support the hypothesis that a modulation of the oxidative homeostasis is at least in part responsible for the French paradox (Freedman et al., 2001; Vinson et al., 2001; Al-Awwadi et al., 2004).

The main sources of vascular ROS are xanthine oxidase, uncoupled eNOS and NADPH oxidases. Xanthine oxidase seems to be involved in cardiovascular diseases like hypertension and atherosclerosis (Erdei et al, 2006; Yamamoto et al., 2006). Nevertheless, research on this enzyme is complicated by the fact that it generates ROS as a by-product of the catalyzation of hypoxanthine / xanthine to uric acid. The role of eNOS, which acts as a contributor to oxidative stress if it is uncoupled, in the French paradox was investigated extensively. Still, the published results are controversial (Bernátová et al., 2002; Ralay Ranaivo et al., 2004). The last of the mentioned ROS sources, NADPH oxidases, represent a young field of research. Their role in the French paradox has not been investigated in detail yet. Nevertheless, the previous results are promising (Orallo et al., 2002; Ying et al, 2003). In this study, we, therefore, tried to illuminate the role of NADPH oxidases as main sources of vascular ROS in the mechanism of the French paradox.

Purple grape juice has been propagated to exhibit the same or even more pronounced beneficial effects than red wine (Vinson et al., 2001). Since it does not contain ethanol with its deleterious side effects, grape juice appears to be more appropriate for

prophylaxis of cardiovascular diseases than red wine. For that reason, we investigated the influence of both red wine and purple grape juice on vascular NADPH oxidases.

The first step was the investigation of possible effects of polyphenolic liquids on Nox expression and activity *in vitro*. Fur this purpose, the rat aortic smooth muscle cell line A7r5 was incubated with the purple grape products red wine and purple grape juice in PBS with the respective controls ethanol and PBS. Since vascular Nox isoforms are main contributors to ROS, their activity was estimated using superoxide measurement. Interestingly, both red wine and purple grape juice significantly suppressed NADPH-derived superoxide production to approximately 50%. A7r5 cells incubated with red wine produced 49.7% of the amount of superoxide measured after incubation with ethanol. Purple grape juice led to the generation of 54.3% superoxide in comparison to PBS.

As described above, Nox are not the only producers of superoxide in vasculature. Thus, a Nox inhibitor added to the cells at the maximum of light emission could indicate Nox activity by the amount of inhibition. The frequently used apocynin was chosen as the appropriate substance, because it was the most specific inhibitor available at the time these experiments were performed. This substance inhibits Nox activity in phagocytic cells by blocking the assembly of the subunits to form the active enzyme complex (Simons et al., 1990). It was also described to inhibit Nox activity in non-phagocytic cells like endothelial cells (Meyer et al., 1999), but newer data are contradictory with the last point. After examining the effect of apocynin on NADPH oxidase activity in leukocytes, endothelial cells and VSMC, Heumüller et al. (2008) supposed that apocynin predominantly acts as an antioxidant in the last two cell types.

The apocynin inhibitable part of the chemiluminescence signal was significantly reduced by treatment with red wine. It reached only 36.8% of the inhibited part after incubation with ethanol. Assuming that apocynin is a Nox inhibitor in vascular smooth muscle cells, these findings indicate that the suppressed ROS generation in A7r5 cells treated with red wine is due to a reduced Nox activity. This result was not surprising, since Deby-Dupont et al. (2005) reported a reduced Nox activity in monocytes infected with *Chlamydia pneumoniae* after 19 h incubation with diverse red wine polyphenols. However, purple grape juice did not statistically significantly

affect the apocynin inhibitable part of chemiluminescence (PGJ: 60.2 ± 13.2, PBS: 100 ± 14.4, $p = 0.07$). Thus, the decrease in ROS production cannot be explained solely by a reduced Nox activity. There are other contributors of vascular ROS, in particular uncoupled eNOS that was discussed to be implicated in the French paradox (Wallerath et al., 2002; 2003; 2005; Bernátová et al., 2002). Possibly, the reduced oxidative stress caused by purple grape products is not due to suppressed Nox activity alone, but to simultaneous effects on different enzyme systems.

We proved that purple grape products suppress the generation of superoxide in vascular smooth muscle cells, and that red wine – if apocynin indeed acts as a Nox inhibitor in this experimental setting – reduces Nox activity in the same cells. To confirm these statements, we quantified the immunoreactive signals of Nox protein in A7r5 cells after incubation with red wine, purple grape juice and the respective controls. Nox4 is the only vascular Nox isoform that is detectable in A7r5 cells with the antibodies established in our working group. We suspected that red wine would suppress Nox4 immunoreactivity[8], because it was able to diminish ROS production by a reduced Nox activity. Indeed, red wine treatment reduced Nox4 signals to 68.2% of the signal obtained after incubation with ethanol. Ethanol itself did not influence Nox4 in comparison to PBS. Surprisingly, purple grape juice led to a significant reduction of Nox4 signals to 60.4% of the levels reached by incubation with only PBS. It seems that vascular NADPH oxidases are at least involved in the effects of purple grape juice on oxidative stress, although the effect on NADPH oxidase activity was not strong enough to reach statistical significance ($p = 0.07$).

Another interesting aspect of this study is the evidence for concentration-dependency of Nox4 down-regulation by purple grape products. Both, red wine and purple grape juice decreased Nox4 immunoreactivity in a concentration-dependent matter. Red wine at a concentration of 1% was sufficient to reduce the signals significantly to 81.8% of control. The strongest reduction (44.2% of control) was achieved by a 10% concentration. In contrast, purple grape juice seems to influence Nox4 with a lower

[8] For the reason mentioned in chapter **3.2.1** we describe the signals obtained with this antibody as immunoreactive signals.

potency. While a 10% concentration down-regulated Nox4 to 60.4% of control, all concentrations up to 5% did not significantly affect Nox4 immunoreactivity.

The findings that grape juice acts with a lower potency than red wine both on protein level and in its influence on Nox activity is contradictory to a study from Fitzpatrick et al. (1993), in which different grape products were tested and compared concerning their vasorelaxing activity *in vitro*, and in which purple grape juice was a much stronger vasodilator than the red wine tested. However, the present work investigated the effects of purple grape products on cells after a 72 h incubation period, whereas Fitzpatrick et al. (1993) examined the immediate effects of the substances on vessel walls.

5.2 Discrepancy between *in vitro* and *in vivo* Findings

Our findings in the *in vitro* part of the study indicated that vascular Nox isoforms, in particular Nox4, play an important role in the French paradox. So we were encouraged to verify these results by *in vivo* application of purple grape products.

Healthy male Wistar unilever rats (WUR) are a widespread used outbred rat strain in multidisciplinary research (see the HsdCpb:WU data sheet from Harlan, www.harlaneurope.com). In addition, the cardiovascular stressed spontaneously hypertensive rats (SHR) are an established model for genetic hypertension. SHR were developed from Wistar rats by selective breeding of hypertensive animals (Lerman et al., 2005). Male SHR develop systolic blood pressures of more than 200 mmHg with an age of 8 – 10 weeks (Stasch et al., 1987). In these animals, hypertension is associated with decreased nitric oxide bioavailability due to excessive ROS generation in the vessel wall (Schnackenberg et al., 1998), and with endothelial dysfunction and impaired vasorelaxation (Zalba et al., 2000). The increased ROS production seems to originate from vascular Nox isoforms, because Nox activity and $p22^{phox}$ mRNA expression are elevated in SHR vessels (Zalba et al., 2000; Cai et al., 2003). Additionally, our working group found evidence for enhanced Nox1 and Nox2 protein levels in SHR compared to Wistar rats (Wind, 2006).

To elucidate the *in vivo* role of Nox proteins in the beneficial effect of purple grape products, we determined the immunoreactivity of three vascular Nox isoforms, Nox1, Nox2 and Nox4, following different feeding conditions including red wine and purple grape juice, with ethanol and sugar solution as additional "active" controls. Moreover, vasofunction was investigated in aortic rings of the same animals.

We chose a 50% concentration for the red wine, because it was described to be effective in literature (Bentzon et al., 2001; Vinson et al., 2001), and because pure red wine certainly does not meet the condition of "moderate alcohol consumption". Since purple grape juice was only half as potent as red wine in down-regulating Nox4 protein levels in the foregoing *in vitro* experiments, we chose a 100% concentration for the juice. Pure purple grape juice was also administered by Osman et al. (1998) without exhibiting any harmful effect in dogs and monkeys.

When healthy WUR were fed with red wine or purple grape juice for 14 days, we found no statistically significant change in the protein expression of any investigated Nox isoform. Thus, our *in vitro* findings, which revealed evidence for a decrease in Nox4 immunoreactive signals after incubation of aortic smooth muscle cells with purple grape products, could not be confirmed in the *in vivo* part of this study.

For the *in vivo* experiments, we used the same type of red wine and purple grape juice that exerted effects in the *in vitro* experiments. Apparently, there is some difference between *in vivo* and *in vitro* application of grape polyphenols. Many studies on the French paradox have investigated different parameters *in vitro* only, without verifying the results *in vivo*. In general, ingestion of polyphenolic beverages leads to polyphenol plasma levels that are much lower than the polyphenol concentrations that are achievable, when these liquids are directly applied to the cells or organ preparations. In fact, it has been reported that the bioavailability of ingested polyphenols was rather low in humans (Duthie et al., 1998) and in rats (Xu et al., 2004). To overcome the low bioavailability of polyphenolic compounds, high orally amounts are needed to achieve plasma concentrations in the range of those used in *in vitro* experiments.

In the present *in vivo* study, the animals ingested 22 ml of a 50% red wine solution per day. The mean content of polyphenols in red wine is very variable, and we do not know the definite amount of polyphenols in the red wine we used. Sarr et al. (2006)

reported that 2.9 g polyphenolic extract was produced from one litre red wine (Corbières A.O.C.). Using these data and taking into account the 1:1 dilution of the red wine in water, the daily intake of polyphenols in our experiments was about 80 mg/kg/d. However, this amount was divided into many small bulks by the drinking behaviour of the animals during 24 h. As a result, the intragastral amounts of polyphenolic compounds remained constantly low in our test animals, and accordingly low were presumably the plasma levels of the polyphenols. Diebolt et al. (2001), who found an enhanced vasorelaxing effect with an extract of red wine polyphenols in Wistar rats, directly administered this extract into the stomach of rats. So the intragastral concentrations of polyphenols had one high peak each day. It cannot be ruled out that in the present study plasma concentrations of the food-derived polyphenols were constantly too low to exhibit significant effects on Nox protein expression.

While we did not find any *down*-regulation in protein expression of the vascular ROS producers Nox1, Nox2 and Nox4 after treating male WUR with purple grape products, we found an *up*-regulation of the Nox4 immunoreactive signals in the ethanol group. This elevation of the Nox4 immunoreactive signals was statistically significant as compared to the water control and, in addition, to red wine (both groups belonged to one treatment cluster together with the ethanol group). To our knowledge, an increase in Nox4 protein expression has not yet been described in the literature. Polikandriotis et al. (2006), who investigated the harmful effects of alcohol, reported an increase in Nox2 protein expression in lung tissue of Spraque Dawley rats after administration of ethanol for six weeks, which had also resulted in enhanced pulmonal superoxide generation. In contrast, we did *not* find a change in Nox2 protein expression. That discrepancy may be attributed to the different organs investigated, i.e. aortic tissue in our study and lung tissue in that of Polikandriotis et al. (2006). Furthermore, the amount of ethanol in the described study was rather high with 36% of the daily-ingested calories in a liquid diet.

Our findings that ethanol but not red wine treatment enhances Nox4 immunoreactive signals suggest that some components of the red wine are able to prevent the harmful effect of ethanol. The most favourite candidates for these components are surely the polyphenols. In this context, the result of a normal Nox4 expression in animals treated

with red wine as compared to ethanol appears to contradict our previous assumption that oral administration of red wine would not result in sufficient polyphenol plasma levels. Possibly, metabolization of ethanol or its effect on Nox protein is altered in the presence of accompanying polyphenols. Another explanation derives from the aforementioned study of Sarr et al. (2006). The authors reported an inhibition of angiotensin II-induced Nox1 protein overexpression in Wistar rats after ingestion of red wine polyphenols (administered in the drinking water), but no change of Nox1 expression in animals not pretreated with angiotensin II. In other words, red wine polyphenols did only affect pathologically enhanced Nox1 protein levels and had no effect on normal Nox1 expression in healthy animals (Sarr et al., 2006). These findings of Sarr et al. (2006) are in agreement with our results. We found elevated Nox4 immunoreactive signals in rats stressed with ethanol, which did not occur when ethanol was accompanied by the non-alcoholic components of red wine. While angiotensin II elevates Nox1, ethanol increases Nox4 signals. In both cases, red wine polyphenols and / or red wine itself are apparently able to prevent the overexpression of these Nox proteins.

If the observed difference in Nox4 immunoreactivity was in fact caused by the polyphenols in red wine, then these polyphenols must have reached the endothelium in levels sufficient to prevent the overexpression of Nox protein. The polyphenol plasma level, however, might not be high enough to significantly reduce the normal expression of the Nox proteins assessed.

SHR, which were used in this study as a model for cardiovascular diseases, show an overexpression of Nox1 and Nox2 protein in aorta (Wind, 2006). In this context, SHR are very similar to angiotensin II-treated rats that were shown to overexpress Nox1 (Sarr et al., 2006). Unfortunately, for animal welfare reasons, we were not allowed to treat these cardially stressed hypertensive animals with the potentially harmful red wine (cf. chapter **3.1**). So we do not know, whether red wine would also reduce the pathological Nox overexpression in these animals. Moreover, by accident[9], we did not

[9] Enough animals were ordered from Harlan Winkelmann GmbH in Borchen, Germany. Half of them arrived dead in one cage. The other twelve animals were healthy, which was attested by the veterinarian of the animal house ("Zentrales Tierlabor"), Dr. Ina Schepp. After the usual adaption period they were assigned to two instead of three treatment groups.

have enough animals to form a third treatment group to control the sugar intake. Since ingestion of the sugar solution did not result in any effect on Nox protein expression in WUR, there would probably be no alteration in protein levels in SHR, too.

Nevertheless, no statistically significant change in Nox protein expression was apparent in SHR treated with purple grape juice when compared to water treatment. There are three possible explanations for this finding, and none of them appears more reasonable than the other: (1) The amount of absorbed polyphenols was to small to exert significant effects on the pathological Nox expression in these animals. (2) Purple grape juice polyphenols are not able to affect Nox overexpression in the same way as red wine polyphenols. (3) SHR do not react like angiotensin II-treated rats with respect to their overexpression of Nox proteins.

5.3 Organ Bath Studies on Aortic Vasofunction

In order to give our findings concerning Nox protein expression functional relevance, we performed *ex vivo* vessel studies using the same animals[10]. NADPH oxidases produce ROS and are involved in oxidative stress, which in turn is linked to endothelial dysfunction. In organ bath experiments, we assessed endothelial dysfunction by impairment of endothelium-derived vasorelaxation in response to carbachol (CCh). This synthetic compound is an analogue of the natural transmitter acetylcholine and leads to an endothelial NO release via muscarinerg M3-receptors. If purple grape polyphenols would have beneficial vascular effects, then vasorelaxation should be improved compared to control. Conversely, in animals not treated with polyphenolic liquids, inferior vasorelaxations should indicate the deleterious effects of ROS-producing enzymes like Nox proteins. In order to confirm, whether potential differences among groups were due to Nox proteins, the tension measurements have been repeated in the presence of the Nox inhibitor apocynin. Thus, Nox-dependent differences would vanish.

[10] For practical reasons, organ bath studies were performed immediately after dissecting the aortae. Protein determination was done afterwards with the rest of the thoracic aortae.

In this experimental setting it was important to verify that precontraction with phenylephrine (PE) was not influenced by different treatments of animals. In fact, we found no significant difference between the groups with regard to PE-induced contraction. Thus, contraction of aortic rings was not influenced by a possibly altered Nox protein activity or other interfering effects, including ethanol.

In a recently published paper, Tirapelli et al. (2006) reported an increased contraction after treating Wistar rats with ethanol for two or more weeks, contrary to our study in which neither ethanol nor red wine had an effect on PE-induced vasocontraction. That discrepancy may be due to the different amounts of ethanol used for feeding. Tirapelli et al. (2006) administered 20% ethanol in water, whereas we burdened the animals just with 6% ethanol, either alone or in the form of red wine. The concentration of 6% was considered to be a relatively realistic model for the simulation of modest red wine consumption. Feeding in this manner corresponds to a drinking behaviour in which half of the ingested fluid is red wine or a comparable alcoholic beverage. In contrast, Tirapelli et al. (2006) tried to mimic alcohol abuse rather than modest consumption – the amount of ethanol corresponding to spirits being half of the ingested fluid.

Since there were no significant differences in PE-induced contraction among the treatment groups, the subsequent experiments on CCh-induced vasorelaxation due to NO could be adequately analysed for real treatment effects. Overall, we found no beneficial effects on aortic vasofunction in association with feeding of purple grape products for 14 days. These findings were in accordance with our later obtained results for Nox immunoreactivity.

Considering our protein expression data, CCh-induced vasorelaxation should only be impaired in the animals treated with ethanol, because only ethanol led to an increase of the expression of ROS-producing Nox4 protein. A measure for the degree of impairment is given by the EC50 value obtained from the dose-response-curve. Hence, EC50 is a measure of the potency of CCh to induce vasorelaxation. If its potency is reduced, higher concentrations of the drug are needed to produce the same degree of vasorelaxation, resulting in a higher EC50 value. A reduction in CCh potency can be due to a decreased NO bioavailability under conditions of oxidative stress. In fact, the concentration of CCh needed to produce half maximal relaxation (EC50) was markedly increased (3.4-fold) solely in the ethanol group as compared to

the water control with no overlap in the 95% confidence interval. Since four parallel treatment groups had to be compared to one control group, a global statistic method including all five groups had to be used. We, therefore, applied Akaike's Information Criterion (AIC) to the five data sets, and found that a global model implying different EC50 values was only slightly more probable (1.3-fold) than an alternative model using the assumption that all EC50 values were the same. These findings might be conflicting with respect to the ethanol group, which showed a significant elevation of Nox4 signals. However, an alteration of protein immunoreactivity determined by Western Blotting is not an evidence for a change in protein activity determined by functional experiments like vessel studies.

The results obtained with SHR correspond to those obtained with WUR, even though at different levels. First of all, PE-induced maximum contraction was lower in SHR than in WUR aortic rings. This well-known observation is related to the thickened and more rigid vessel walls of the hypertensive animals (Gendron et al., 2004). When endothelium-derived vasorelaxation was investigated, the EC50 value, but not the maximum relaxation, was considerably lower than in WUR. The increased sensitivity to CCh was accompanied by an inverse effect of CCh on vasorelaxation at concentrations higher than 1 µM. The drug itself possibly caused this decline in relaxation. These findings confirm a study published by Yang et al. (2004). In the course of their studies on endothelium-derived contractions due to the parasympathic acetylcholine in high concentrations in aortae from SHR and normotensive controls, they found acetylcholine-induced vasocontraction in aortic rings from SHR but not from Wistar rats (Yang et al., 2004). Nevertheless, our results clearly show that treatment with purple grape juice for 14 days did not improve NO-dependent vasorelaxation in SHR aortic rings as compared to water control.

While our findings did not indicate a change in endothelium-derived vasorelaxation caused by red wine or purple grape juice, different other studies have demonstrated an improved vasorelaxation evoked by red wine with and without alcohol as well as different other polyphenolic grape products, but not ethanol alone (Fitzpatrick et al., 1993; Zenebe et al., 2003; Duarte et al., 2004). However, these experiments were all performed *in vitro*, and the examined substances were directly applied to the vessel rings. Thus, polyphenols were present at relatively high concentrations and, because

of their antioxidant properties (Tapiero et al., 2002), could act directly against ROS, thereby enhancing NO bioavailability. In our study, a change in vasorelaxation would result from diet-induced intracellular modification of the protein expression, which in turn would affect the function of the endothelium.

In other studies, red wine extracts were orally administered to Wistar rats. These extracts were given either by intragastral gavage at a dose of 20 mg/kg/d for 7 d (Diebolt et al., 2001) or in the drinking water at a dose of 150 mg/kg/d for three weeks (Sarr et al., 2006). Diebolt et al. (2001) could demonstrate an ameliorated *ex vivo* vasorelaxation in healthy rats. Sarr et al. (2006) reported such an improvement in animals showing endothelial dysfunction due to angiotensin II treatment, but not in healthy rats, although they treated with a very similar red wine extract as Diebolt et al. (2001) for a period three times as long. As considered in chapter **5.2**, the different outcomes with respect to endothelium-derived vasorelaxation in healthy rats of these two studies and our work might be due to the different manners of administration (intragastral application with daily peaks [Diebolt et al., 2001] versus administration in drinking water with constantly low intragastral levels [this work; Sarr et al., 2006]). In addition, there is some doubt that results obtained with polyphenolic extracts are directly comparable to those obtained with native red wine. These extracts, which are produced by methods like freeze-drying from selected vine varieties (van Golde et al., 2004), may show a higher effectiveness than native red wine polyphenols that are embedded in a complex system of hundreds of different compounds.

In literature, there is evidence for a beneficial effect of purple grape juice on vasculature, but only few papers report on both purple grape juice and red wine. Fitzpatrick et al. (1993) found a similar endothelium-dependent vasorelaxation following direct *in vitro* application of both substances on rat aortic rings. We could not confirm these findings in *ex vivo* organ bath studies. In this study, purple grape juice did not influence vasofunction in WUR and SHR, neither regarding PE-induced contraction nor CCh-induced endothelium-derived relaxation.

5.4 Relevance of NADPH Oxidases in the French Paradox

In this study, we provided evidence for the *in vitro* effects of the purple grape products red wine and purple grape juice on Nox activity and Nox4 immunoreactivity. However, we could not confirm these findings *in vivo*, neither in healthy Wistar rats nor in cardiovascular stressed SHR. Moderate consumption of red wine or purple grape juice did not reduce the immunoreactive signals of Nox1, Nox2 or Nox4 protein in rat aortae. Moreover, both red wine and purple grape juice failed to exert a beneficial effect on endothelium-derived vasorelaxation. The only potential benefit of red wine polyphenols observed was the prevention of an overexpression of Nox4, which was induced when ethanol alone was given to Wistar rats.

As mentioned above, many publications concerning the French paradox investigated different parameters *in vitro* without verifying them *in vivo*. Especially in vasofunction experiments, it is easier to apply the substances of interest directly on the vessels. Since polyphenols have antioxidant properties and thereby enhance NO bioavailability, it is not surprising that they improve vasorelaxation. This may also occur *in vivo* after oral administration. However, in experimental settings designed to show a persistent effect of the ingested polyphenols on vasofunction, the direct actions of antioxidants are irrelevant. In these cases, an improvement in vasofunction can be derived from certain changes in the vessel wall. Such an improved vasorelaxation in rat aortic rings *ex vivo* has been reported by Diebolt el al. (2001) and Sarr et al. (2006). However, in these studies polyphenolic extracts were used. In this context it seems noteworthy that many biological properties of polyphenols and their metabolites are not precisely known yet. Thus, a systematic research on the impact of single polyphenols or special combinations of them on the French paradox is still difficult. That will become apparent if we bring to mind the great and almost confusing variety of investigated substances in French paradox research. In many cases it is not clear, why just these specific polyphenols have been used (e.g. Leikert et al., 2002; Benito et al., 2004), suggesting that the selection was done rather at random than scientifically justified.

In the present study, the animals were treated with native red wine instead of polyphenolic extracts. Moreover, the amounts delivered were comparable rather to

modest than to excessive red wine consumption, thus mimicking the drinking customs of the French more realistically. In doing so, we found no indications of an improved vasorelaxation of rat aortic rings *ex vivo*, and there was also no significant decrease in aortic Nox protein expression *in vivo*. Hence, the question arises, whether vascular NADPH oxidases are in fact involved in the beneficial effects that have been attributed to the red wine. Despite of this, there are many findings which may support this assumption, even though Nox isoforms are a very young field of research. The scientific rationale for the research on Nox isoforms is based on the generally accepted concept that oxidative stress plays a crucial role in cardiovascular morbidity.

Orallo et al. (2002) were the first who implicated NADPH oxidases in the French paradox. They reported that the polyphenol resveratrol has caused a decrease of NADPH-dependent superoxide production in aortic tissue homogenates. Shortly after, Ying et al. (2003) demonstrated that green and black tea polyphenols reduced $p22^{phox}$ and $p67^{phox}$ protein expression *in vitro*. The subunit $p22^{phox}$ is known to be essential for activity of all vascular Nox isoforms (Azumi et al., 1999), but $p67^{phox}$ only seems to be required for Nox2 activation (Nisimoto et al., 1999; Takeya et al., 2003; Ambasta et al., 2004; Martyn et al., 2006). Although regulatory subunits are required for the activation of vascular Nox isoforms, their expression alone is not sufficient to produce ROS. Furthermore, an enhanced $p22^{phox}$ expression can be associated with all vascular Nox isoforms, so an exact characterization of the specific involved Nox isoform is hard to prove. Thus, the interpretation of the findings of Ying et al. (2003) is difficult. Recently, new antibodies raised against the catalytic vascular subunits Nox1 and Nox4 were generated (Wingler et al., 2001). Additionally, there are some different commercial Nox2 antibodies. Hence, it seems reasonable to examine the impact of the catalytic Nox isoforms itself instead of only that of their regulatory subunits. Apart from our present study, there is just one other work that has assessed the role of a catalytic subunit (Nox1) in the French paradox (Sarr et al., 2006). With the present study, we investigated the *in vitro* and *in vivo* effect of the polyphenolic beverages red wine and purple grape juice on *all* catalytic Nox isoforms that were known to occur in vessels at this time.

The fact that we could not confirm the findings of our *in vitro* experiments, which indicated an involvement of NADPH oxidases in the French paradox, *in vivo* might

favourite a totally different hypothesis for the French paradox. Law and Wald (1999), for example, demonstrated that mortality from cardiovascular diseases was mainly correlated to the intake of animal fat. Some decades before, the consumption of animal fat was rather low in France. But the fraction of animal fat in the typical French diet has enhanced over the last decades. Law and Wald (1999) stated that there is a time lag between the increase in animal fat intake and the following increase in cardiovascular mortality. This time lag could be responsible for the apparent paradox, because today's mortality corresponds to animal fat intake of 30 years ago (Law and Wald, 1999). Provided that the time lag hypothesis is true, then the French paradox should diminish within the next few decades.

Although the French paradox is commonly related to red wine consumption, this association may be confounded by other parameters, because red wine drinkers tend to be less fat, to exercise more, and to drink with meals (Goldberg et al., 2001). The latter aspect seems to be of importance, because wine consumption has been shown to reduce the postprandial endothelial dysfunction induced by a high fat meal (Blanco-Colio et al., 2000). The acute administration of red wine along with a high-fat meal reduced the increase in NF-κB, a nuclear transcription factor that induces several genes for inflammatory proteins including C-reactive protein (CRP). This effect was not seen with vodka, the other alcoholic beverage investigated in this study (Blanco-Colio et al., 2000).

Finally, the data of two large studies strongly exclude a selective benefit from red wine in the American population (Klatsky et al., 1997; Gaziano et al., 1999). Klatsky et al. (1997) followed mortality in 128934 Californians enrolled in a health maintenance organization for 13 years with respect to their total and type of alcohol consumption. At 13 years of follow-up, there was no evidence for any selective effect of red wine (Klatsky et al., 1997). In a Boston area case-control study of 340 subjects, Gaziano et al. (1999) followed the incidence of myocardial infarction according to the type of alcohol consumed. Again, no significant differences were observed by the type of alcohol.

Nevertheless, there is cumulating evidence that the ingestion of polyphenolic foodstuff and beverages has some beneficial impact on cardiovascular health, at least if these compounds are ingested in pharmacological effective doses. Polyphenols have

been shown to exhibit specific effects on different vascular parameters. One of these effects is the inhibition of Nox activity and Nox4 protein expression *in vitro*, as demonstrated in this study. Modest consumption of red wine alone may not be sufficient to produce significant cardioprotective effects on the investigated parameters *in vivo*. This assumption is supported by our experiments.

Thus, the origin of the French paradox cannot be explained by vascular NADPH oxidases. Hitherto, other experimental designs failed to conclusively identify possible pathways, too. Further investigation is required in this appealing field of research to bring light in the molecular mechanisms of the French paradox.

6 SUMMARY

The term French paradox stands for the epidemiologically proven fact that the French suffer less from cardiovascular morbidity and mortality than other people with a comparable fat intake. This paradox was attributed to the rather high red wine consumption of the French. Several studies have stated a beneficial influence of purple grape products on oxidative stress. Since NADPH oxidases are one of the main contributors to vascular reactive oxygen species, we experimentally examined the effect of the purple grape products red wine and purple grape juice on Nox protein and activity *in vitro* and *in vivo*.

The immunoreactivity of the vascular Nox isoforms Nox1, Nox2 and Nox4 in rat aortic tissue homogenates and of Nox4 in rat vascular smooth muscle cells (VSMC) following administration of red wine and / or purple grape juice was assessed. Nox activity was determined via measurement of superoxide production in VSMC and by performing isometric tension measurements on isolated rat thoracic aorta segments. The *in vivo* experiments were performed in healthy Wistar unilever rats (WUR) and in spontaneously hypertensive rats (SHR), which serve as a disease model for genetic hypertension.

In vitro incubation with red wine and / or purple grape juice led dose-dependently to decreased Nox4 immunoreactive signals and to a lower superoxide production in VSMC. The apocynin inhibitable part of superoxide generation, a measure for Nox activity, was reduced by red wine only. However, oral treatment with both purple grape products did not significantly affect Nox protein levels in WUR. Surprisingly, ingestion of ethanol resulted in Nox4 overexpression, which did not occur with red wine. *Ex vivo* endothelium-derived vasorelaxation in organ bath chambers was not influenced by ingestion of purple grape products in WUR. Treating SHR with purple grape juice affected neither Nox protein expression nor vasofunction.

The findings of this study strongly point out the discrepancy between *in vitro* and *in vivo* experiments in French paradox research. While direct application of purple grape products on VSMC had distinct effects on both Nox activity and protein expression,

in vivo administration of the same liquids did not affect Nox protein expression and vasofunction. Thus, the origin of the French Paradox cannot be explained by NADPH oxidases. Since other experimental designs also were not able to conclusively identify possible pathways, the molecular mechanism of the French paradox is still unclear.

7 ZUSAMMENFASSUNG

Das „Französische Paradox'" bezeichnet die Beobachtung, dass Frankreichs Bevölkerung im Vergleich zu anderen Nationen trotz eines ähnlich hohen alimentären Fettanteils eine geringere kardiovaskuläre Morbidität und Mortalität aufweist. Dieses Paradox wird mit dem ausgeprägteren Rotweinkonsum in Frankreich erklärt. Diverse Studien konnten eine positive Wirkung von Rotwein auf den für kardiovaskuläre Erkrankungen mitverantwortlichen oxidativen Stress nachweisen. NADPH-Oxidasen stellen eine der Hauptquellen für reaktive Sauerstoffspezies im Gefäßsystem dar. Daher untersuchten wir in dieser Studie den Einfluss von Rotwein und rotem Traubensaft auf die Expression und Aktivität von NADPH-Oxidasen *in vitro* und *in vivo*.

Nach Inkubation glatter Gefäßmuskelzellen mit Rotwein, Traubensaft bzw. den entsprechenden Kontrollen wurde die NADPH-abhängige Bildung von Superoxid in An- und Abwesenheit des Nox-Inhibitors Apocynin sowie die Immunoreaktivität von Nox4 im Western Blot bestimmt. Des Weiteren wurden gesunde Wistar-Unilever-Ratten (WUR) mit den oben genannten Substanzen behandelt, spontan hypertensive Ratten (SHR) erhielten Traubensaft bzw. Wasser als Kontrolle. Die Aktivität der NADPH-Oxidasen wurde auf indirektem Wege *ex vivo* an Aortensegmenten im Organbad untersucht. Ferner wurde die Proteinexpression der vaskulären Nox-Isoformen Nox1, Nox2 und Nox4 quantifiziert.

Die Inkubation glatter Gefäßmuskelzellen sowohl mit Rotwein als auch mit rotem Traubensaft führte dosisabhängig zu verminderten Nox4-Signalen im Western Blot sowie zu einer verringerten Bildung von Superoxid. Der Anteil der Superoxidbildung, der durch zusätzliche Applikation von Apocynin gehemmt werden konnte – ein Maß für die Aktivität der NADPH-Oxidasen –, wurde lediglich durch Rotwein reduziert, während roter Traubensaft keinen signifikanten Einfluss zeigte. Nach Behandlung gesunder Ratten mit den gleichen Substanzen konnte hingegen keine Veränderung der Expression der vaskulären Nox-Isoformen Nox1, Nox2 und Nox4 nachgewiesen werden. Überraschenderweise führte jedoch die Gabe von Ethanol zu einer

signifikanten Überexpression von Nox4, die nach Rotweingabe nicht auffiel. Auch auf die Ergebnisse der *ex vivo* durchgeführten Gefäßstudien wirkte sich eine vorherige Behandlung mit Rotwein oder Traubensaft nicht aus. Die Behandlung von SHR mit Traubensaft beeinflusste im Vergleich zur Kontrolle weder die Nox-Proteinexpression noch die Gefäßreaktivität.

Die Ergebnisse dieser Studie verdeutlichen die Diskrepanz zwischen *in vitro*- und *in vivo*-Experimenten, inbesondere in der Frage nach möglichen Ursachen für das „Französische Paradox". Während die direkte Applikation von Rotwein und Traubensaft sowohl die Aktivität als auch die Proteinexpression der NADPH-Oxidasen beeinträchtigte, konnten wir nach *in vivo*-Gabe derselben Substanzen keinerlei Reaktionen nachweisen. Somit scheint das „Französische Paradox" über NADPH-Oxidasen nicht erklärbar zu sein. Auch andere experimentelle Ansätze konnten bisher nicht schlüssig mögliche, zu Grunde liegende Wege aufdecken. Die molekularen Mechanismen des „Französischen Paradox" bleiben daher weiterhin offen.

8 REFERENCES

1. Afanas'ev I (2004). "Interplay between superoxide and nitric oxide in aging and diseases." Biogerontology 5(4): 267-70.

2. Al-Awwadi NA, Bornet A, Azay J, Araiz C, Delbosc S, Cristol JP, Linck N, Cros G, Teissedre PL (2004). "Red wine polyphenols alone or in association with ethanol prevent hypertension, cardiac hypertrophy, and production of reactive oxygen species in the insulin-resistant fructose-fed rat." J Agric Food Chem 52(18): 5593-7.

3. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP (2004). "Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase." J Biol Chem 279(44): 45935-41.

4. Ames BN, Shigenaga MK, Hagen TM (1993). "Oxidants, antioxidants, and the degenerative diseases of aging." Proc Natl Acad Sci USA 90(17): 7915-22.

5. Aziz MH, Kumar R, Ahmad N (2003). "Cancer chemoprevention by resveratrol: *in vitro* and *in vivo* studies and the underlying mechanisms (review)." Int J Oncol 23(1): 17-28.

6. Azumi H, Inoue N, Takeshita S, Rikitake Y, Kawashima S, Hayashi Y, Itoh H, Yokoyama M (1999). "Expression of NADH/NADPH oxidase p22phox in human coronary." Circulation 100(14): 1494-8.

7. Babior BM (2002). "The leukocyte NADPH oxidase." Isr Med Assoc J 4(11): 1023-4.

8. Bánfi B, Malgrange B, Knisz J, Steger K, Dubois-Dauphin M, Krause KH (2004). "NOX3, a superoxide-generating NADPH oxidase of the inner ear." J Biol Chem 279(44): 46065-72.

9. Bánfi B, Molnár G, Maturana A, Steger K, Hegedûs B, Demaurex N, Krause KH (2001). "A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes." J Biol Chem 276(40): 37594-601.

10. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990). “Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide.“ Proc Natl Acad Sci USA 87(4): 1620-4.

11. Beckman KB, Ames BN (1998). “The free radical theory of aging matures.“ Physiol Rev 78(2): 547-81.

12. Benito S, Buxaderas S, Mitjavila MT (2004). “Flavonoid metabolites and susceptibility of rat lipoproteins to oxidation.“ Am J Physiol Heart Circ Physiol 287(6): H2819-24.

13. Bentzon JF, Skovenborg E, Hansen C, Møller J, de Gaulejac NS, Proch J, Falk E (2001). “Red wine does not reduce mature atherosclerosis in apolipoprotein E-deficient mice.“ Circulation 103(12): 1681-7.

14. Bernátová I, Pechánová O, Babál P, Kyselá S, Stvrtina S, Andriantsitohaina R (2002). ”Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension.“ Am J Physiol Heart Circ Physiol 282(3): H942-8.

15. Blanco-Colio LM, Valderrama M, Alvarez-Sala LA, Bustos C, Ortego M, Hernández-Presa MA, Cancelas P, Gómez-Gerique J, Millán J, Egido J (2000). “Red wine intake prevents nuclear factor-kappaB activation in peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia.” Circulation 102(9): 1020-6.

16. Byrne JA, Grieve DJ, Bendall JK, Li JM, Gove C, Lambeth JD, Cave AC, Shah AM (2003). “Contrasting roles of NADPH oxidase isoforms in pressure-overload versus angiotensin II-induced cardiac hypertrophy.“ Circ Res 93(9): 802-5.

17. Cai H, Griendling KK, Harrison DG (2003). “The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases.“ Trends Pharmacol Sci 24(9): 471-8.

18. Cheng G, Cao Z, Xu X, van Meir EG, Lambeth JD (2001). “Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5.“ Gene 269(1-2): 131-40.

19. Cheng G, Lambeth JD (2004). “NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain.“ J Biol Chem 279(6): 4737-42.

20. Christon R, Marette A, Badeau M, Bourgoin F, Mélançon S, Bachelard H (2005). “Fatty acid-induced changes in vascular reactivity in healthy adult rats.” Metabolism 54(12): 1600-9.

21. Davis KL, Martin E, Turko IV, Murad F (2001). “Novel effects of nitric oxide.“ Annu Rev Pharmacol Toxicol 41: 203-36.

22. De Lorgeril M, Salen P, Martin JL, Boucher F, Paillard F, de Leiris J (2002). “Wine drinking and risks of cardiovascular complications after recent acute myocardial infarction.“ Circulation 106(12): 1465-9.

23. Deby-Dupont G, Mouithys-Mickalad A, Serteyn D, Lamy M, Deby C (2005). “Resveratrol and curcumin reduce the respiratory burst of Chlamydia-primed THP-1 cells.“ Biochem Biophys Res Commun 333(1): 21-7.

24. Díaz V, Cumsille MA, Bevilacqua JA (2003). “Alcohol and hemorrhagic stroke in Santiago, Chile. A case-control study.“ Neuroepidemiology 22(6): 339-44.

25. Diebolt M, Bucher B, Andriantsitohaina R (2001). “Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression.“ Hypertension 38(2): 159-65.

26. Duarte J, Andriambeloson E, Diebolt M, Andriantsitohaina R (2004). “Wine polyphenols stimulate superoxide anion production to promote calcium signaling and endothelial-dependent vasodilatation.“ Physiol Res 53(6): 595-602.

27. Dupuy C, Ohayon R, Valent A, Noël-Hudson MS, Dème D, Virion A (1999). “Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cdnas.“ J Biol Chem 274(52): 37265-9.

28. Duthie GG, Pedersen MW, Gardner PT, Morrice PC, Jenkinson AM, McPhail DB, Steele GM (1998). “The effect of whisky and wine consumption on total phenol content and antioxidant capacity of plasma from healthy volunteers.” Eur J Clin Nutr 52(10): 733-6.

29. Erdei N, Tóth A, Pásztor ET, Papp Z, Edes I, Koller A, Bagi Z (2006). "High-fat diet-induced reduction in nitric oxide-dependent arteriolar dilation in rats: role of xanthine oxidase-derived superoxide anion." Am J Physiol Heart Circ Physiol 291(5): H2107-15.

30. Estler CJ, Schmidt HH (2006). "Pharmakologie und Toxikologie. Lehrbuch für Studium und Praxis." Schattauer-Verlag, ISBN: 3-7945-2295-8.

31. Ferrario CM, Strawn WB (2006). "Role of the renin-angiotensin-aldosterone system and proinflammatory mediators in cardiovascular disease." Am J Cardiol 98(1): 121-8.

32. Fitzpatrick DF, Hirschfield SL, Coffey RG (1993). "Endothelium-dependent vasorelaxing activity of wine and other grape products." Am J Physiol 265(2 Pt 2): H774-8.

33. Freedman JE, Parker C 3rd, Li L, Perlman JA, Frei B, Ivanov V, Deak LR, Iafrati MD, Folts JD (2001). "Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release." Circulation 103(23): 2792-8.

34. Frémont L, Gozzélino MT, Franchi MP, Linard A (1998). "Dietary flavonoids reduce lipid peroxidation in rats fed polyunsaturated or monounsaturated fat diets." J Nutr 128(9): 1495-502.

35. Furchgott RF (1998). "Endothelium-Derived Relaxing Factor: Discovery, Early Studies, and Identification as Nitric Oxide." Nobel Lectures, Physiology or Medicine 1996-2000, World Scientific Publishing Co., ISBN 981-238-005-1.

36. Gaziano JM, Hennekens CH, Godfried SL, Sesso HD, Glynn RJ, Breslow JL, Buring JE (1999). "Type of alcoholic beverage and risk of myocardial infarction." Am J Cardiol 83(1): 52-7.

37. Geisterfer AA, Peach MJ, Owens GK (1988). "Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells." Circ Res 62(4): 749-56.

38. Geiszt M, Kopp JB, Várnai P, Leto TL (2000). "Identification of renox, an NAD(P)H oxidase in kidney." Proc Natl Acad Sci USA 97(14): 8010-4.

39. Gendron G, Gobeil F Jr, Morin J, D'Orléans-Juste P, Regoli D (2004). "Contractile responses of aortae from WKY and SHR to vasoconstrictors." Clin Exp Hypertens 26(6): 511-23.

40. Goldberg IJ, Mosca L, Piano MR, Fisher EA (2001). "Wine and your heart: a scientific advisory for healthcare professionals from the Nutrition Committee, Council on Epidemiology and Prevention, and Council on Cardiovascular Nursing of the American Heart Association." Circulation 103(3): 472-5.

41. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW (1994). "Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells." Circ Res 74(6): 1141-8.

42. Griendling KK, Sorescu D, Ushio-Fukai M (2000). "NAD(P)H oxidase: role in cardiovascular biology and disease." Circ Res 86(5): 494-501.

43. Griffith OW, Stuehr DJ (1995). "Nitric oxide synthases: properties and catalytic mechanism." Annu Rev Physiol 57: 707-36.

44. Groemping Y, Lapouge K, Smerdon SJ, Rittinger K (2003). "Molecular basis of phosphorylation-induced activation of the NADPH oxidase." Cell 113(3): 343-55.

45. Grønbaek M, Becker U, Johansen D, Gottschau A, Schnohr P, Hein HO, Jensen G, Sørensen TI (2000). "Type of alcohol consumed and mortality from all causes, coronary heart disease, and cancer." Ann Intern Med 133(6): 411-9.

46. Guthikonda S, Sinkey C, Barenz T, Haynes WG (2003). "Xanthine oxidase inhibition reverses endothelial dysfunction in heavy smokers." Circulation 107(3): 416-21.

47. Hemmens B, Mayer B (1998). " Enzymology of nitric oxide synthases." Methods Mol Biol 100: 1-32.

48. Heumüller S, Wind S, Barbosa-Sicard E, Schmidt HH, Busse R, Schröder K, Brandes RP (2008). "Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant." Hypertension 51(2): 211-7.

49. Hines LM, Stampfer MJ, Ma J, Gaziano JM, Ridker PM, Hankinson SE, Sacks F, Rimm EB, Hunter DJ (2001). "Genetic variation in alcohol dehydrogenase and the beneficial effect of moderate alcohol consumption on myocardial infarction." N Engl J Med 344(8): 549-55.

50. Hwang J, Ing MH, Salazar A, Lassègue B, Griendling K, Navab M, Sevanian A, Hsiai TK (2003). "Pulsatile versus oscillatory shear stress regulates NADPH oxidase subunit expression: implication for native LDL oxidation." Circ Res 93(12): 1225-32.

51. Iso H, Baba S, Mannami T, Sasaki S, Okada K, Konishi M, Tsugane S; JPHC Study Group (2004). "Alcohol consumption and risk of stroke among middle-aged men: the JPHC Study Cohort I." Stroke 35(5): 1124-9.

52. Klatsky AL, Armstrong MA, Friedman GD (1997). "Red wine, white wine, liquor, beer, and risk for coronary artery disease hospitalization." Am J Cardiol 80(4): 416-20.

53. Kritharides L, Stocker R (2002). "The use of antioxidant supplements in coronary heart disease." Atherosclerosis 164(2): 211-9.

54. Kuribayashi F, Nunoi H, Wakamatsu K, Tsunawaki S, Sato K, Ito T, Sumimoto H (2002). "The adaptor protein p40(phox) as a positive regulator of the superoxide-producing phagocyte oxidase." EMBO J 21(23): 6312-20.

55. Kusaka I, Kusaka G, Zhou C, Ishikawa M, Nanda A, Granger DN, Zhang JH, Tang J (2004). "Role of AT1 receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury." Am J Physiol Heart Circ Physiol 286(6): H2442-51.

56. Laemmli UK (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature 227(5259): 680-5.

57. Lambeth JD (2004). “NOX enzymes and the biology of reactive oxygen.“ Nat Rev Immunol 4(3): 181-9.

58. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG (2003). “Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension.” J Clin Invest 111(8): 1201-9.

59. Lapouge K, Smith SJ, Walker PA, Gamblin SJ, Smerdon SJ, Rittinger K (2000). “Structure of the TPR domain of p67phox in complex with Rac.GTP.“ Mol Cell 6(4): 899-907.

60. Lassègue B, Clempus RE (2003). “Vascular NAD(P)H oxidases: specific features, expression, and regulation.“ Am J Physiol Regul Integr Comp Physiol 285(2): R277-97.

61. Lassègue B, Sorescu D, Szöcs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK (2001). “Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways.“ Circ Res 88(9): 888-94.

62. Law MR, Wald NJ (1999). “Why heart disease mortality is low in France: the time lag explanation.“ BMJ 318(7196): 1471-6.

63. Law MR, Wald NJ (2002). ”Risk factor thresholds: their existence under scrutiny.“ BMJ 324(7353): 1570-6.

64. Leikert JF, Räthel TR, Wohlfart P, Cheynier V, Vollmar AM, Dirsch VM (2002). “Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells.“ Circulation 106(13): 1614-7.

65. Leiro J, Alvarez E, Arranz JA, Laguna R, Uriarte E, Orallo F (2004). “Effects of cis-resveratrol on inflammatory murine macrophages: antioxidant activity and down-regulation of inflammatory genes.“ J Leukoc Biol 75(6): 1156-65.

66. Lerman LO, Chade AR, Sica V, Napoli C (2005). “Animal models of hypertension: an overview.“ J Lab Clin Med 146(3): 160-73.

67. Li JM, Shah AM (2001). “Differential NADPH- versus NADH-dependent superoxide production by phagocyte-type endothelial cell NADPH oxidase.” Cardiovasc Res 52(3): 477-86.

68. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). “Protein measurement with the Folin phenol reagent.“ J Biol Chem 193(1): 265-75.

69. Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG (2006). “Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases.“ Cell Signal 18(1): 69-82.

70. Mayer B, John M, Heinzel B, Werner ER, Wachter H, Schultz G, Böhme E (1991). “Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase.“ FEBS Lett 288(1-2): 187-91.

71. McCord JM (2000). “The evolution of free radicals and oxidative stress.“ Am J Med 108(8): 652-9.

72. Meyer JW, Holland JA, Ziegler LM, Chang MM, Beebe G, Schmitt ME (1999). “Identification of a functional leukocyte-type NADPH oxidase in human endothelial cells: a potential atherogenic source of reactive oxygen species.” Endothelium 7(1): 11-22.

73. Motulsky HJ, Christopoulos A (2003). “Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting.” GraphPad Software Inc., San Diego CA, www.graphpad.com.

74. Mukamal KJ, Conigrave KM, Mittleman MA, Camargo CA Jr, Stampfer MJ, Willett WC, Rimm EB (2003). “Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men.“ N Engl J Med 348(2): 109-18.

75. Niederlaender E (2006). “Causes of death in the EU.” Statistics in focus 10/2006, European Communities, ISSN 1024-4352.

76. Nisimoto Y, Motalebi S, Han CH, Lambeth JD (1999). "The p67(phox) activation domain regulates electron flow from NADPH to flavin in flavocytochrome b(558)." J Biol Chem 274(33): 22999-3005.

77. Opitz N, Drummond GR, Selemidis S, Meurer S, Schmidt HH (2007). "The 'A's and 'O's of NADPH oxidase regulation: a commentary on "Subcellular localization and function of alternatively spliced Noxo1 isoforms"." Free Radic Biol Med 42(2): 175-9.

78. Orallo F, Alvarez E, Camiña M, Leiro JM, Gómez E, Fernández P (2002). "The possible implication of trans-Resveratrol in the cardioprotective effects of long-term moderate wine consumption." Mol Pharmacol 61(2): 294-302.

79. Osman HE, Maalej N, Shanmuganayagam D, Folts JD (1998). "Grape juice but not orange or grapefruit juice inhibits platelet activity in dogs and monkeys." J Nutr 128(12): 2307-12.

80. Polikandriotis JA, Rupnow HL, Elms SC, Clempus RE, Campbell DJ, Sutliff RL, Brown LA, Guidot DM, Hart CM (2006). "Chronic ethanol ingestion increases superoxide production and NADPH oxidase expression in the lung." Am J Respir Cell Mol Biol 34(3): 314-9.

81. Pritchard KA Jr, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB (1995). "Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion." Circ Res 77(3): 510-8.

82. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG (1996). "Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone." J Clin Invest 97(8): 1916-23.

83. Ralay Ranaivo H, Diebolt M, Andriantsitohaina R (2004). "Wine polyphenols induce hypotension, and decrease cardiac reactivity and infarct size in rats: involvement of nitric oxide." Br J Pharmacol 142(4): 671-8.

84. Rapola JM, Virtamo J, Ripatti S, Huttunen JK, Albanes D, Taylor PR, Heinonen OP (1997). "Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction." Lancet 349(9067): 1715-20.

85. Renaud S, de Lorgeril M (1992). ”Wine, alcohol, platelets, and the French paradox for coronary heart disease." Lancet 339(8808): 1523-6.

86. Rimm EB, Williams P, Fosher K, Criqui M, Stampfer MJ (1999). “Moderate alcohol intake and lower risk of coronary heart disease: meta-analysis of effects on lipids and haemostatic factors.“ BMJ 319(7224): 1523-8.

87. Ross R (1999). “Atherosclerosis - an inflammatory disease.“ N Engl J Med 340(2): 115-26.

88. Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, Baehner RL, Cole FS, Curnutte JT, Orkin SH (1986). “Cloning the gene for an inherited human disorder - chronic granulomatous disease - on the basis of its chromosomal location.“ Nature 322(6074): 32-8.

89. Ruano-Ravina A, Figueiras A, Barros-Dios JM (2004). “Type of wine and risk of lung cancer: a case-control study in Spain.“ Thorax 59(11): 981-5.

90. Rueckschloss U, Quinn MT, Holtz J, Morawietz H (2002). “Dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II in human endothelial cells: protective effect of angiotensin II type 1 receptor blockade in patients with coronary artery disease.“ Arterioscler Thromb Vasc Biol 22(11): 1845-51.

91. Sarr M, Chataigneau M, Martins S, Schott C, El Bedoui J, Oak MH, Muller B, Chataigneau T, Schini-Kerth VB (2006). “Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase.“ Cardiovasc Res 71(4): 794-802.

92. Schaefer EJ (2002). “Lipoproteins, nutrition, and heart disease.” Am J Clin Nutr 75(2): 191-212.

93. Schnackenberg CG, Welch WJ, Wilcox CS (1998). “Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: role of nitric oxide.“ Hypertension 32(1): 59-64.

94. Schoonen WM, Salinas CA, Kiemeney LA, Stanford JL (2005). “Alcohol consumption and risk of prostate cancer in middle-aged men.“ Int J Cancer 113(1): 133-40.

95. Sies H (1991). “Oxidative stress: from basic research to clinical application.” Am J Med 91(3C): 31S-38S.

96. Simons JM, Hart BA, Ip Vai Ching TR, Van Dijk H, Labadie RP (1990). “Metabolic activation of natural phenols into selective oxidative burst agonists by activated human neutrophils.” Free Radic Biol Med 8(3): 251-8.

97. Singh U, Jialal I (2006). “Oxidative stress and atherosclerosis.“ Pathophysiology 13(3): 129-42.

98. Sonta T, Inoguchi T, Tsubouchi H, Sekiguchi N, Kobayashi K, Matsumoto S, Utsumi H, Nawata H (2004). “Evidence for contribution of vascular NAD(P)H oxidase to increased oxidative stress in animal models of diabetes and obesity.“ Free Radic Biol Med 37(1): 115-23.

99. Sorescu D, Weiss D, Lassègue B, Clempus RE, Szöcs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR, Griendling KK (2002). “Superoxide production and expression of nox family proteins in human atherosclerosis.“ Circulation 105(12): 1429-35.

100. Stasch JP, Kazda S, Hirth C, Morich F (1987). “Role of nisoldipine on blood pressure, cardiac hypertrophy, and atrial natriuretic peptides in spontaneously hypertensive rats.“ Hypertension 10(3): 303-7.

101. Stocker R, Keaney JF Jr (2004). ”Role of oxidative modifications in atherosclerosis." Physiol Rev 84(4): 1381-478.

102. Stocker R, O’Halloran RA (2004). “Dealcoholized red wine decreases atherosclerosis in apolipoprotein E gene-deficient mice independently of inhibition of lipid peroxidation in the artery wall.“ Am J Clin Nutr 79(1): 123-30.

103. Stokes KY, Clanton EC, Russell JM, Ross CR, Granger DN (2001). “NAD(P)H oxidase-derived superoxide mediates hypercholesterolemia-induced leukocyte-endothelial cell adhesion.“ Circ Res 88(5): 499-505.

104. Suh YA, Arnold RS, Lassègue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD (1999). "Cell transformation by the superoxide-generating oxidase Mox1." Nature 401(6748): 79-82.

105. Takayama T, Wada A, Tsutamoto T, Ohnishi M, Fujii M, Isono T, Horie M (2004). "Contribution of vascular NAD(P)H oxidase to endothelial dysfunction in heart failure and the therapeutic effects of HMG-CoA reductase inhibitor." Circ J 68(11): 1067-75.

106. Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H, Sumimoto H (2003). "Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases." J Biol Chem 278(27): 25234-46.

107. Taniyama Y, Griendling KK (2003). "Reactive oxygen species in the vasculature: molecular and cellular mechanisms." Hypertension 42(6): 1075-81.

108. Tapiero H, Tew KD, Ba GN, Mathé G (2002). "Polyphenols: do they play a role in the prevention of human pathologies?" Biomed Pharmacother 56(4): 200-7.

109. Tirapelli CR, Al-Khoury J, Bkaily G, D'Orléans-Juste P, Lanchote VL, Uyemura SA, de Oliveira AM (2006). "Chronic ethanol consumption enhances phenylephrine-induced contraction in the isolated rat aorta." J Pharmacol Exp Ther 316(1): 233-41.

110. Tojo A, Onozato ML, Kobayashi N, Goto A, Matsuoka H, Fujita T (2002). "Angiotensin II and oxidative stress in Dahl Salt-sensitive rat with heart failure." Hypertension 40(6): 834-9.

111. Van Golde PH, van der Westelaken M, Bouma BN, van de Wiel A (2004). "Characteristics of piraltin, a polyphenol concentrate, produced by freeze-drying of red wine." Life Sci 74(9): 1159-66.

112. Vinson JA, Teufel K, Wu N (2001). "Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in a hamster model." Atherosclerosis 156(1): 67-72.

113. Wallerath T, Deckert G, Ternes T, Anderson H, Li H, Witte K, Förstermann U (2002). "Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase." Circulation 106(13): 1652-8.

114. Wallerath T, Li H, Gödtel-Ambrust U, Schwarz PM, Förstermann U (2005). "A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase." Nitric Oxide 12(2): 97-104.

115. Wallerath T, Poleo D, Li H, Förstermann U (2003). "Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects." J Am Coll Cardiol 41(3): 471-8.

116. Warnholtz A, Nickenig G, Schulz E, Macharzina R, Bräsen JH, Skatchkov M, Heitzer T, Stasch JP, Griendling KK, Harrison DG, Böhm M, Meinertz T, Münzel T (1999). "Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system." Circulation 99(15): 2027-33.

117. Wind S (2006). "NADPH oxidase inhibition and endothelial dysfunction in aged spontaneously hypertensive rats." PhD Dissertation, Philipps-University, Marburg, Germany.

118. Wingler K, Wünsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH (2001). "Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo." Free Radic Biol Med 31(11): 1456-64.

119. Wollny T, Aiello L, Di Tommaso D, Bellavia V, Rotilio D, Donati MB, de Gaetano G, Iacoviello L (1999). "Modulation of haemostatic function and prevention of experimental thrombosis by red wine in rats: a role for increased nitric oxide production." Br J Pharmacol 127(3): 747-55.

120. World Health Organization (1989). "World health statistics annual 1989." World Health Organization, Geneva.

121. Xu JZ, Yeung SY, Chang Q, Huang Y, Chen ZY (2004). "Comparison of antioxidant activity and bioavailability of tea epicatechins with their epimers." Br J Nutr 91(6): 873-81.

122. Yamamoto Y, Ogino K, Igawa G, Matsuura T, Kaetsu Y, Sugihara S, Matsubara K, Miake J, Hamada T, Yoshida A, Igawa O, Yamamoto T, Shigemasa C, Hisatome I (2006). "Allopurinol reduces neointimal hyperplasia in the carotid artery ligation model in spontaneously hypertensive rats." Hypertens Res 29(11): 915-21.

123. Yang D, Gluais P, Zhang JN, Vanhoutte PM, Félétou M (2004). "Endothelium-dependent contractions to acetylcholine, ATP and the calcium ionophore A 23187 in aortas from spontaneously hypertensive and normotensive rats." Fundam Clin Pharmacol 18(3): 321-6.

124. Ying CJ, Xu JW, Ikeda K, Takahashi K, Nara Y, Yamori Y (2003). "Tea polyphenols regulate nicotinamide adenine dinucleotide phosphate oxidase subunit expression and ameliorate angiotensin II-induced hyperpermeability in endothelial cells." Hypertens Res 26(10): 823-8.

125. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P (2000). "Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators." N Engl J Med 342(3): 154-60.

126. Zalba G, Beaumont FJ, San José G, Fortuño A, Fortuño MA, Etayo JC, Díez J (2000). "Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats." Hypertension 35(5): 1055-61.

127. Zenebe W, Pechánová O, Andriantsitohaina R (2003). "Red wine polyphenols induce vasorelaxation by increased nitric oxide bioactivity." Physiol Res 52(4): 425-32.

128. Zhang X, Shan P, Jiang G, Cohn L, Lee PJ (2006). "Toll-like receptor 4 deficiency causes pulmonary emphysema." J Clin Invest 116(11): 3050-9.

129. Ziegler S, Kostner K, Thallinger C, Bur A, Brunner M, Wolzt M, Joukhadar C (2005). "Wine ingestion has no effect on lipid peroxidation products." Pharmacology 75(3): 152-6.

9 ABBREVIATIONS

A	Ampere
aa	Amino acid
AA-Bis	Acrylamide Bisacrylamide
AIC	Akaike's Information Criterion, statistical test
ANOVA	Analysis of variances
APS	Ammonium peroxodisulphate
BSA	Bovine serum albumine
$CaCl_2$	Calcium chloride
CCh	Carbachol (carbamoylcholine)
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CO_2	Carbon dioxide
$CuSO_4$	Cupric sulphate
EC50	Required concentration to reach 50% of maximum effect
ECL	Enhanced chemiluminescence
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylene diamine tetraacetic acid
e.g.	Exempli gratia
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
G	Gravity
$gp91^{phox}$	Glycoprotein with a size of 91 kDa, subunit of phagocytic oxidase
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HDL-C	High density lipoprotein cholesterol
HEPES	Hydroxyethyl piperazin ethan sulfonic acid
H_2O_2	Hydrogenperoxide
HOCl	Hypochlorous acid
HRPO	Horse radish peroxidase
i.e.	Id est
KCl	Potassium chloride
kDa	Kilodalton
KHB	Krebs Henseleit Buffer
KH_2PO_4	Potassium hydrogenphosphate
LB	Lysis buffer

LDL-C	Low density lipoprotein cholesterol
M	Molar (mol/l)
$MgSO_4$	Magnesium sulphate
mRNA	Messenger ribonucleic acid
N	Newton
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
$NaHCO_3$	Sodium hydrogencarbonate
NaOH	Sodium hydroxide
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	Catalytic subunit of NADPH oxidases
n.s.	No statistic significance ($p \geq 0.05$)
O_2^-	Superoxide anion
^-OCl	Hypochlorite
OH^-	Hydroxyl radical
$ONOO^-$	Peroxynitrite
OxLDL-C	Oxidized low density lipoprotein cholesterol
p	Probability
$p47^{phox}$	Protein with a size of 47 kDa, subunit of phagocytic oxidase
PAGE	Polyacrylamide gel electrophoresis
PE	Phenylephrine
pH	Pondus / potentia hydrogenii
ROS	Reactive oxygen species
SDS	Sodium dodecylsulphate
SEM	Standard error of means
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
TBS-T	Tris-buffered saline with Tween
TCA	Trichloroacetic acid
Temed	N,N,N,N-tetramethyl ethylene diamine
Tris	2-Amino-2-hydroxymethyl-1,3-propanediol
VSMC	Vascular smooth muscle cell
WUR	Wistar unilever rats

10 DANKSAGUNG

Ich möchte einigen Menschen danken, die mich auf meinem Weg hierher begleitet und unterstützt haben:

- Harald Schmidt für die Bereitstellung des Themas, für die Betreuung bei der Planung der Experimente sowie für die intensive Diskussion der Ergebnisse.

- Knut Beuerlein für die konstruktive Kritik beim Anfertigen sowie für das mehrfache Korrekturlesen dieser Arbeit.

- Sven Wind für die enorme Hilfe bei der Erstellung der *in vitro*-Daten.

- Petra Kronich für das Beibringen des Western Blottings.

- Bärbel Fühler für ihre Hilfe bei den ersten Versuchen am Organbad.

- Helmut Müller für den technischen Support bei allen erdenklichen Problemen.

- Dem Rest meiner Arbeitsgruppe (Daniel Janowitz, Henrike Grund, Sanchaita Sonar, Arun Kumar, Thomas Eucker, Dorit Gertkens, Martin Deile, Ashraf Taye) sowie den AGs Dreyer und Teschemacher für die große Hilfsbereitschaft im RBI.

- Meinen Eltern und explizit meinem Vater für das Korrekturlesen dieser Arbeit, die konstruktive Kritik und vor allem die stetige moralische Unterstützung.

- Stanislav Kaschinski nicht nur für die Hilfe bei der graphischen Gestaltung dieser Arbeit.

11 CURRICULUM VITAE

Anna Bertram
geboren am 22.03.1981 in Limburg a.d. Lahn

Schulische Ausbildung

1987 – 1991	Grundschule Elz Süd
1991 – 2000	Fürst-Johann-Ludwig-Schule Hadamar
2000	Abitur

Hochschulbildung

2000 – 2007	Studium der Humanmedizin an der Justus-Liebig-Universität Gießen
09/2002	Physikum
2005 – 2006	Praktisches Jahr
06/2007	2. Ärztliche Prüfung und Approbation zur Ärztin

Promotion

Seit 03/2004	Promotion bei Prof. Dr. H. H. H. W. Schmidt im Rudolf-Buchheim-Institut für Pharmakologie der Justus-Liebig-Universität Gießen

Berufstätigkeit

Seit 09/2007	Assistenzärztin in der Klinik für Nieren- und Hochdruckerkrankungen, Zentrum Innere Medizin, Medizinische Hochschule Hannover

ERKLÄRUNG

Ich erkläre:

Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht.

Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

Gießen, den _____

_____

Anna Bertram