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Xanthine oxidoreductase in essential hypertension and metabolic syndrome

EXPERIMENTAL STUDIES ON RODENT MODELS

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ACADEMIC DISSERTATION

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Abstract

Essential hypertension, a major public health concern, is associated with increased cardiovascular mortality. High dietary salt (NaCl) is the best studied and most important predisposing risk factor. Excessive salt intake leads to renal and cardiac dysfunction by blood pressure dependent and independent mechanisms. Sedentary lifestyle and overnutrition contribute to the increasing prevalence of hypertension, obesity, atherogenic dyslipidemia and dysglycemia. These constitute a pathophysiological state referred to as metabolic syndrome, and are associated with cardiovascular mortality.

Elevated circulating urate is a common finding in these disorders, and it has been suggested to be an independent risk factor to cardiovascular diseases. Being the terminal metabolite of human purine metabolism, urate levels reflect the balance between intake, production and excretion. While dysregulation of urate excretion has been reported in association with cardiovascular disorders, present studies aimed to clarify the role of the urate producing enzyme, xanthine oxidoreductase (XOR), i.e. the gene product xanthine dehydrogenase (XDH) and its post-translationally formed isoform xanthine oxidase (XO), in cardiovascular diseases.

XOR converts hypoxanthine and xanthine, derived from ATP and other purine nucleotides, to urate. In the rat liver, but not in the kidney, uricase converts urate to allantoin. While urate is an endogenous antioxidant, XO can produce reactive oxygen species (ROS), which are involved in cell signalling and associated with organ dysfunction if the production rate is high. Among other factors, low oxygen levels upregulate XOR, and if prolonged, also increase the conversion of XDH to XO.

Cross-transplantation studies between hypertension-prone and -resistant rat strains have revealed a major role for the kidney in salt-sensitive hypertension. Salt overload and resulting hypertension induce hemodynamic changes and energy demand which in turn may lead to renal hypoxia.

In present studies hypertension-prone rat strains were found to have higher renal XOR activity than salt-resistant strains. Furthermore, NaCl intake upregulated dose-dependently renal XOR isoforms only in hypertension-prone rats. Salt-sensitive and -resistant rats were then kept on ‘low’ and ‘high’ NaCl diets for different periods of time, with or without the specific XOR inhibitor allopurinol, to clarify whether renal XOR induction precedes or follows the development of hypertension. Renal XOR induction appeared concomitantly with blood pressure elevation. While allopurinol did not significantly alleviate hypertension,

it prevented left-ventricular and renal hypertrophy indicating a possible role for XOR in salt-induced target organ damage.

Nitric oxide synthases (NOS) catalyze the main pathways for the production of nitric oxide (NO). NO is an endothelium-derived relaxing factor with pleiotropic paracrine effects and is able to modify renal sodium excretion. ROS produced by XO can scavenge NO. XOR resembles bacterial nitrate reductases and may be able to recycle nitrate to NO. Therefore the impact of a paucity or surplus of NO on XOR regulation was investigated. NOS inhibition by L-NAME aggravated salt-induced hypertension and induced renal XOR, whereas NO prodrug, isosorbide-5-mononitrate, alleviated salt-induced hypertension without modulating renal XOR.

XOR expression and activity were further studied using Zucker fa/fa strain, which is a prediabetic rodent model of metabolic syndrome. These rats developed substantial obesity and modest hypertension. In contrast to lean controls, hepatic and renal XOR activities were upregulated. XOR activities were modified by diet and downregulated by the angiotensin II receptor blocking agent, valsartan.

Cyclosporine (CsA) is a fungal cyclic undecapeptide and one of the first-line immunosuppressive drugs used in the management of organ transplantation. Renal vasoconstriction and malignant hypertension and other renal side effects ensue high CsA doses. As a result, CsA nephrotoxicity limits its use and is aggravated by dietary NaCl. Substantial CsA-induced renal XO upregulation was found in salt-sensitive rats kept on high NaCl diet, indicating a possible role for this ROS producing isoform in CsA-induced nephrotoxicity.

In conclusion, the present studies demonstrated upregulation of XOR in obese and salt-sensitive rat strains. Furthermore, renal XOR was induced by dietary salt. NOS and angiotensin II participate in XOR regulation. XO upregulation followed drug-induced nephrotoxicity. Renal hypoxia, common to these models, is one of the plausible XOR inducing factors. Although XOR inhibition did not alleviate hypertension, present experimental data indicate that XOR plays a role in in the pathology of salt-induced cardiac and renal damage.

Contents

List of original publications

This thesis is based on the following publications referred to in the text by their Roman numerals, and reprinted with the permission of the copyright holders (I–III), and on unpublished data

I LAAKSO J., MERVAALA E., HIMBERG J.J., TERÄVÄINEN T.L., KARPPANEN H., VAPAATALO H. and LAPATTO R. (1998). Increased kidney xanthine oxidoreductase activity in salt-induced experimental hypertension. Hypertension; 32: 902–6.

II LAAKSO J., VASKONEN T., MERVAALA E., VAPAATALO H. and LAPATTO R. (1999). Inhibition of nitric oxide synthase induces renal xanthine oxidoreductase activity in spontaneously hypertensive rats. Life Sci., 65: 2679–85.

III LAAKSO J., TERÄVÄINEN T.L., MARTELIN E., VASKONEN T. and LAPATTO R. (2004). Renal xanthine oxidoreductase activity during development of hypertension in spontaneously hypertensive rats. J Hypertens., 22:1333–40.

IV LAAKSO J., VASKONEN T., LAPATTO R. (2009). Renal and hepatic xanthine oxidoreductase activities in obese Zucker rats. Submitted.

In addition some previously unpublished data are presented.

Main abbreviations

11βHSD2	11 ß-hydroxysteroid-dehydrogenase (EC 1.1.1.146)
ACE	Angiotensin convertase (EC 3.4.15.1)
ALDH	Aldehyde dehydrogenase (EC 1.2.1.3
Ang II	Angiotensin II
ARB	Angiotensin receptor blocker
ANOVA	Analysis of variance
AO	Aldehyde oxidase (EC 1.2.3.1)
AP	Allopurinol
AT_1	Angiotensin receptor type 1
AT_2	Angiotensin receptor type 2
ATP	Adenosine triphosphate
BP	Blood pressure
BH_4	5,6,7,8-Tetrahydrobiopterin
CsA	Cyclosporine
Dahl SS/Jr	Dahl salt sensitive rat strain
Dahl SR/Jr	Dahl salt resistant rat strain
DOCA	Deoxy-corticosterone acetate
DPI	Diphenylene iodonium
FAD	Flavin adenine dinucleotide
G6PDH	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
GSH	Glutathione, reduced form
GSSG	Glutathione, oxidized form
IL-1-β	β-interleukin 1
I/R	Ischemia-reperfusion
IS-5-MN	Isosorbide-5-mononitrate
L-NAME	N^{ω}-nitro-L-arginine methyl ester
LDH	Lactate dehydrogenase (EC 1.1.1.27)
LVH	Left ventricular hypertrophy
MAP	Mean arterial pressure (also Mitogen-activated protein kinase)
Moco	Molybdenum cofactor
mRNA	Messenger ribonucleic acid
$NAD(P)^+$	Nicotine adenine dinucleotide (phosphate), oxidized form
NAD(P)H	Nicotine adenine dinucleotide (phosphate), reduced form
NFAT	Nuclear factor of activated T-cells
NOS	Nitric oxide synthase (L-Arginine, NADPH: oxygen oxido reductase, EC 1.14.13.39)
NYHA	New York Heart Association
QTL	Quantitative trait locus
RA(A)S	Renin angiotensin (aldosterone) system
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SOD	Superoxide dismutase (EC 1.15.1.1)
TAL	Thick ascending loop
TNF-α	Tumor necrosis factor α
XDH	Xanthine dehydrogenase (EC 1.17.1.4)
XO	Xanthine oxidase (EC 1.1.3.22)
XOR	Xanthine oxidoreductase
WKY	Wistar Kyoto

1 Introduction

Arterial blood pressure is regulated by cardiac output and peripheral resistance. Sustained increase in arterial pressure is defined as hypertension and is usually the result of increased resistance in resistance vessels. In up to 95% of subjects with hypertension, the underlying cause for high blood pressure is not known (Staessen *et al.*, 2003). The remaining five percent of cases, in which the underlying cause is known, are referred to as having secondary hypertension. Despite the progress in prevention and treatment, the prevalence of essential hypertension remains high, more than 25% of adult population worldwide (Kearney *et al.*, 2005).

Cut-off values for blood pressure levels in the diagnosis of hypertension are related to occurrence of other cardiovascular risk factors, prognostic information, and to the capacity of health care systems. Generally a blood pressure exceeding 140/90 mmHg (systolic/diastolic) is considered high (Arguedas *et al.*, 2009).

Essential hypertension is an established risk factor for atherosclerosis and stroke, which are considered among the leading causes of death. In addition to genetic predisposition, diet is an important multifactorial determinant of blood pressure (Miller *et al.*, 1999). Epidemiological studies have indicated that high salt (NaCl) intake is an independent risk factor for cardiovascular and renal disease (Boero *et al.*, 2002). Obesity, sedentary life-style and a low intake of vegetables are also independent risk factors for hypertension. These factors further contribute to the aggregation of hypertension with abdominal obesity, dyslipidemia and insulin resistance that together constitute metabolic syndrome.

Metabolic syndrome is now recognized as one of the key factors resulting in the increased prevalence of diabetes and cardiovascular disease worldwide. The prevalence of diabetes in EU region is currently 6.8% of total population and is expected to reach 7.8% in 2025. Similar trend is also predicted in developing countries (The International Diabetes Foundation, Diabetes atlas, 2008). In developed countries type 2 diabetes accounts for 85 to 95% of all diabetes cases and in developing countries the percentage is even higher.

Systolic blood pressure (SBP) response to dietary NaCl is not homogenous. Salt-sensitive individuals have an exaggerated SBP response to changes in NaCl intake. In hypertension population, the percentage of salt-sensitive individuals has been estimated to be as high as 70% (Weinberger, 1996). Experimental studies on kidney transplantation between salt-

resistant and salt-sensitive rats have demonstrated the crucial role of the kidney in salt-sensitivity (Rettig and Grisk, 2005).

Association of high serum urate levels with the development of essential hypertension has been known for decades, but the underlying mechanisms are still not fully understood. Urate is a biochemical marker, which has even been suggested to be an independent risk factor for essential hypertension, metabolic syndrome and cardiovascular diseases (Strasak *et al.*, 2008a and 2008b). Circulating urate levels reflect the balance between its elimination and production, and the availability of endogenous and dietary precursors.

In humans, urate (uric acid) is the end-product of purine metabolism because of the genetic silencing of the enzyme uricase (Oda *et al.*, 2002). In the rat, hepatic uricase converts circulating urate to the more water soluble allantoin. Increased levels of urate have been shown to be nephrotoxic in rats (Mazzali *et al.*, 2002). The nephrotoxic properties of urate have been proposed to contribute to the development of salt-sensitive hypertension (Watanabe *et al.*, 2002).

Urate is produced by the enzyme xanthine oxidoreductase (XOR) from xanthine and hypoxanthine, both of which are degradation products of ATP and other purine nucleotides. Xanthine dehydrogenase (XDH, EC 1.1.1.204) is the translated protein. Post-translational modifications can transform XDH into xanthine oxidase (XO, EC 1.1.3.22), which yields electrons to oxygen instead of NAD^+, resulting in increased ROS production (Hille and Nishino, 1995).

While XOR is one of the main sources of reactive oxygen species (ROS), XOR is also an enzyme, which produces one of the most powerful endogenous antioxidative molecules, urate. ROS are by-products of oxygen metabolism and are important cell signalling molecules.

Increased ROS production, by XOR and other enzymes, has been shown to associated with hypertension in animal models and in severe forms of human hypertension. ROS production, particularly H_2O_2, has been shown to participate in the regulation of renal sodium excretion and endothelial function. ROS may thus contribute to salt-sensitive hypertension (Taylor *et al.*, 2005). ROS may also react with NO and regulate NO availability. NO is a potent endothelium-derived vasodilatating factor and has multiple roles including regulation of renal sodium excretion (Lahera *et al.* 1991) and regulation of oxygen consumption at cellular level (Victor *et al.* 2007). Recently, XOR has been reported to contribute to recycling of NO metabolite nitrate back to NO (Jansson *et al.*, 2008). The physiological and pathophysiological significance of this pathway with respect to cardiovascular homeostasis has not been fully evaluated yet. ROS, reactive nitrogen species and urate have been shown to play a role in innate immune function (reviewed by Vorbach *et al.*, 2003; Bianchi, 2007).

The aim of the present studies was to characterize the possible role of XOR in salt-sensitive rat models of hypertension and metabolic syndrome using specific hypertension-

prone and obesity-prone rat strains and their genetically close control strains. Possible association of XOR with salt-sensitivity was studied in rats on different levels of salt intake for different periods of time. Effects of XOR inhibition on the development of salt-induced hypertension were also studied to clarify whether regulation of renal XOR activity had any causal role. Because XOR has potential interrelationships with NO homeostasis, one of the aims was to clarify whether NO paucity or excess would regulate XOR activity. Cyclosporine (CsA) belongs to first-line drugs for immunomediated diseases and it is particularly useful to prevent rejection after organ transplantation. However, CsA use is limited by its nephrotoxicity, which is associated with renal vasoconstriction and functional changes resulting in malignant hypertension. In addition, CsA toxicity has been reported to be aggravated by dietary NaCl (Pere *et al.*, 1998).

2 Review of the literature

2.1 LONG-TERM REGULATION OF BLOOD PRESSURE

Several physiological mechanisms are involved in the long-term regulation of blood pressure. The complex array includes regulation by endocrine and paracrine hormonal mechanisms, sympathetic nerve system, central nervous system, cardiac output, peripheral resistance and renal mechanisms.

The kidney regulates long-term blood pressure by adjusting sodium, chloride and volume homeostasis with high precision. The relationship between chronic intake level of sodium chloride, and resulting blood pressure response, has been defined by Guyton and co-workers as an S-shaped curve, or alternatively, the chronic pressure-natriuresis curve (Guyton *et al.*, 1972), as depicted in Figure 1. Mechanisms, which regulate long-term balance of blood pressure and volume homeostasis, modify the chronic pressure-natriuresis curve. The curve indicates that the increased NaCl excretion in salt-sensitive subjects is achieved at increased blood pressure levels. All types of hypertension are associated with a 'right shift' in the pressure-natriuresis curve.

The renin-angiotensin system (RAS) or renin-angiotensin-aldosterone system (RAAS), are the most important long-term blood pressure regulating mechanisms. Aspartyl protease renin (EC 3.4.99.19) is released from the kidney and acts on the circulating hepatic protein angiotensinogen converting it to a decapeptide, angiotensin I. This peptide is then further converted in the vascular bed to the octapeptide, angiotensin II (Ang II) by the membrane bound zinc metallopeptidase, angiotensin convertase (ACE, EC 3.4.15.1) (reviewed by Le *et al.*, 2008). The effects of Ang II in blood pressure regulation are mediated by AT_1 receptors. The second receptor class, AT_2, has opposing effects compared with AT_1. Ang II is a vasoconstrictor and participates in volume regulation by liberating mineralocorticoid, aldosterone. ACE also cleaves other peptides. It inactivates bradykinin, which is a strongly vasodilatating peptide and opposes the effects of Ang II. The importance of these classical pathways (Figure 2) became evident after the discovery of ACE inhibitors, which are still widely used antihypertensive drugs. The pathway provided also other important targets for antihypertensive treatments, including AT_1 receptor blockers. Recently, renin inhibitors

have been introduced as a novel class of antihypertensive drugs (reviewed recently by Jensen *et al.*, 2008). Further cleavage of Ang II produces hepta- (Ang III and $Ang_{1–7}$) and hexapeptides (Ang IV), which also exert biological activity. Ang II is the most potent of these peptides. While so called classical RAS pathways involve circulating components, there is growing interest in intracellular RAS, which involves alternative Ang II and $Ang_{1–7}$ generating systems, such as catepsin and chymase, and possibly also nuclear receptors.

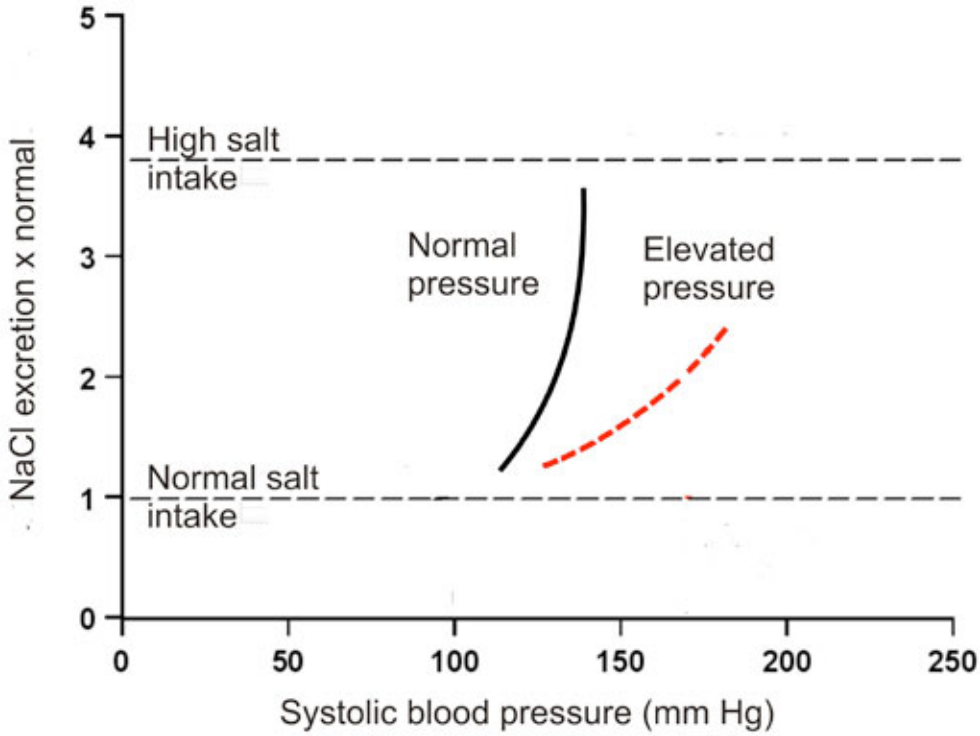

Figure 1. Schematic overview on arterial pressure in relation to NaCl intake (i.e. NaCl excretion). To excrete all sodium at a given intake level, salt-sensitive subjects (red line) require higher arterial pressure levels than salt-resitant (black line). In salt-sensitive individuals the slope of the curve is decreased. Salt-sensitivity is determined by a variety of genetic and endocrine factors (see text). While natriuretic hormones and antihypertensive drugs cause left-shift in the curve, the activation of RAAS increases circulating aldosterone level and, sodium reabsorption, and results in a shift to the right. Rodent models indicate that salt-sensitivity can be induced by urate exposure, reactive oxygen species or by inhibition of NO production (see text). Oxygen availability in the kidney is also one of the factors which can modify the pressure-natriuresis curve. Modified from Guyton *et al.* (1972).

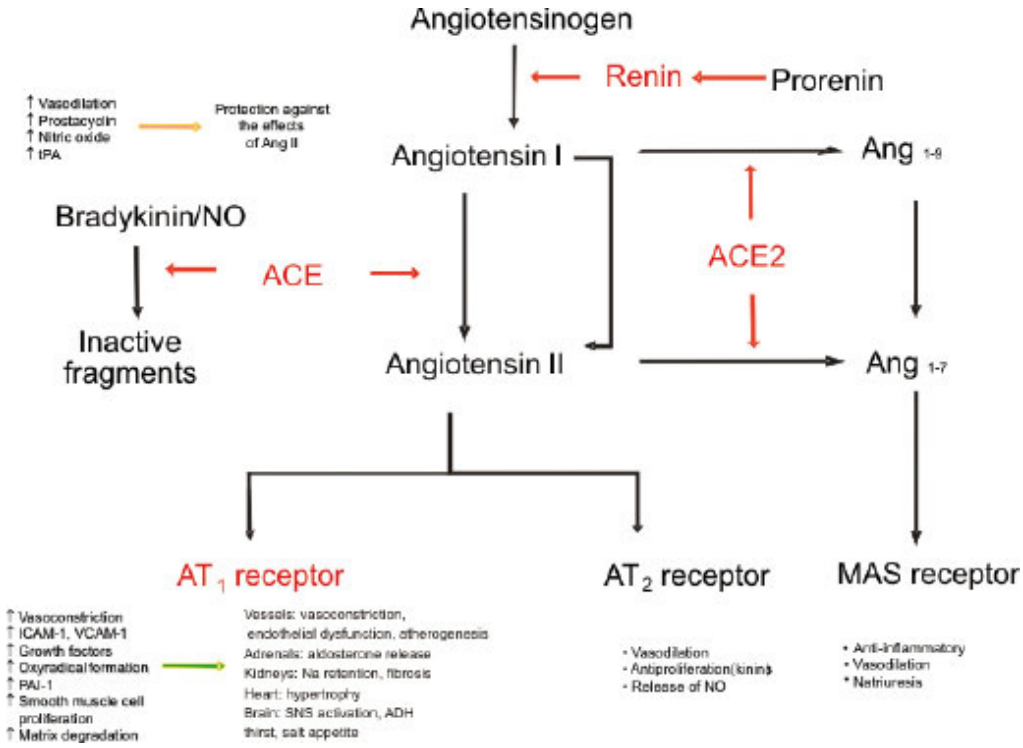

Figure 2. Classical pathways of the renin-angiotensin system, and the kallikrein-kinin system are depicted. Recently alternative pathways have gained much interest. In these Ang II is converted by ACE2 to $Ang_{1–7}$ and to $Ang_{1–9}$, which exert their effects through MAS receptors.Collated from different sources cited in the text.

Growing interest on RAS began already in 1898, when Finnish physiologist Robert Tigerstedt discovered a renal blood pressure increasing protein, renin, and the field is still expanding (Fyhrquist and Saijonmaa, 2008). The kidney is unique in having all components of RAS within one tissue. In addition to the liver, the kidney is able to synthesize angiotensinogen. Renal Ang II concentrations are much higher than can be anticipated from levels delivered by circulation. Furthermore, Ang II levels are higher in specific parts of the kidney. Consequently locally generated Ang II in the kidney may be more important than circulating Ang II (reviewed by Kobori *et al.* 2007). It is also possible that Ang II receptor blockers exert their effects by upregulating $Ang_{1–7}$, rather than by inhibiting Ang II effects directly (reviewed by Lubel *et al.* 2008).

Recently uric acid has been reported to increase dose-dependently Ang II production in vascular smooth cell cultures, linking urate to RAS and cardiovascular effects (Corry *et al.*, 2008).

2.2 THE ROLE OF THE KIDNEY IN THE DEVELOPMENT OF HYPERTENSION

Abnormal kidney function has a pivotal role in the predisposition to NaCl induced hypertension. In order to study the role of the kidney in the development of hypertension, renal cross-transplantation experiments were carried out in the 1980s by Rettig and Grisk using salt-resistant and hypertension-prone salt-sensitive rat strains (Grisk *et al.*, 2002; Rettig and Grisk, 2005). Kidney transplants derived from normotensive salt-resistant rats were shown to result in sustained decrease the systolic blood pressure of an adult salt-sensitive recipient and abolish the predisposition to hypertension in young animals (Morgan *et al.*, 1990; Roman, 1986). These observations, based on studies on the Dahl salt-sensitive (Dahl SS/Jr) and salt-resistant (Dahl SR/Jr) rat, established the crucial importance of the kidney in the pathogenesis of hypertension. In the spontaneously hypertensive rat (SHR) and its normotensive control, the Wistar-Kyoto rat (WKY), renal homografts were rejected, but similar experiments with F1 hybrids were successful and confirmed the previous results on Dahl strains.

The renal cross-transplantation experiments were carried out with rats kept on a 'low' dietary NaCl level. Repeating the experiments with rats kept on a 'high' dietary NaCl diet indicated that in addition to renal mechanisms also extrarenal mechanisms play a role, since the hypertension-prone, salt-sensitive Dahl SS/Jr rat with a kidney homograft from salt-resistant rat still developed hypertension (Morgan *et al.*, 1990).

The kidney has a major role in the development of hypertension and at the same time is one of the target organs of the harmful effects of hypertension As such, a vicious cycle is formed and further emphasizes renal involvement in the development of hypertension.

Reduced renal blood flow is one of the characteristic findings in hypertension (Welch *et al.*, 2001). Generally, in all tissues, lower blood flow means decreased oxygen delivery. In the case of the kidney, lower blood flow could be partly compensated by lower energy expenditure, since the workload for reabsorption of solutes is decreased. Tubular ischemia has been recognized to participate in the pathological renal changes resulting in the development of salt-sensitive hypertension (Johnson *et al.*, 2002).

2.3 SALT-SENSITIVITY

Pressure response to dietary NaCl varies with genetic background. Individuals who have high blood pressure responses to an acute change in salt-intake have been referred to as salt-sensitive. The division is categorical. In human studies a change of 10% or more in mean arterial blood pressure (MAP) has been used to define salt-sensitivity. A <5% change in MAP defines salt-resistance. While salt-sensitivity refers to an acute response, dietary NaCl has delayed effects on blood pressure, which are associated with the development of hypertension (Van Vliet *et al.*, 2008).

The prevalence of salt-sensitivity among hypertensive patients has been estimated to be high, approximately 50% (Poch *et al.*, 2001). Compelling evidence has accrued that high dietary NaCl intake markedly contributes to the development of arterial hypertension. There is also a substantial amount of evidence for a universal recommendation to restrict the intake of NaCl to 5 grams per day in order to prevent or decrease the prevalence of hypertension and its harmful consequences (Sacks *et al.*, 2001).

Sustained increase of arterial blood pressure results from insufficient renal NaCl excretion in relation to dietary load. Increases in systolic blood pressure are accompanied with increased NaCl excretion, which tends to correct the blood pressure change (Guyton *et al.*, 1972).

The renin-angiotensin system is closely associated with salt-sensitivity. In addition, several other factors modify salt-sensitivity including nitric oxide, the kallikrein-kinin system and eicosanoids. In clinical studies, salt restriction resulted in compensatory activation of RAAS, which tends to oppose changes in blood pressure. If RAAS is blocked, e.g. by ACE inhibitors, blood pressure becomes more dependent on salt intake (He and MacGregor, 2003). Other studies have shown that infusion of angiotensin II to the salt-resistant dogs results in a profound blood pressure response to salt-loading (Hall *et al.*, 1980).

In experimental animals with or without genetic predisposition to hypertension, salt overload suppresses renin release into circulation from juxtamedullary cells and subsequent RAAS response. Salt-sensitivity can also be seen as an inability to suppress RAAS in response to increased NaCl intake.

In spite of blunted RAAS response to NaCl load, Ang II receptor blockers attenuate salt-induced experimental hypertension effectively. These phenomena have led to a hypothesis involving local renin-angiotensin system (for review, see Fyhrquist and Saijonmaa, 2008). Salt-loading inactivates RAAS and consequently, salt-induced sustained increase in blood pressure has been referred to as low renin hypertension. In contrast, the local renin angiotensin system is activated by NaCl and results in an increase of cardiac AT_1 receptors. Furthermore, the local renin angiotensin system is involved in stimulation of growth and may participate in hypertension associated cardiac hypertrophy (Liang *et al.*, 2007).

2.3.1 *Sodium and chloride*

The human body has adapted to ancient low sodium diets and tends to keep sodium and chloride. As such, the homeostasis is challenged by the high salt intake, provided by modern diets. Most NaCl in human diets is derived from industrially processed food. It is considered practically impossible to compose an otherwise balanced diet, which would not provide adequate amounts of sodium chloride.

Only few studies have attempted to clarify which one of the ions, sodium or chloride is more important in relation to harmful cardiovascular effects of dietary NaCl. In several experimental studies sodium loading with other anions except chloride has

failed to increase blood pressure (Boegehold and Kotchen, 1989 and 1991). While in hypertension-prone, Dahl SS/Jr rats, NaCl is most potent, varying the anion component does not completely abolish the increases in blood pressure (Roman and Kaldunski, 1991).

Ang II stimulates the release of mineralocorticoid aldosterone from the adrenal gland resulting in an increased sodium reuptake. Cortisol also has mineralocorticoid activity, which become significant in high concentrations. Salt-sensitive individuals show increased cortisol/cortisone ratios. Cortisol is inactivated mainly by renal 11β-hydroxysteroid dehydrogenase-2 (11βHSD2), an enzyme, which is easily inhibited by liquorice saponin, glycyrrhizin, resulting in markedly increased cortisol levels and blood pressure (Quinkler and Stewart, 2003). Experimentally similar condition can be generated in rats by the administration of deoxycortisone acetate with NaCl (DOCA-NaCl). In this hypertension model, dietary sodium overload without chloride (as acetate etc.) does not increase blood pressure (Ziomber *et al.*, 2008).

2.3.2 *Interrelationships with other dietary ions*

Observational and intervention studies have consistently indicated that vegetarian diets are associated with beneficial effects on blood pressure, even when intake of NaCl is not different from omnivores (Berkow and Barnard, 2005). In the DASH trial (The Dietary Approaches to Stop Hypertension) marked positive cardiovascular effects of a diet comprising of increased amounts of fruits, whole grains, nuts, vegetables, fish, poultry and reduced amounts of red meats, sweets and sugared beverages has been reported (Sacks *et al.*, 2001). Akita *et al.* (2003) found that the DASH diet decreases the slopes of steady-state pressure-natriuresis curves. This finding indicates that salt-sensitivity is decreased. At low Na^+ -intake levels, the effects of the DASH diet vanished.

While the exact mechanisms have not been determined, the positive effects of vegetarian diets have been attributed to the low levels of sodium and chloride, to high contents of potassium, calcium and magnesium, to high levels of fibre, polyphenols, to the content of polyunsaturated fatty acids and to low energy density. One of the recent hypotheses is that the beneficial effects of these type of diets are related to the high NO_3^- levels of vegetables (Webb *et al.*, 2008b), as is further discussed in Section 2.6 Nitric oxide.

The effect of dietary NaCl in the development of hypertension and subsequent cardiovascular disorders is modified by other mineral ions present in the diet. In rodent models supplementation of potassium and magnesium has attenuated development of hypertension (Mervaala *et al.*, 1992). Mg^{2+} is an important cofactor in reactions requiring ATP and probably acts as a mild calcium antagonist by promoting vascular relaxation. Mg-deficiency promotes inflammation in rodent studies. Although experimental and epidemiological studies indicate a role for Mg^{2+} in hypertension, data from clinical studies have been less convincing (Bo and Pisu, 2008).

Increased intake of calcium lowers blood pressure in rodent models of hypertension (Hatton *et al.*, 1994). Dietary NaCl increases calcium loss in the urine (Mervaala *et al.* 1994b). Data from intervention studies have been controversial, although some of the clinical studies show significant positive effects (Sabbagh and Vatanparast, 2009).

To summarize preceding Sections 2.1 to 2.3: Several overlapping mechanisms are involved in long-term regulation of blood pressure. RAAS is one of the most important systems involved in chronic blood pressure regulation. Experimental cross-transplantation studies indicate that salt-sensitivity and hypertension 'go with the kidney', highlighting the importance of the kidney in the development of hypertension. Hypertension is always associated with a 'right-shift' of pressure natriuresis curve and salt-sensitivity with a decrease of its slope. While sodium intake in human nutrition mostly translates to the intake of NaCl, sensitivity to sodium and chloride may vary among subjects. Beneficial effects of vegan diets have been attributed to low NaCl levels and high levels of other ions, particularly potassium. Also the high nitrate levels found in vegetables has been suggested to mediate these effects, an issue, which remains to be clarified.

2.4 SALT-INDUCED TARGET ORGAN EFFECTS

2.4.1 *Hypertrophy*

High blood pressure is associated with left ventricular hypertrophy and renal enlargement in addition to impaired endothelial function. The left ventricular hypertrophy may exist even independently of increased blood pressure in response to increased intake of NaCl. Salt-induced increase in renal mass has been described both in the presence and absence of increased systolic blood pressure (Radin *et al.*, 2008). Several environmental and genetic risk factors have emerged. At the moment it is not known, which genetically determined structural or biochemical disorders explain the development of essential hypertension.

Increasing salt intake results in salt-sensitive rats in marked hypertension and left ventricular and renal enlargement within weeks. The genetically related salt-resistant rats develop these harmful, NaCl-induced effects to a much lesser extent (McCormick *et al.*, 1989; Mervaala *et al.*, 1994a and 1994b).

Increased renal mass, due to renal hypertrophy or hyperplasia, can occur in response to electrolyte overload (Mervaala *et al.*, 1994a and 1994b). It also occurs in several different physiological and pathophysiological conditions, which include increased blood pressure, metabolic syndrome, diabetes mellitus and compensatory growth. One of the most important factors contributing to hypertension induced renal enlargement is Ang II. In the kidney effects of Ang II are mediated by the AT_1 receptor (Wolf and Wenzel, 2004). Both uric acid and Ang II have been reported to stimulate vascular smooth cell proliferation in culture and, furthermore, urate has been reported to upregulate intracellular levels of Ang II (Corry *et al.*, 2008).

2.4.2 *Renal hypoxia*

All tissues depend on adequate oxygen supply. The two kidneys represent approximately 1% of body weight, but receive 20% of the resting cardiac output. Renal outer medulla and cortex are, however, remarkably susceptible to hypoxia because of effective oxygen shunting in the kidneys (Schurek *et al.*, 1990). In spite of an abundant blood supply, the kidney is sensitive to changes in O_2 availability. This sensitivity enables the kidney to regulate O_2 transport, e.g. by controlling the blood erythrocyte count via erythropoietin secretion. At the same time, the possibility of hypoxia is increased.

The medullary thick ascending limb has high Na^+/K^+-ATPase activity and oxygen demand. NaCl load results in renal enlargement. It can be hypothetized that NaCl-induced renal hypertrophy may increase the diffusion distances for oxygen and result in lowered oxygen availability in the most susceptible areas. Hemodynamic changes in the kidney, e.g., increased vascular resistance, during the development of hypertension may also alter oxygen tension in the kidney (Matavelli *et al.*, 2007).

Tubular ischemia has been recognized to contribute to the development of salt-sensitive hypertension (Johnson *et al.*, 2002). Decreased number of renal glomeruli, as well as structural difference in the glomeruli, may partially explain changes in renal function in prehypertensive SHR (Skov *et al.*, 1994). The excretion of concentrated urine requires energy and high amounts of oxygen (Brezis and Rosen, 1995).

The ability to produce concentrated urine is challenged by increased sodium intake. In a normal physiological state renal medulla is at the brink of hypoxia (Schurek *et al.*, 1990). High NaCl intake may result in increased energy demand and subsequent reduction in oxygen availability. Development of hypertension results in pathologic hemodynamic changes, which may contribute to the development of areas of relative hypoxia in the kidney. Hemoglobin levels in SHRs are higher than in the WKY rats (Welch *et al.*, 2001), which lends support to the hypothesis of renal hypoxia. High hemoglobin levels may also increase peripheral resistance and contribute to hypertension.

Decreased renal NAD^+/NADH ratios have been found in several rodent models of hypertension including salt-induced hypertension (Orbetzova *et al.*, 1976). Isolated tubular cells from SHR, during the development of hypertension are reported to have 15% to 25% higher oxygen consumption and slightly lower ATP levels than cells from WKY, even when hypertension was treated with drugs (Brazy *et al.*, 1989). The SHR kidney may therefore be more susceptible to hypoxia than kidney of WKY rat during the excretion of excess sodium.

Renal ability to produce concentrated urine is also based on high osmolyte concentration in renal medulla (Eckstein and Grünewald, 1996). In order to increase urine osmolarity to meet the challenges associated with high NaCl intake, it is anticipated that higher osmolyte levels are needed, the generation of which also requires energy. Renal circulation has effective shunting, which may contribute to the stability of osmolar gradients, but renders the kidney susceptible to decreased oxygen availability.

Decreased capillary density have been shown to contribute to the development of hypertension in the SHR model, since stimulation of angiogenesis by subjecting young SHRs to hypoxic conditions has been shown not only to increase capillary density, but also prevent development of hypertension (Vilar *et al.*, 2008) in rats kept on a standard diet.

There is experimental evidence that decreased oxygen tension is found in association with diabetes (Palm, 2006). Recent studies indicate that renal hypoxia upregulates the renin-angiotensin system, which in turn causes vasoconstriction of efferent arterioles and increases ROS production (Nangaku *et al.*, 2007) which likely occurs at the expense of O_2 available for energy production. As hypoxia upregulates XOR activity, tissue XOR levels may reflect oxygen availability (Terada *et al.*, 1997).

Wang and co-authors (2008) recently found that hypoxia increases production of IL-6, a proinflammatory cytokine, in cultures of lung microvascular endothelial cells concomitantly with XDH/XO upregulation. It was also found that IL-6 antibodies nearly reversed XOR upregulation (Wang *et al.*, 2008). Furthermore, it was also shown in the same study that IL-6 is upregulated by increasing NaCl intake in Dahl SS rats, but not in the salt-resistant Dahl SR rats.

Chronically increased blood pressure can contribute to renal damage by several mechanisms leading to inflammation and infiltration of immunocompetent cells. In animal models renovascular hypertension, association of hypertension with inflammation has been a constant finding (Rodriguez-Iturbe *et al.*, 2002a, Rodriguez-Iturbe *et al.*, 2002b).

2.4.3 *Cyclosporine* (CsA) *nephrotoxicity*

CsA is a common immunosuppressive agent used in organ transplantation. CsA binds to cyclophilin and the complex inhibits calcineurin, a proteinphosphatase involved in dephosphorylation of members of the nuclear factors of T-cells (NFAT), which then translocate into the nucleus (Beals *et al.*, 1997). NFATs induce synthesis of interleukins. Inhibition of calcineurin by CsA inhibits gene expression of activated T-cells via NFAT pathway (Matsuda *et al.*, 2000a). In addition to calcineurin inhibition, there is evidence that CsA exert its immunosuppressive effects also by blocking JNK and p38 signaling pathways during T-cell activation (Matsuda *et al.*, 2000b). Interestingly p38 has been reported also to phosphorylate XDH and XO (Kayyali *et al.*, 2001). Recent reports indicate that calcineurin is involved in the development of cardiac hypertrophy and, furthermore, CsA has been shown to reverse cardiac hypertrophy in rats (Hongzhuan *et al.*, 2008).

Nephrotoxicity limits CsA use in organ transplantation. Therapeutic CsA doses can result in malignant hypertension by impairing renal function. Development of CsA-induced renal damage proceeds with several mechanism leading e.g. to hyperuricemia as renal function becomes compromised. On the other hand hyperuricaemia has been shown to exacerbate CsA-induced nephropathy (Mazzali *et al.*, 2001b). These phenomena relate to levels of circulating urate and renal elimination mechanisms and provide one possible vicious

cycle, which is associated with CsA toxicity. In the context of XOR, it is however interesting to note that CsA administration results in renal hypoxia by marked Ang II induced renal vasoconstriction. Furthermore, CsA toxicity is exacerbated by increased dietary intake of NaCl (Pere *et al.*, 1998). Allopurinol, a specific XOR inhibitor, has been added into some of the cold storage solutions used to preserve kidney transplants (Baatard *et al.*, 1993).

In theory, CsA induced vasoconstriction and other factors resulting in hypoxia can lead to XOR induction, generation of ROS and NO metabolites, add to the urate load and finally contribute to harmful renal effects of CsA. Hypoxic adaptation involves upregulation of angiogenesis, glycolysis and erythropoiesis. In many tissues decreasing oxygen consumption by induction of the pentose phosphate shunt is one of the adaptive mechanisms. The hypoxia inducible factor (HIF-1α) is one of the regulating factors involved. Inhibition of HIF-1α by CsA has been reported in cell cultures of glioma cells, and has been suggested to contribute to CsA toxicity (D'Angelo *et al.*, 2003). CsA inhibits calcineurin, which in turn regulates HIF-1α expression (Liu *et al.*, 2007). Induction of XOR by hypoxia is however not mediated by HIF-1α (Linder *et al.*, 2003).

To summarize the Section 2.4: While the kidney has a crucial role in the development of hypertension, it is also one of the target-organs for harmful effects of sustained increases in SBP. In a normal physiological state, renal medulla is at the brink of hypoxia. High NaCl intake may result in compromised oxygen availability. NaCl induced renal hypertrophy may increase diffusion distances for oxygen. Decreased capillary density may contribute to the development of hypertension in hypertension prone rats. Nephrotoxic effects of CsA are in part mediated by renal vasoconstriction. CsA nephrotocity is aggravated by dietary NaCl.

2.5 OXIDATIVE STRESS AND HYPERTENSION

Vascular endothelium plays a crucial role in hemodynamic homeostasis. Endothelial cells synthesize NO, which is a vasodilator and contributes to endothelial health. ROS, in particular superoxide, have been suggested to react with NO, decreasing its availability and thus contributing to endothelial dysfunction. This phenomenon has been associated also with clinical hypertension (Landmesser and Drexler, 2007a). An increased endothelial production of superoxide has been found in several experimental models of hypertension including Dahl SS rats (Zicha *et al.*, 2001) and the SHR model (Schnackenberg *et al.*, 1998). In contrast, vascular production of superoxide was not associated with increased blood pressure induced by 5-day infusion of adrenaline (Kawazoe *et al.*, 2000; Laursen *et al.*, 1997).

Potential sources of reactive oxygen species in the circulation include XO, cyclooxygenases, NAD(P)H oxidase, also NADH oxidase activity of XDH, uncoupled NO synthases, mitochondrial respiratory enzymes and lipoxygenases (Szasz *et al.*, 2007).

XO, which is a post-translationally modified form of XDH, may pass electrons to oxygen in favour over NAD^+ and generate ROS. XO has been identified as one of the major H_2O_2 and O_2^- generating enzymes in the endothelium along with NAD(P)H oxidase, and the mitochondrial electron transport chain (Iuchi *et al.*, 2003). Once formed, O_2^- gives rise to other ROS molecules, such as H_2O_2, by the action of superoxide dismutase (SOD).

Both XDH and XO (to a lesser extent) exhibit NADH oxidase activity with simultaneous ROS production. This activity is inhibited by diphenylene iodonium (DPI), but it is not inhibited by oxopurinol or allopurinol (Zhang *et al.*, 1998a).

NAD(P)H oxidase and reactive oxygen species have also been shown to associated with dexamethasone induced hypertension in mice (Hu *et al.*, 2006). Angiotensin II infusion increases ROS production, a process which can be prevented by apocynin.

Studies on NAD(P)H oxidase have to a great extent relied on two putative inhibitors, apocynin and DPI. While the former has been shown to be an antioxidant with no inhibiting activity, the inhibiting activity of the latter requires dimerization by myeloperoxidase, an enzyme, which is not present e.g. in vascular endothelium. Currently there are no specific inhibitors of NAD(P)H oxidase. NAD(P)H oxidase is a complex consisting of several protein components, which hampers interpretation of mRNA based studies. Consequently, some of the studies on NAD(P)H oxidases should be interpreted cautiously (Heumuller *et al.*, 2008).

While NADPH oxidase is a membrane-bound protein and produces superoxide anions in the extracellular space, XOR is cytosolic and is therefore associated with intracellular redox homeostasis. As a cytosolic enzyme XO is released under pathophysiological conditions into circulation and is able to bind to the extracellular matrix of cells via glycosaminoglycan residues. Cell-bound XO retains its ability to produce superoxide anions (Adachi *et al.*, 1993; Houston *et al.*, 1999).

ROS have a well-established role in the regulation of sodium retention in the kidney (Taylor *et al.*, 2005), which is mediated by ROS induced degradation of NO. NO has a major role in controlling renal hemodynamics and sodium excretion (Zou and Cowley, 1999). Furthermore, O_2^- is able to directly induce sodium reabsorption (Ortiz *et al.*, 2002). The major demand for oxygen in the kidney is related to the active transport of sodium ions. Hypertonic infusion in the medullary thick ascending limb has been reported to increase oxidative stress (Mori and Cowley, 2004).

ROS are potentially harmful and scavenged by specific enzymes or endogenous and exogenous redox active molecules. Inhibition of uricase by oxonic acid to the extent which produces only modest increase in urate levels, not high enough to produce microcrystals, results in the development of salt-sensitivity and hypertension in salt-resistant rats (Mazzali *et al.*, 2001a). These findings point towards that urate, although being an antioxidant, contributes to development of hypertension rather than alleviates it.

There is an ongoing discussion on the role of oxidative stress in clinical hypertension. It

has not yet been clarified whether increased oxidative stress is a pathological consequence of hypertension, a predecessor of it, or whether is involved in the development of it. Most of epidemiological studies (involving up to 250 000 subjects) do not lend support to the idea that supplementation with common antioxidative vitamins would prevent cardiovascular diseases (Howes, 2006).

More recent studies seem to indicate that oxidative stress contributes to hypoxia in the kidney and compromise renal function (Welch, 2006). Treatment of hypertension with antihypertensive drugs has been reported to decrease ROS production (Landmesser *et al.*, 2007b).

To summarize: Large epidemiological studies on association of cardiovascular diseases with dietary antioxidative agents have been negative. ROS are indispensable physiological signal transducers, which may become harmful only if their production rate exceeds endogenous scavenger capacity of metabolizing enzymes and redox active compounds. Association of ROS with hypertension is well-established in experimental models. It is not known whether increased ROS production precedes hypertension.

2.6 NITRIC OXIDE (NO)

Nitric oxide is an established vasodilator and has a wide range of biological functions, which link reduced availability of NO with pathogenesis of hypertension, atherosclerosis and diabetes. NO plays a crucial role in sodium retention (Zou and Cowley, 1999).

The role of vascular endothelium in vasoregulation became apparent only after identification of the endothelial relaxing factor, which is synthesized from L-arginine by NO synthases (NOS) mainly in the endothelium (Moncada and Higgs, 2006). In mammals three different NOS enzymes exist: constitutive, inducible and neuronal NOS.

NO has been shown to play a crucial role in renal medullary oxygenation (Brezis and Rosen, 1995). As reviewed recently by Palm and co-workers (2009), NO competes with oxygen for the binding site in cytochrome oxidases and inhibit mitochondrial oxygen expenditure. This occurs, when levels of oxygen are low in comparison to NO, i.e. in hypoxia. NO is able to increase the efficiency of electrolyte transport per consumed oxygen molecule in the renal tubulus. In Sprague-Dawley rats increasing dietary salt intake upregulates endothelial, inducible and neural NOS in renal medulla, indicating the importance of NOS in adaptation to dietary salt load (Mattson and Higgins, 1996).

Chronic, unselective inhibition of NOS by N^{ω}-nitro-L-arginine methyl ester (L-NAME) was first described as a model of hypertension by Oliveira-Ribeiro *et al.* in 1992. L-NAME was shown to produce a marked renal vasoconstriction and hypoperfusion, which could partially be reversed by L-arginine infusion, but not by D-arginine (Ribeiro *et al.*,

1992). While renal cortical blood flow was reported to remain unaltered, renal medullary blood flow was markedly decreased (Nakanishi *et al.*, 1995). L-NAME has been shown to decrease NO production in mice from 70% to 90% (Wickman *et al.*, 2003). We have previously shown that chronic NOS inhibition downregulates renal glutathione synthesis (Levonen *et al.*, 2000), which may render the kidney more vulnerable to harmful effects of increased ROS production. Increased levels of the endogenous NOS inhibitor, asymmetric dimethyl arginine have been associated with acute coronary events in epidemiological studies (Valkonen *et al.*, 2001).

Chronic NOS inhibition results in sodium and water retention leading to hypertension, not only in genetically hypertension-prone experimental animals, but also in normotensive, salt-resistant rats (Mattson *et al.*, 1994). Low dose L-NAME treatment, not sufficient to increase blood pressure, has been reported to transform salt-resistant dogs to salt-sensitive (Salazar *et al.*, 1993).

Generation of peroxynitrite from reaction of NO with ROS can inactivate NO, and it has been suggested as one of the mechanisms explaining why the inhibition of ROS producing enzyme XO can lead to the improvement of endothelial function (George *et al.*, 2006). Sickle cell disease, which is caused by a mutation in β-globin, results in polymerization of the mutated hemoglobin and subsequent episodes of ischemia/reperfusion in tissues e.g. in the liver. An increased level of circulating XO, which originates from the liver, has been reported in sickle cell disease. Circulating XO binds avidly to cells of the vascular lumen. Markedly increased XO-dependent vascular oxidative stress has been observed to contribute to vascular dysfunction, which is a hallmark of the disease. Clinically, the disease is not associated with hypertension. Mice models of sickle cell disease do not show increased blood pressure, but they are fivefold more sensitive to blood pressure increasing effects of the NOS inhibitor L-NAME than controls (Aslan *et al.*, 2001).

To summarize Section 2.6: NO is a potent endothelium derived vasodilator, which has several other roles including regulation of renal oxygenation. NO is produced by NOS. NOS inhibition results in malignant hypertension. Low doses, not enough to produce increased SBP, can render salt-resistant animals to salt-sensitive.

2.7 URATE

2.7.1 *Urate as an antioxidant*

XOR catalyzes the rate limiting step of purine metabolism producing urate. Recent prospective large epidemiological studies suggest a role for urate as an independent risk factor for cardiovascular diseases. Strasak *et al.* (2008b) studied more than 28 000 elderly Australian women and reported serum uric acid as an independent predictor of all major forms of cardiac deaths. In an even larger study, more than 83 000 Australian men, had

similar outcome (Strasak *et al.*, 2008a). The association of high serum urate levels with the development of essential hypertension has been known for decades (Cannon *et al.*, 1966, Prebis *et al.*, 1981). The underlying biochemical mechanisms have still not been fully elucidated. A significant association of plasma urate and XOR activity with mean arterial blood pressure has been observed in clinical studies among normotensive individuals (Newaz *et al.*, 1996).

In most mammals circulating urate levels are kept low by hepatic microsomal uricase, which degrades urate to the more water-soluble allantoin. In the rat, renal uricase is not expressed and the kidney produces at least 7% of total excreted urate (Chin *et al.*, 1980). Uricase is silenced in humans and other primates as a consequence of two independent mutations (Oda *et al.*, 2002). Urate excretion is relatively low in humans, and consequently, its levels in the circulation are high, approximately 50 times higher than in the rat. In humans, urate filtered through glomerulus is reabsorbed effectively by the URAT1 transporter while only 5–10% is excreted (Anzai *et al.*, 2007). Several agents including nonsteroidal anti-inflammatory drugs and losartan, an Ang II receptor antagonist and antihypertensive drug, exhibit uricosuric effects by inhibiting the URAT1 transporter (Enomoto *et al.*, 2002). The kidney can regulate changes in circulating urate levels, which may result from increases in urate production induced by dietary purine intake or from an increase in endogenous production (Rieselbach *et al.*, 1964).

Clinical studies on lowering urate with the uricosuric agent, probenicid, did not result in an improvement of endothelial function (George *et al.*, 2006). Systemic administration of uric acid to healthy men, in order to temporarily increase circulating urate, did not exert any effect on hemodynamic variables or NO dependent endothelial function (Waring *et al.*, 2004). However, in other experimental settings contradictory results were obtained. Intravenous urate infusions in rodents have been reported to result in endothelial dysfunction (Khosla *et al.*, 2005). In human studies urate infusion improved endothelial function in subjects with type 1 diabetes and in smokers (Waring *et al.*, 2001).

In experimental studies, increasing circulating urate levels in rats by the uricase inhibitor, oxonic acid, have been shown to result in renal arteriolopathy and hypertension (Mazzali *et al.*, 2001a; Mazzali *et al.*, 2002; Sanchez-Lozada *et al.*, 2002; Johnson *et al.*, 2003). The increased urate levels were reported to induce salt-sensitivity in rats (Mazzali *et al.*, 2001a) with stimulation of the renin angiotensin system. Based on these studies uric acid has been proposed to participate in the development of human salt-sensitivity (Watanabe *et al.*, 2002).

Urate is one of the most potent water-soluble antioxidants, even more effective than ascorbate (Waring *et al.*, 2001). In addition, urate is able to chelate transition metals, such as iron, thus protecting its environment from Fenton reactions, which generate ROS. Urate has been shown to protect ROS inactivating superoxide dismutase enzymes SOD1 and SOD3 from ROS-induced loss of activity (Hink *et al.*, 2002).

While various treatments with antioxidative compounds have been effective in alleviating hypertension in rodent models (Tian *et al.*, 2005), clinical studies relating to treatment of hypertension with antioxidants have not been encouraging. The finding that urate rather worsens rather than alleviates hypertension in rodent models does not support the view of urate having a role as an antioxidant preventing increased blood pressure through the scavenging ROS.

In humans urate shows a circadian rhythm in close association with NO (Kanabrocki *et al.*, 2000). Urate scavenges peroxynitrite ($OONO^-$), an oxidative compound derived from the reaction between NO and O_2^-, which is also capable of reacting with proteins and other cellular constituents (Squadrito *et al.*, 2000). Urate has been reported to exert neuroprotective effects against inflammation in rodent models of multiple sclerosis by scavenging peroxynitrite (Hooper *et al.*, 1998, Spitsin *et al.*, 2000). Urate can also react directly with NO, producing nitrated urate. In this form, it may decompose back to NO and act as NO reservoir or vehicle (Suzuki, 2007). The finding points towards the possibility that locally generated urate might be able to modify the effects of NO.

2.7.2 *Urate modifies immune function*

Immunization with a protein can lead to tolerance unless an adjuvant, such as aluminum oxide, is co-administered and resulting in a stimulated immune response. Bacterial wall components also act as adjuvants. Dead cells from the body itself are immunogenic, indicating that some endogenous components may also act as adjuvants. Several molecules, including monosodium urate, purinergic metabolites, heat shock proteins and nuclear proteins, have been postulated to stimulate innate immunological response and together form a damage-associated molecular pattern (Bianchi, 2007).

Tissue damage increases local degradation of nucleotides, for example ATP, DNA and RNA. Subsequently dying cells liberate xanthine and hypoxanthine, which are then converted to urate by XOR. The liberated uric acid acts as a danger signal and endogenous adjuvant (Shi *et al.*, 2003). Urate crystals have been shown to activate dendritic cells, which upon activation orchestrate the immune system (Shi *et al.*, 2006). In humans circulating urate levels are high and increased local production may result in the precipitation of microcrystals. Precipitation of urate and consequent activation of the immune system is the mechanism responsible for the clinical manifestation of gout. High urate concentration in humans is therefore a sensitive trigger for the immune system to act in the event of tissue damage, and, as an endogenous adjuvant. In solution uric acid or urate is also a modulator of the immune system (Shi *et al.*, 2006).

Local production of urate by XOR may initiate an immune response. In a recent double-blind clinical trial, the XOR inhibitor allopurinol reduced the rise of inflammatory markers after acute ischemic stroke, most notably the proinflammatory surface adhesion molecule ICAM-1 (Muir *et al.*, 2008). Experimentally ICAM-1 has been shown to be

important in hypertension related end-organ damage (Mervaala *et al.*, 1999). Rodent models of hypertension have been shown to involve recruitment of macrophages and other immunocompetent cells into the kidney. Immunosuppression has been reported to alleviate hypertension in Dahl salt-sensitive rats (Tian *et al.*, 2007). However, the role of urate has not been clarified in these experimental settings.

To summarize Section 2.7: Urate is produced by XOR in a reaction, which is rate limiting in purine metabolism. As an antioxidant it may protect NO against ROS. Urate may also protect NO from degradation by forming a complex with it. In rats, moderately increased urate levels result in hypertension and salt-sensitivity. Urate acts also as an adjuvant and may contribute to dietary NaCl and hypertension-induced target organ inflammation. Increased urate levels may have nephrotoxic properties.

2.8 xanthine oxidoreductase (xor)

As early as 1902, an enzyme fraction present in whole milk was found to decolorize methylene blue in the presence of formaldehyde by Schardinger (1902). Twenty years after this discovery, the same enzyme fraction was found to produce urate (Morgan *et al.*, 1922). Later urate production was shown to belong to the terminal rate-limiting steps in human purine catabolism from the purine nucleotide degradation products hypoxanthine and xanthine by the same 'Schardinger enzyme' or xanthine oxidoreductase. These pathways are depicted schematically in Figure 3. In humans, urate is a terminal metabolite, unlike in the rat liver where urate is further oxidized to allantoin.

XOR exists in two interconvertible isoforms, xanthine dehydrogenase (XDH, EC 1.2.1.37) and xanthine oxidase (XO, EC 1.1.3.22). XO is a post-translationally modified form of the initial gene product XDH. In the rat, the gene coding XDH protein is located on chromosome 6 (at 6q13) and the human sequence on chromosome 2 (at 2p23.1, Rytkönen *et al.*, 1995).

In the reaction with hypoxathine or xanthine, the XO isoform transfers electrons to molecular oxygen generating reactive oxygen species, such as superoxide anion and hydrogen peroxide. XDH in turn transfers the electrons, generated from production of urate from hypoxanthine and xanthine, to NAD^+, which is subsequently reduced to NADH (Fridovich, 1970, Harris and Massey, 1997; Hille and Nishino, 1995).

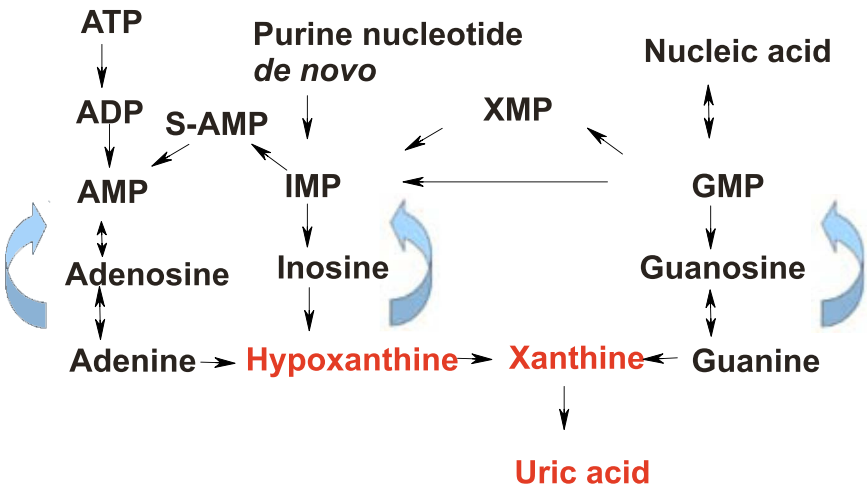

Figure 3. Schematic presentation of purine metabolism. Reactions involving XOR activity are highlighted with red colour. Purines are expensive in terms of the number of ATPs used in their synthesis. Blue colour refers to salvage pathways, which are energetically important as purine synthesis consumes high amount of ATP molecules. IMP, inosine monophosphate; S-AMP, succinyl monophosphate; XMP, xanthine monophosphate.

As is depicted in the three-dimensional presentation in Figure 4, XOR is a nonglycosylated metalloflavoprotein which contains two identical 145 kDa subunits. Each subunit has one molybdopteridin site, one FAD site and two Fe-S centers. The XOR sequence shows remarkable similarity with other molybdoenzymes, and it has partially overlapping substrate specificity with aldehyde oxidase (AO) (Garattini *et al.*, 2008).

Mutation of two amino acids in the amino acid cluster that determines the conformation around the FAD prosthetic group, favours the XO conformation and increases superoxide: hydrogen peroxide ratio from 1:3 to 6:1 (Asai *et al.*, 2007).

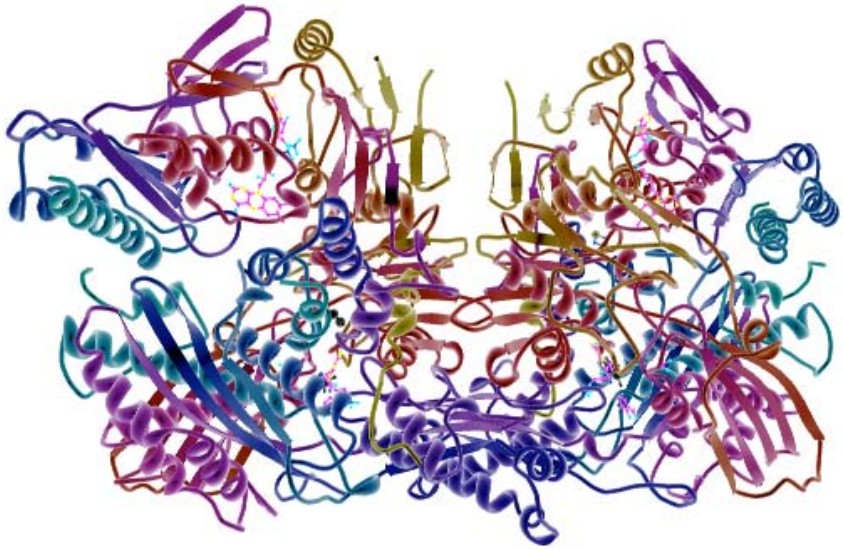

Figure 4. Three dimensional model of bovine xanthine oxidase showing α-helixes and β-pleated sheets. The enzyme is a 300 kDa homodimer. FAD site is visible upper left. Data from Enroth *et al.* (2000) depicted using Molecular Visualization Applications by Moreland *et al.*(2005).

2.8.1 *Distribution*

XOR has been detected in all mammalian cells as well as vertebrates, invertebrates, higher plants, green algae, fungi and bacteria. It is an integral part of the purine catabolism in all cells. In mammals highest activities are found in the intestine, liver, mammary gland and bile ducts.

As an enzyme capable to produce ROS, reactive nitrogen species and the endogenous adjuvant, urate, XOR is related to innate immunity and, along with another antimicrobial enzyme lysozyme, is expressed in capillary endothelial surfaces (Spiekermann *et al.*, 2003), in mammary gland epithelia and other epithelial surfaces, epithelial secretions, milk and phagocytes (Vorbach *et al.*, 2003).

In circulation, the XO isoform prevails. The highest levels of circulating levels of XO have been found in rats. Circulating antibodies against XOR exist in healthy individuals. The antibodies do not inhibit the enzyme, but may have a role in accelerating the turnover of the ROS producing enzyme (Benboubetra *et al.*, 1997).

Khan *et al.* (2004) found that deficiency of neuronal NOS (NOS1 isoform), but not endothelial NOS (NOS3), induced XOR in murine cardiac myocytes. Increased ROS

production by XOR was found with depression of myocardial excitation-contraction coupling, which was reversed by XOR inhibition with allopurinol. Neuronal NOS and XOR were found in the same immunoprecipitate and also in the same sarcolemmal fraction obtained by sucrose gradient chromatography. These results indicate that XOR and NOS1 are located in close proximity to each other in cardiac sarcolemma (Khan *et al.*, 2004).

XOR is an abundant protein in milk, from where it was first isolated. In mice and cattle XOR represents 1–2% of total soluble protein of lactating glands. In milk XOR is present predominantly in XO isoform and does not contain molybdenum. XO is a structural element participating in the enveloping of fat droplets in milk, where it also has an antimicrobial role. In lipid globules of milk, XOR is associated with butyrophilin suggesting a role in the formation and secretion of milk lipid (Murphy and Vance, 1999).

2.8.2 *Regulation of XOR activity and expression*

XOR is subject to post-translational modifications. For a summary of modifying factors and inductors, see Table 1.

Hypoxia is one of the best known factors that regulate XOR (Lanzillo *et al.*, 1996, Terada *et al.*, 1997). XOR activity is rapidly changed by hypoxia (within 4 hours), indicating that protein synthesis is not initially required (Linder *et al.*, 2003). However, at a later stage (24 hours or more) protein synthesis also becomes involved. The p38 kinase, or the 'stress-induced kinase', is involved in events resulting in phosphorylation of XDH/XO and is regulated by hypoxia (Kayyali *et al.*, 2001; Kinugawa *et al.*, 2005). Tissue XOR activity may increase in areas of hypoxia as XOR activity is suppressed in normoxia.

Proinflammatory mediators, such as lipopolysaccharide and cytokines, have been reported to increase endothelial XO expression (Brandes *et al.*, 1999). XOR expression is induced by inflammatory cytokines, such as TNF-α, IL-1β and IFN-γ, suggesting a role for XOR in inflammatory processes (Page *et al.*, 1998). IL-3 also contributes to XDH to XO conversion (Friedl *et al.*, 1989). In human monocyte cultures, XOR inhibition by allopurinol decreases ICAM-1 expression (Mizuno *et al.*, 2004). Along with I/R process and subsequent ROS production by XO (described in the previous Chapter), these phenomena have an association with defence against bacterial invasion, as such an occurrence could eventually obstruct circulation.

Sulfuration of Moco (see Section 2.8.3) is essential for human XOR activity. Deficiency of the Moco sulfurase enzyme results in XOR and aldehyde oxidase deficiency (Ichida *et al.*, 2001).

XOR is an abundant protein in milk. XOR expression in the mammary gland is tightly regulated by secretory activity (McManaman *et al.*, 2002). Lactogenic hormones, prolactin and cortisol, increase XOR expression and activity by up to 500% as demonstrated by incubating mammary epithelial cells with these hormones. This effect was inhibited by genistein, a tyrosine kinase inhibitor and by MAP kinase pathway inhibitor. (McManaman

Table 1. Factors, which regulate and modify XOR activity and/or expression

Regulating factor	Effects	Reference
Hypoxia	An apparent increase in activity which does not initially require translation or transcription.	Linder *et al.*, 2003
XDH/XO phosphorylation	Casein kinase II, p38 kinase ('stress induced kinase' activated by hypoxia).	Kayyali *et al.*, 2001
Sulfuration of Moco	Moco sulfuration can be removed with cyanide and resulfured with molybdenum sulfurase. Moco sulfuration gives the cell an opportunity to rapidly regulate the amount of active XOR.	Ichida *et al.*, 2001
Disulphide-S-monoxides	Oxidation of cystein residues, reversible XDH to XO conversion.	Sakuma *et al.*, 2008
Irreversible XDH to XO conversion	Proteolysis, inhibited by leupeptin.	Amaya *et al.*, 1990
Intracellular iron	Translational upregulation.	Martelin *et al.*, 2002
Nuclear factor Y	Trasnscriptional activation of human xdh gene.	Martelin *et al.*, 2000
Proinflammatory mediators, lipopolysaccharide, IL-3	XDH to XO conversion.	Friedl *et al.*, 1989
Circulating spontaneous XOR antibodies	Endogenous inhibition of XOR or turnover acceleration of XOR protein.	Bruder *et al.*, 1984
Neuronal NOS	XOR down-regulation.	Khan *et al.*, 2004
Tumor suppressor pathway including OSM-1 and SAFB1	XOR induction.	Lin *et al.*, 2008
Angiotensin II	XOR induction in a culture of bovine endothelial cells.	Landmesser *et al.*, 2007b
NAD(P)H oxidase	XOR induction in a culture of bovine endothelial cells.	Landmesser *et al.*, 2007b
Toxic chemicals	Dioxines, such as TCDD, induce XOR activity in mouse liver.	Sugihara *et al.*, 2001

et al., 2000). Mice, made deficient of XOR by gene knockout, do not live beyond 6 weeks. XOR +/- mice have normal appearance, but are not able to lactate normally, and their pups die (Vorbach *et al.*, 2002).

2.8.3 *Molybdenum cofactor (Moco)*

The oxidation of xanthine and hypoxanthine takes place at the Mo center of the enzyme. Concomitant reduction reactions are catalyzed by the two FAD centers of the molecule. Molybdenum cofactor is an essential for all molybdenum enzymes (Nason *et al.*, 1971). Known mammalian molybdoenzymes include in addition to XOR, aldehyde oxidase and sulphite oxidase. In this family of proteins, Mo occurs as Moco, in which Mo is bound to a unique tricyclic pterin structure (Schwarz, 2005). Mo is the only transition metal of the second series of Periodic Table known to be essential for mammals. Its chemistry is perhaps the most complex of all transition metals. Mo has found its way to biochemical systems most likely because of its easy redox conversions in aqueous environments (ranging from oxidation state IV to VI). Unlike many other transition metals Mo appears in biological systems as an anion, e.g. MoO_4^{2-}.

Molybdenum is generally less toxic in comparison with other transition metals. Excessive Mo intake has occurred in geologically distinct areas of Russia and results in increased xanthine oxidoreductase activity, high urate levels and gout-like symptoms. These effects are opposed by copper ions, and more specifically by tungstate. Tungstate can replace molybdate in the enzyme during Mo deficiency, which has been used experimentally to inhibit XOR *in vivo* (Brown *et al.*, 1988).

Because of its vulnerability to degradation by oxygen, Moco is not believed to exist as a free compound in mammalian cells. Without a protective environment provided by an apoenzyme, the half-life of Moco at neutral pH is only a few minutes (Nason *et al.*, 1971). Enzymes involved in Moco synthesis are highly conserved in all cells. In humans Moco deficiency (MIM 252 150), leading to deficiency of XOR, AO and sulphite oxidase, is fatal in early childhood due to sulphite accumulation in the central nervous system (Schwarz *et al.*, 2004).

2.8.4 *Conversion of XDH to XO*

Initially the XOR protein is translated as XDH, which is subject to post-translational modifications converting XDH to the XO isoform with concomitant changes in enzyme activity and specificity. The conversion is reversible when it involves the formation of a disulfide bridge by oxidation of -SH moieties of critical cysteine residues. XOR has a cluster of cysteins, which makes the enzyme redox sensitive.

There are data to suggest that XDH and XO are at thermal equilibrium, and proteolysis or thiol oxidation can then lock the protein in XO isoform. (Nishino *et al.*, 2005). The irreversible conversion of XDH to XO results from proteolysis of a linker peptide, which

connects the molybdenum and NAD domains. For example, Ca-proteases and trypsin can cleave a 20 kDa fragment from the enzyme resulting in an irreversible conversion. These changes may take place even *in vitro*, for example by the disruption of lysosomes during purification, thus leading to artificially high XO activity. This type of conversion can be prevented *in vitro* by the use of protease inhibitors, such as leupeptin.

Proteolytic cleavage of the enzyme takes place in a variety of conditions. During ischemia XOR is converted to the ROS producing isoform XO. Restoration of circulation increases oxygen availability, which is accompanied with XO catalyzed ROS production (Parks *et al.*, 1988). During ischemia ATP is degraded to AMP and then further to xanthine and hypoxanthine. This process provides an increased substrate availability for XO. Low oxygen tension has been reported not only to induce XOR, but also to increase XDH to XO conversion. This type of setting is referred to as ischemia-reperfusion or I/R, and is often associated with tissue damage (McCord, 1985). As the lack of oxygen is a powerful regulator of metabolic and cellular events, I/R is a complex process involving a plethora of stress induced factors.

Transformation may take place in mucosa in association with the common cold caused by *Rhinoviruses*. The *Rhinovirus* infection has been reported to induce conversion of XDH to XO. Serine proteases are involved as the conversion was inhibited by aprotinin and leupeptin, but not by inhibitors of metalloproteinases. During *rhinovirus* infection of primary human bronchial epithelial cells, ROS generated by XO depleted cellular GSH stores thus shifting the redox balance to favor oxidative XDH to XO conversion. This in turn creates a vicious cycle which was found to be harmful to the infected cells (Papi *et al.*, 2008). The authors reported that oxypurinol, a specific XOR inhibitor, not only decreased ROS production substantially, but also prevented virus proliferation in cell culture.

2.8.5 *XOR, nitrate, nitrite and nitric oxide*

Although it was already known in 1924 that XOR reduces nitrate to nitrite, there has afterwards been a long-lived tenet, according to which nitrate is not metabolized by mammalian cells. Nitrite has traditionally been regarded as a bacterial metabolite of nitrate. Circulating nitrate is derived from dietary sources and NO metabolism (Lundberg and Weitzberg, 2005). In clinical studies, nitrate levels correlated negatively with plasma levels of an endogenous NOS inhibitor, asymmetric dimethyl arginine (Päivä *et al.*, 2006).

Jansson *et al.* (2008) have recently demonstrated that germ-free mice are also able to produce nitrite from nitrate. Allopurinol treatment was shown to reduce 40 – 80% of nitrate reductase activity in mouse, rat and human liver homogenates. In murine tissues greatest effects were found in the liver, the intestine and the kidney, while in the lung and cardiac tissues allopurinol did not have any effect on nitrate reduction (Jansson *et al.*, 2008, Tripatara *et al.*, 2007). These results are in line with XOR distribution, which has been covered in Section 2.9.4.

Evidence has been obtained indicating that even NO can be generated enzymatically from nitrite. Zhang *et al.* (1997, 1998b) were first to show that XO is able to convert nitrite ions into NO. Furthermore, XDH tertiary structure was noted to resemble that of bacterial nitrate reductase (EC 1.6.6.1), which is also a Moco enzyme.

Human studies have suggested generation of NO by erythrocyte bound XOR (Webb *et al.*, 2008a). Recent reports by Li *et al.* (2008) demonstrated that along with XOR, AO is another enzyme capable in producing NO. Li *et al.* (2008) provided evidence indicating that nitrite reduction does not take place in blood, but in tissues. The generation of NO from nitrite by AO and XOR is regulated by pH, oxygenation, nitrite levels and redox balance. These features indicate that the nitrite pathway could be an alternative NO source in hypoxic conditions. The oxygen in NO molecules are derived from molecular oxygen when NO is generated from L-arginine by NOS (Leone *et al.*, 1991).

Experimentally, the reaction producing NO from nitrite has been demonstrated within (or near) physiological levels of nitrate and nitrite, and also when tissue oxygen levels are not compromised (Jansson *et al.*, 2008). In liver homogenates from rats nitrite formation from nitrate was not inhibited by L-NAME, suggesting that nitric oxide synthases were not involved. In line with these observations, nitrate has been reported to transiently lower SBP in rats even during coadministration of the NOS inhibitor, L-NAME (Kanematsu *et al.*, 2008). Casey *et al.* (2008) found that i.v. administration of nitrite i.v. decreased transiently both pulmonary and systemic arterial pressure and increased cardiac output in Sprague-Dawley rats by allopurinol-sensitive mechanisms, which were not blocked by L-NAME.

Recent studies by Webb *et al.* (2008b) demonstrated a transient blood pressure lowering effect from beet juice, which contained high levels of nitrate. Nitrate is continuously excreted into saliva. The decrease in SBP was blocked by collection of saliva, which impedes conversion of nitrate to nitrite by nitrate reductases of oral anaerobic bacteria.

The use of organic nitrates is a cornerstone in the treatment of ischemic heart disease. Development of nitrate tolerance limits the usefulness of organic nitrates, including glycerol trinitrate. Development of nitrate tolerance is a complex phenomenon. Mitochondrial aldehyde dehydrogenase (ALDH2) is a redox sensitive enzyme, which yields nitrate from the nitro moiety of many organonitrates. Downregulation of ALDH2 has been associated with nitrate tolerance. There is evidence that also XOR is capable to activate organic nitrates to NO in the presence of thiols and ascorbate (Doel *et al.*, 2001, Li *et al.*, 2005). As nitrite has been shown to be an active metabolite of organonitrates, it has been suggested as a therapeutically useful agent to bypass tolerance to organic nitrates (Dejam *et al.*, 2007). In their human study, the authors reported that oxopurinol failed to inhibit vasodilation induced by nitrite infusion, but on the contrary potentiated vasodilation, indicating that the role of XOR in NO production has not been fully elucidated.

NO exerts most of its effects principally by nitrosating molecules, e.g. generating nitrated derivative of guanylyl cyclase, reacting with glutathione residues in hemoglobin

and producing covalently nitrated thiols in cystein moieties in many proteins (Foster *et al.*, 2003; Bohlen *et al.*, 2009). NO has been reported to inhibit XOR, but whether this takes place also *in vivo* has not been clarified (Khan *et al.*, 2004). Urate has been reported to react with NO. Nitrosated urate can transfer NO to glutathione and may act as a carrier molecule for NO and prolong NO half-life (Suzuki, 2007). The reaction of a superoxide anion with NO produces peroxynitrate, which is able to react with tyrosine moieties of proteins. Urate was shown to effectively compete with tyrosine, and to protect the heart from protein nitration (Teng *et al.*, 2002). Nitrate is a stable molecule and it has been suggested to participate in the long-term regulation of NO via XOR mediated pathways (Lundberg *et al.*, 2008). So far all experimental and human cardiovascular studies on nitrite have reported transient SBP lowering effects.

2.8.6 *Fructose and XOR*

Fructose is one of the dietary factors, which increases urate production. In high acute doses fructose can induce acidosis and hypoglycaemia in subjects with fructose intolerance and to a lesser extent also in tolerant subjects. Fructose and its congeners are rapidly phosphorylated to fructose-1-phosphate, a reaction which consumes ATP and increases ATP metabolism with a concomitant increase in purine substrates for XOR and subsequent increases in urate production (Laakso et. al. 2003).

In animal models fructose feeding induces hypertension. In contrast to glucose, fructose does not exert normal insulin and leptin response. Chronic fructose feeding in rats has been reported to induce leptin resistance and substantial obesity, thus providing an animal model for metabolic syndrome (Oron-Herman *et al.*, 2008). Obesity is associated with sustained increases in blood pressure. In addition, obesity is accompanied with altered renal function, increased reabsorption of sodium, sympathetic outflow and compression of the kidney which is anatomically surrounded by adipose tissue (reviewed by Hall 2003). Experimental studies have indicated that hypercholesterolemia can modify renal function (Minami *et al.*, 2005).

As fructose and its congeners are widely used in food industry, they have been suggested as one of the underlying factors contributing to increased prevalence of metabolic syndrome (Johnson *et al.*, 2007). In contrast to experimental studies, the first epidemiological studies have failed to confirm an association of fructose intake with SBP (Forman *et al.*, 2009).

2.8.7 *Roles of XOR beyond purine metabolism*

While XOR is the key enzyme involved in the last steps of purine metabolism, it has become increasingly evident that the enzyme also has many other biological functions. Upon its discovery XOR or the 'Schardinger enzyme', was found to catalyze (in milk) the discoloration of methylene blue in the presence of aldehydes. Twenty years later there was vivid discussion in the literature concerning whether nitrate reduction (in the presence

of aldehydes, hypoxanthine and xanthine) could be catalyzed by the same 'Schardinger enzyme' (Dixon and Thurlow, 1924). Since then new features relating to biological roles of XOR have been discovered. Established and emerging biological roles for XOR are summarized in Table 2.

XOR has a broad specificity towards reducing substrates, including purines, numerous aromatic heterocycles and aldehydes. The role of XOR in pteridine metabolism is depicted in Figure 5, including the reaction which has often been used to determine XOR activity.

In addition to its most important endogenous substrates hypoxanthine and xanthine, XOR is involved in the metabolism of ethanol and a variety of xenobiotic compounds. Thiopurines are also good substrates for XOR. Azathioprine is an immunosuppressive prodrug, which is first converted to 6-mercaptopurine in the liver and subsequently transformed to a pharmacologically active purine species. XOR is able to metabolize 6-mercaptopurine to 6-thiouric acid and inactivate the pharmacological effects of azathioprine. The co-administration of allopurinol has allowed lower azathioprine doses with less hepatotoxicity (Ansari *et al.*, 2008).

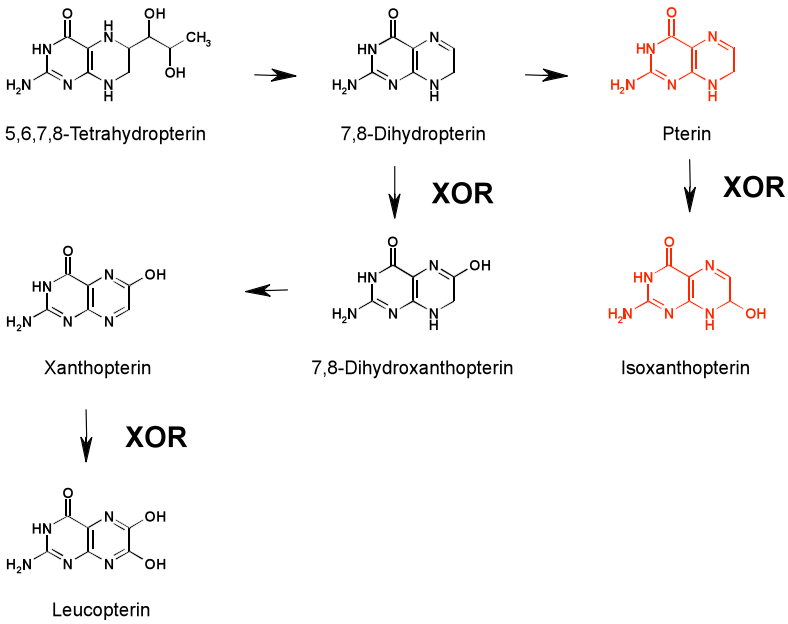

Figure 5. Xanthine oxidoreductase catalyzed reactions in pterin metabolism, which can be inhibited by allopurinol. The conversions not labelled with XOR are nonenzymatic. The reaction which has been used in determination of XDH and XO is marked in red (Beckman *et al.*, 1989). BH_4, i.e. 5,6,7,8-tetrahydropterin is a cofactor of NOS (Hurshman and Marletta 2002).

2.8.8 *Human xanthine oxidoreductase*

Table 2. Established and emerging physiological roles of XOR.

Physiological function	Features	Reference
Purine metabolism	Production of uric acid. Inhibited by AP or oxopurinol, febuxostat and high WO^{4-}/low MoO^{4-} diet.	Morgan *et al.*, 1922
Metabolism of (BH_4)	Degradation of 5,6,7,8 tetrahydro-biopterin (BH_4) to pterin to isoxanthopterin. BH_4 is NOS cofactor.	Blair, 1957; Beckman *et al.*, 1989; Blau *et al.*, 1996
Xenobiotic metabolism	Aldehydes, several drugs, nitrofuran derivatives, heterocycles, thiopurines.	Kitamura *et al.*, 2006; Ansari *et al.*, 2008
Ferreoxidase,	Incorporation of Fe into transferring 1000-fold more active than caeruloplasmin. High WO^{4-}/low MoO^{4-}, HCN and MeOH inhibits. XO has higher activity than XDH. pH optimum corresponds to intestinal mucosa.	Topham *et al.*, 1980; Hall-Sizemore *et al.*, 1994
Nitric reductase	NO_3^- to NO_2^- to NO, nitrosothiols to NO. Inhibition by high O_2 levels, allopurinol, oxypurinol. Organic nitrates to NO. Inhibition by DPI, but not by AP.	Li *et al.*, 2005; Jansson *et al.*, 2008; Webb *et al.*, 2008a
Retinol metabolism	all-*trans*-retinol to all-*trans*-retinaldehyde in human mammary epithelial cell line, inhibits viral replication	Taibi and Nicotra, 2007
NADH oxidase	DPI inhibits, AP does not, indicating that Moco is not involved. XDH has higher activity than XO.	Sanders *et al.*, 1997; Zhang *et al.*, 1998a; Maia *et al.*, 2007
Interaction with nNOS	nNOS -/- mice have upregulation of XOR in cardiac tissue. XOR and nNOS have the same localization in cardiac myocytes.	Khan *et al.*, 2004
Regulation of renal COX-2	30-fold upregulation of XOR during renal development concomitantly with COX-2. XOR -/- mice have strikingly similar renal phenotype as COX-2 knockout mice.	Ohtsubo *et al.*, 2004
Adipogenesis	Regulation of PPARγ by XOR	Cheung *et al.*, 2007
Structural element in milk	XDH and butyrophilin are indispensable for milk fat excretion. Fat excretion impaired in XOR-/+ mice (XOR-/- mice died). XOR colocalizes with adipophilin.	McManaman *et al.*, 2002
Innate immune function	XOR is expressed in epithelial and endothelia surfaces and closely associated with TLR-NF-kappa-B pathways and directly activated by NF-κ-B and AP-1.	Vorbach *et al.*, 2003
Immune function	Production of bactericidial ROS and adjuvant urate in epithelium. Virus-induced protelolysis of XDH to XO.	Vorbach *et al.*, 2003; Papi *et al.*, 2008

In spite of high circulating urate levels, human XOR activities are approximately one hundredfold lower than in rodents. Post mortem studies have indicated that XOR is occasionally found in the kidney and the lung, whereas in the human plasma, brain and heart XOR is undetectable (Sarnesto *et al.*, 1996; Kinnula *et al.*, 1997; Saksela *et al.*, 1998). These and similar studies led to a decline of interest in clinical studies, which had concentrated on XOR as a potential drug target in myocardial ischemia (Brown *et al.*, 1988).

Analyses have been carried out in whole tissues, leading to dilution of the enzyme, if most activity is found only on the endothelial wall of blood vessels. XOR binds to endothelium via glycosaminoglycans. Endothelium bound XOR can be liberated to circulation by heparin bolus and can subsequently be determined from plasma by monitoring O2- generation with and without oxopurinol by using electron spin trapping techniques (Spiekermann *et al.*, 2003).

XOR activity in human milk peaks during first weeks post partum and declines thereafter. At this later phase it has been shown to have equivalent NADH oxidase activity as bovine enzyme, indicating that the FAD site of the enzyme is comparable. Mo content of human XOR is less than 10% in comparison with bovine enzyme and it contains only 30% of the Fe-S clusters found in the bovine enzyme. Also xanthine molecular activity by human enzyme was found to be less than 10% that of bovine enzyme (Godber *et al.*, 2005).

Human XOR deficiency (MIM 278300) is not fatal. Literature addressing other characteristics other than xanthinuria, for example immune function in XOR deficient humans, is scarce.

2.8.9 *Cardiovascular studies on XOR inhibition*

Clinical and experimental studies on the effects of XOR inhibition published before 2006 have been recently reviewed (Pacher *et al.*, 2006). Most experimental studies have modelled myocardial infarction by using an I/R setting. Of these studies 19 reported positive effects when infarct size was chosen as an endpoint, whereas 7 studies reported failures of reduction in infarct size. Clinical studies reviewed in the same article have more disconcordant results, partly because of different clinical settings and surrogate end-points. In six studies out of eight, some level of cardioprotection was reported.

Although XOR activity in human tissues is low, recent studies with electron spin resonance techniques indicate that XOR activity is inducible also in human tissues. Spiekerman *et al.* (2003) reported a marked (>200%) increase in endothelial XOR activity in patients with angiographically documented coronary artery disease (CAD), in comparison with age-matched controls. Furthermore, the authors demonstrated XOR and NAD(P)H oxidase induced production of ROS.

In line with studies reviewed by Pacher *et al.* (2006), recent experimental studies

with rodent models point towards that XOR inhibition may exert beneficial effects on cardiac function, see Table 3. There are ongoing clinical studies in which potential use of allopurinol in cardiovascular disease will be investigated. While studies on rodent models have given a positive overall picture, the outcome in reported clinical studies have been variable, although some of them show positive effects. Generally, the studies so far have been relatively limited, except the OPT-CHF study in which 405 patients with cardiac failure of NYHA class II-IV were studied. The outcomes of most recent studies are summarized in Table 4.

Relative high allopurinol or oxopurinol doses have been used in experimental and clinical studies on cardiovascular effects of XOR inhibition. Dose levels have been more than would be needed for XOR inhibition. Consequently the effects of these drugs on other metabolic pathways may become important. Studies employing other XOR inhibitors (except allopurinol or oxopurinol) are scarce. Recently Sánches-Lozada *et al.* (2008) have reported SBP lowering effects of Febuxostat, a potent non-purine XOR inhibitor, in an experimental hyperuricemic rodent model of hypertension.

Microparticles are circulating lipid vesicles, generated by blebbing of plasma membrane from apoptotic cells and also from non-apoptotic cells in response to various stimulae. The concentration of microparticles is increased in various pathophysiological states including myocardial infarction and end-stage renal disease. Microparticles derived from human lymphoid cell culture induced endothelial dysfunction with increases in ROS production and decreases in NO availability. These effects are inhibited by XOR inhibitors and also by inhibitors of phosphoinositol-3-kinase (Mostefai *et al.*, 2008).

To summarize Section 2.8: XDH is a protein derived from translation and is subject to several post-translational modifications. The interconversion of XDH and XO isoforms has been suggested to have a biological role, because the conversion is a complex phenomenon and appears to involve thermal equilibrium. Several factors, including Ang II, cytokines, hypoxia, intracellular iron and lactogenic hormones, regulate XDH expression. In addition to its important house-keeping role in purine and pterin metabolism, XOR has several other functions, including detoxification of exogenous compounds. XOR derived ROS can also scavenge NO and may participate in the activation of organic nitrates. Human XOR has lower Moco content than that found in rodents. XOR protein levels and human XOR activities are also generally much lower than those found in rodents. Evaluation of the role of XOR in cardiovascular phenomena is complex due to multiple roles of the enzyme. XOR can produce NO and ROS, which together generate reactive nitrogen species, important for innate immunity. Recent experimental studies have indicated positive cardiovascular effects of XOR inhibition, including reduction of infarct size and alleviation of hypertension induced left ventricular hypertrophy. However, results from clinical studies have been more variable.

Table 3. Experimental studies on XOR inhibition since 2006. Earlier studies have been reviewed by Pacher *et al.* (2006).

Experimental studies	Effects	Reference
Pressure overload-induced (transverse aortic constriction) LVH and dysfunction in C57/BL6 mice Febuxostat 5 mg/kg/d 8d.	Attenuation of TAC-induced changes in LV ejection fraction, LV end-diastolic posterior wall thickness, LV diameter at end systole. Plasma uric acid levels decreased from approx 0.75 mg/L to approx. 0.1 mg/L.	Xu *et al.*, 2008
TO-2 hamster (deficient in δ-sarcoglycan). Model of dilated cardiomyopathy. Allopurinol (30 mg/kg/d, 6 weeks).	Attenuation of LV dysfunction and dilation, myocardial fibrosis and the upregulation of a fetal-type cardiac gene. Inhibition of decrease in GSH/GSSG ratio and the increase in malondialdehyde levels in the heart.	Hayashi *et al.*, 2008
L-NAME induced hypertension and LVH in Wistar rats. Allopurinol 40 mg/kg/d 5 weeks.	Allopurinol alleviated left ventricular hypertrophy without interfering with L-NAME induced hypertension	Kasal *et al.*, 2008
Cocaine-induced cardiac dysfunction in Wistar rats. Allopurinol (50 mg/kg, 7 days) resulted in 60% decrease in XOR activity.	Inhibition restored fractional shortening, cardiac output and stroke volume. Apocynin (50 mg/kg) also tested with positive results. Apocynin downregulated XOR, but did not influence NAD(P)H.	Isabelle *et al.*, 2007
Nitrite induced cardioprotection of in vivo and ex vivo rat (SD) models of myocardial ischemia. Oxypurinol 10 µmol/L or DPI 2 µmol/L in perfusate.	Recovery of left ventricular developed pressure and reduction of infarct size. XOR inhibitors abolished nitrite induced cardioprotection, while NOS inhibitor did not. Putative NAD(P)H oxidase inhibitor, apocynin, had the same effect as allopurinol. NAD(P)H oxidase not measured.	Baker *et al.*, 2007
Pigs fed with high-cholesterol diet for 12 weeks. Intrarenal oxypurinol infusion at 300 mg/min/kg for 15 min.	Transient intrarenal infusion of oxypurinol resulted in significantly improved renal perfusion response to an endothelium-dependent challenge as compared with controls.	Daghini *et al.*, 2006
Transgenic TnI-(1–193), which display myocardial stunning, a model of progressive cardiomyopathy. Allopurinol in drinking water 260 mg/L, 1-2 months	Abolishment of relative left ventricular weight increase. Cardiac function determined by electrocardiography, marked improvement of Ca^{2-}-activated twitch tension. Nearly complete inhibition of XOR in cardiac tissue.	Duncan *et al.*, 2005
Reversal of CsA side effects by allopurinol	Alleviation of CsA-induced arterial blood pressure and renal blood flow in rats.	Kobelt *et al.*, 2002

Table 4. A few recent clinical studies involving XOR inhibitors. Earlier studies prior to 2006 have been reviewed by Pacher *et al.* (2006)

Clinical studies	Effects	Reference
Randomized, double-blind, placebo controlled study; patients recruited within 72 hrs of radiologically confirmed ischemic stroke. Two allopurinol dose regimen: 100 mg/d and 300 mg/d for 6 weeks.	Positive changes in surrogate markers with higher allopurinol dose: attenuation of ICAM-1 rise after stroke. 17 patients per group, mean age 70 yrs.	Muir *et al.*, 2008
405 patients with NYHA class III–IV heart failure and LVEF ≤40% were randomly assigned to control and treatment groups. Double-blind treatment with either oxypurinol (600 mg/day) or placebo, for 24 weeks.	No improvement in unselected patients in spite of decrease in plasma urate levels in a composite end-point. Post-hoc analyses suggested benefits in a patient subgroup with elevated SUA and correlated in this group with degree of serum urate decrease. Evaluation of patient status was based on composite clinical end-points describing whether status was improved, unchanged or worsened. Worsening of the condition was based on lethal event, hospitalization and need for emergency treatment. Patients in both groups received all appropriate medications, including ACE etc.	Hare *et al.*, 2008
Patients with systolic, symptomatic heart failure. Oxypurinol	No clinical improvement in a clinical composite score of unselected patients with moderate to severe heart failure. Benefits occurred in patients with high urate levels as revealed by post-hoc analyses.	Chaves *et al.*, 2007
60 patients (30 per group) of NYHA class II-III CHF. Double-blind 1 month placebo controlled treatment with oxypurinol (600 mg/day).	Left ventricular ejection fraction (LVEF) improved, but did not reach statistical significance ($p<0.08$). When patients with LVEF <40% were excluded from analyses LVEF was significantly ($p<0.02$) improved in oxypurinol group. Tendency of decreased left ventricular mass in oxypurinol group. No effects in exercise capacity (6-minute walking test).	Cingolani *et al.*, 2006

3 Aims of the Study

Experimental studies were designed to clarify *in vivo* the possible role of XOR in genetic, salt-induced hypertension and in metabolic syndrome.

Specifically the aims of the present studies were as follows:

1. To compare hypertension-prone, salt-sensitive rats and their genetically close control strains in terms of XDH and XO activities and expression particularly in the kidney.
2. To investigate whether renal XDH and XO activities are modified by dietary intake of NaCl.
3. To resolve whether NO regulates XOR in vivo.
4. To clarify whether XOR has any causative role in hypertension.
5. To find out whether chronic administration of immunosuppressive drug, CsA, at nephrotoxic dose levels, with or without increased NaCl intake, regulates renal XOR activity.
6. To investigate the role of XOR in metabolic syndrome using obesity-prone rat models.

4 Material and methods

4.1 ANIMALS AND TREATMENTS

All animal experiments were carried out according to study protocols approved by the Animal Experimentation Committee of the Institute of Biomedicine at University of Helsinki to meet the standards set by the American Physiological Society and by Helsinki Agreement.

In all experiments, the rats were obtained from (Harlan-Spraque-Dawley, Indianapolis, IN, USA) and kept four animals per cage in standardized conditions (illuminated from 6.30 a.m. until 6.30 p.m., room temperature 22 ± 1 °C).

Experimental and control groups were matched on basis of their blood pressure and body weight. In all experiments the rats had free access to tap water and chow. In the following studies a 'low NaCl' diet refers to mean standard laboratory rat diets which generally contained 0.2 – 0.7% NaCl. In addition a 'high' NaCl diet in this context refers to a standard diet, which has been supplemented with NaCl. The levels have been specified separately in each study.

Study I

Forty-five, three-week-old Dahl salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) male rats were assigned to four weight-matched subgroups of each strain to be kept for seven weeks on diets differing in sodium content. Diets containing 0.05%, 0.20%, 3.25% and 6.50% NaCl (w/w) (corresponding to 0.02, 0.08, 1.28, and 2.56% as Na^+), were prepared by mixing NaCl into a special low sodium laboratory chow (TD90228, Harlan Teklad division, Indianapolis, IN, USA).

Twelve male eight-week-old SHR Okamoto rats and 10 WKY male rats were assigned to two groups, respectively, to be kept for eight weeks on low (0.20% w/w NaCl, corresponding to 0.08% Na^+ w/w) or high (6.50% NaCl w/w, i.e. 2.56% as Na^+) NaCl diets.

In a separate, previously unpublished (Laakso, Pere, Lapatto) experiment fourteen SHRs were assigned into two groups and were kept six weeks on low (0.20% w/w NaCl) or high (6.2% w/w NaCl) NaCl diets. Kidney and left ventricular samples were frozen immediately after excision and stored at -70 °C prior to analysis.

Study II

Forty-seven, nine-week old male SHRs were randomly allocated to six subgroups. The groups were kept three weeks on three different diets containing 0.08, 0.4 or 2.4% of Na^+ (w/w of the diet, added as NaCl, JozoTM, Akzo Salt, Amersfoort, the Netherlands) with or without N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co, St Louis, USA) L-NAME was given at a concentration of 250 mg/kg (w/w) of the chow providing approximately 20 mg/kg bw/day.

Another forty male SHRs, nine weeks of age, were randomly allocated to six groups and kept for eight weeks on either 0.3%, or 1.4% Na^+ diet [0.76% and 3.56% NaCl (w/w) in the diet], and respective groups kept on the same diets with 100 mg/kg of chow isosorbide-5-mononitrate (IS-5-MN), providing approximately 65 mg/kg body weight/day.

Study III

Fifty Wistar–Kyoto (WKY) rats and 49 Okamoto-Aoki spontaneously hypertensive rats, five weeks of age, were acclimatized for one week and fed with standard rat chow containing 0.3% NaCl (w/w). At the onset of the experiment, six rats of each strain were killed and the remaining animals were divided into groups of low and high NaCl diets with or without allopurinol. Animals were killed three, five and eight weeks after the onset of the study. Immediately after death, the liver and the kidneys were removed.

The low NaCl diet (Finnewos Aqua, Helsinki, Finland) contained 0.3% NaCl (w/w). The high NaCl diet was prepared by adding NaCl to a final concentration of 6% (w/w). Respective diets containing 62.5 mg of allopurinol / kg of chow (E. Merck, Darmstadt, Germany) were prepared. As calculated from the chow consumption, the diets containing allopurinol provided approximately 21 mg / kg body weight at the onset and 10 mg / kg of allopurinol at the end of the experiment.

Study IV

Thirty-three female obesity-prone Zucker rats (Harlan, Oxon, UK) were used. Rats were matched for blood pressure and serum cholesterol, and were divided into three groups to receive different diets. Eight lean Zucker rats served as control group and consumed basic dry rat food (Altromin, Lage, Germany). The same control diet was given to the obese Zucker controls. The second obese group received valsartan, which was added into the diet at a level of 12 mg/kg of chow. The atherogenic diet group consumed the same food into which 18% butter was incorporated (Valio, Helsinki, Finland), 1% cholesterol (Sigma, St. Louis, MO, USA), and 6% NaCl (w/w) (University Pharmacy, Helsinki, Finland). Valsartan was a generous gift from Novartis Ltd., Basel, Switzerland.

Cyclosporine experiment

Fifty SHRs, eight-week of age, were divided into seven blood pressure and body weight matched groups of 6–8 animals to be kept on different diets for six weeks with or without s.c. administration of CsA (Sandimmun). The dose level was 5 mg/kg or in case of controls, an equivalent amount of diluted Intralipid solution used as the vehicle. Three dietary protocols were used. A low NaCl diet comprising of standard laboratory chow (R36, Finnewos Aqua, Helsinki, Finland) containing 0.76% NaCl (w/w), a high NaCl diet containing 6.5% NaCl (w/w) and a Mg-supplemented diet containing the same amount of NaCl, 6.5%, as the high NaCl diet, but had three times higher Mg^{2+} level,

0.6% (w/w), than the standard diet. $MgCl_2$ was obtained from E. Merck, Darmstadt, Germany.

Functional parameters, such as blood pressure, renal function data and tissue electrolyte levels have been published earlier in detail (Mervaala *et al.*, 1997).

Tissue samples

At the end of the experiments, the rats were anesthetized with a mixture of CO_2 (70%) and O_2 (30%), (Aga Ltd, Riihimäki, Finland) and decapitated. Blood samples were obtained following decapitation and blood plasma with EDTA as anticoagulant was separated by centrifugation at room temperature. The kidneys and the heart were removed and washed with ice-cold saline solution, frozen immediately in liquid nitrogen, then lyophilized and kept at –20 °C in vacuo (Study I and CsA study) or kept at –80 °C (Studies II, III and IV).

4.2 BLOOD PRESSURE MEASUREMENT

The systolic blood pressures of unanesthetized rats were measured with a tail-cuff pressure analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179–2AB, IITC Life Science). The analog signals obtained were converted to digital values by an on-line microprocessor. Before collecting the recordings, the rats were warmed for 10 to 15 minutes at 28 °C to make the pulsations of the tail artery detectable. Values for systolic blood pressure and heart rate were obtained by averaging results from 3 to 5 measurements. To minimize stress-induced fluctuations in blood pressure, all measurements were randomly taken by the same person at the same time of day (9 to 12).

4.3 BIOCHEMICAL DETERMINATIONS

4.3.1 *Assays of enzyme activities of XO and XDH*

Lyophilized (Study I and CsA study) or frozen (Study II, III and IV) kidneys and other tissues were homogenized in phosphate buffer, pH 7.4. Buffer compositions can be found in the Material and Method Sections of Studies I–IV. EDTA, DTT, and proteinase inhibitors were used to protect proteins from oxidation and free metal ions. Homogenates were ultracentrifuged (100 000g, one hour), and supernatants were passed through a gel filtration column (Biogel P10, Bio-Rad, CA, USA) to remove small interfering molecules.

XO and XDH were measured fluorometrically (Beckman *et al.*, 1989) in Studies I, II III and in the CsA study. The determination was based on the xanthine oxidase-catalyzed conversion of pterin (2-amino-4-hydroxypteridine) to isoxanthopterin.

Radio-HPLC assay was employed in Study IV. XOR activity was measured by using [^{14}C]xanthine as the substrate. After separation of the produced uric acid by HPLC (Shimazu Co, Kyoto, Japan), its radioactivity was recorded with a Radiomatic Flow

Scintillation Analyzer (Packard Instrument, CT, USA) as described elsewhere (Saksela *et al.*, 1998). For total XOR activity (XDH + XO), NAD^+ (400 µM) was present, whereas for XO assay, NAD^+ was omitted. In all studies, total protein was determined with the biuret protein assay (Bio-Rad, CA, USA).

Intra-assay imprecision of the total activity was not more than 5%. One unit of XOR, XDH or XO activity is defined as 1 µmol isoxanthopterin formed per minute and 1 µmol uric acid formed in the case of radio-HPLC method.

4.3.2 *RNA analyses by Northern blot*

Total liver and kidney RNA was extracted from randomly selected specimens of 10-week-old rats by the guanidinium thiocyanate–caesium chloride method (Chirgwin *et al.*, 1979). Subsequently, mRNA was extracted using the Oligotex mRNA kit (Qiagen, Hilden, Germany). To quantify XOR mRNA, Northern blot analysis was performed as described elsewhere (Martelin *et al.*, 2002). Membranes were hybridized using standard procedures (Sambrook *et al.*, 1989) with ^{32}P-labelled complementary RNA probe, including two fragments corresponding to nucleotides 118–2985 of the rat XOR cDNA (kindly provided by Professor T. Nishino, Nippon Medical School, Tokyo, Japan) (Amaya *et al.*, 1990). The same membranes were re-probed with ^{32}P -labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe transcribed from the pTRI-GAPDH plasmid (Ambion, Austin, TX, USA) to control the RNA loading, except for Study IV, where S18 ribosomal RNA was used instead. The X-ray films were scanned with a digital scanner and analysed with Scion Image beta 4.0.2 analysis software (Scion, Frederick, MD, USA).

4.3.3. *Plasma arginine*

Plasma arginine was determined in Study II as described in detail previously (Päivä *et al.*, 2002). Briefly, arginine was determined fluorometrically as a o-pthalaldehyde derivative after reversed phase HPLC with homoarginine as an internal standard. Arginine was isolated with Bond-Elut silica solid phase extraction columns prior to derivatization.

4.3.4. *Other biochemical assays*

Several $NAD(P)^+$ dependent enzymes were also determined spectrophotometrically from the kidney and liver samples as described in detail in Study I and II. These enzymes included G6PDH, LDH and ALDH. ALDH has several isoenzymes, as described at www.aldh.org . As far as photometric activity determination is concerned, there are two ALDH isoforms, cytosolic ALDH1 and mitochondrial ALDH2. The level of substrate was chosen according to the enzyme with the highest Km, to determine total activity of both isoforms from tissue homogenates. The Km for ALDH1 is 500–600 times higher than that of ALDH2.

In Study IV serum lipids and glucose from the samples taken at the end of the experiments were analyzed by an accredited laboratory, United Laboratories Ltd., Helsinki,

Finland (Hitachi 912 Automatic Analyzer, Hitachi Ltd. Tokyo, Japan, all reagent kits were obtained from Boehringer Mannheim). Serum total cholesterol was determined with an enzymatic method (CHOD-PAP -method), serum HDL cholesterol with an enzymatic direct method (HDL-Chol Plus), LDL cholesterol with an enzymatic direct method (LDL-Chol Plus), and triglycerides with an enzymatic method (CHOD-PAP -method). All reagents wre obtained from Boehringer Manheim, Darmstadt, Germany.

4.4 STATISTICAL ANALYSES

The results are given as the mean ± SEM. ANOVA was applied to test the statistical significance for strain and diet effects followed by Duncan's multiple comparison test for comparison of pairs of groups. Linear regression analysis was used to calculate correlation coefficients. SPSS/PC statistical software (SPSS version 11.0, SPSS Inc, SPSS Inc Chicago, IL, USA) was used for statistical computations.

5. Results

5.1 NaCl, systemic blood pressure and organ weights (studies I and III)

Keeping Dahl SS/Jr rats on both the moderately high and high NaCl diets for seven weeks markedly elevated systolic blood pressure (Figure 6). Body weights were decreased by 25% in Dahl SS/Jr but not in Dahl SR/Jr rats kept on highest NaCl intake level. NaCl restriction to 0.05%, w/w (Na^+ content of 0.02%) resulted in markedly decreased growth rate in both in Dahl SS (112 g) and SR rats (155 g) compared with the groups on the low (0.20%, w/w) NaCl diet (0.08% w/w of Na^+ in the diet) and having body weights from 272 g to 309 g.

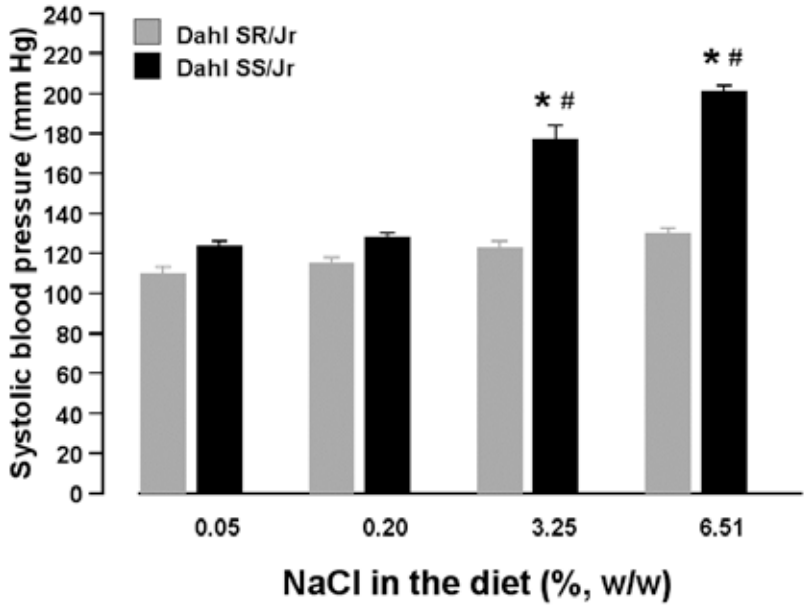

Figure 6. Dose response of NaCl on SBP of salt-sensitive Dahl SS/Jr and salt-resistant Dahl SR/Jr rats. Statistical significance has been denoted as follows: *, $p<0.05$ compared with same strain kept on 0.20 NaCl diet; #, $p<0.05$ as compared to rats of Dahl SR/Jr strain kept on 0.20% NaCl diet (Study I).

The Dahl SS/Jr rats kept on low NaCl diet (i.e. on standard diet) had approximately 20 to 30 grams higher body weights, while absolute and relative kidney weights were not different from Dahl SR/Jr rats on the same standard diet (Study I).

The SHR on the low NaCl diet had markedly higher SBP compared with the WKY rats (Study I). The absolute and relative kidney weights were similar in SHRs and WKY rats kept on a low NaCl diet. SHRs kept on high NaCl diets had increased relative renal and cardiac weights compared with WKY, whereas body weights did not differ between these strains (Study III).

5.2 Renal XOR in hypertension-prone salt-sensitive rats (studies I and III)

Dahl rats: Dahl SS/Jr and SR/Jr rats kept for seven weeks on low NaCl diet (0.08% Na^+ w/w in the diet, i.e. 0.2% NaCl w/w) showed no difference either in blood pressure or in renal activity of XDH, XO or XOR (Figure 7). Increased NaCl intake (3.25 and 6.50% w/w NaCl) resulted in increased renal XDH and XO activity in Dahl SS/Jr rats in a dose-dependent manner ($p<0.001$, ANOVA). In contrast, increased NaCl intake had no effect on renal XDH, XO activity in the Dahl SR rats (Figure 7). NaCl restriction to deficient levels, i.e. 0.05% (w/w NaCl in the diet) resulted in markedly decreased renal XO activity both in Dahl SS/Jr and SR/Jr rats compared with the groups kept on a low (0.20%, w/w) NaCl diet (Study I).

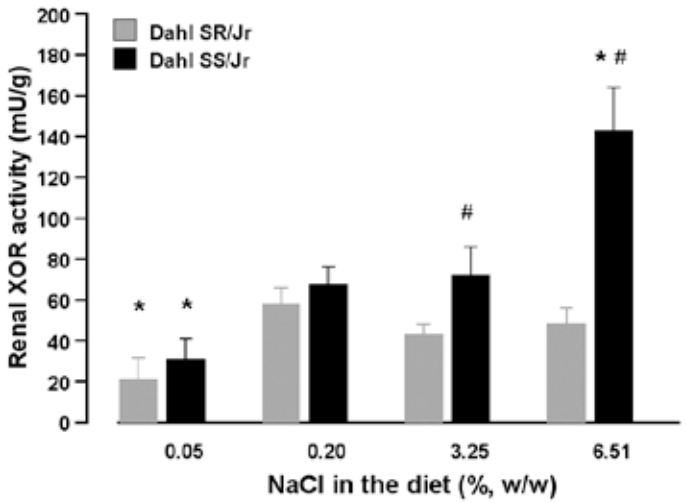

Figure 7. Effect of different levels of dietary NaCl on kidney XOR in (Dahl SS/Jr) salt-sensitive and salt-resistant (Dahl SR,/Jr) rat strains. *, $p<0.05$ as compared to rats of same strain at 0.2% NaCl (i.e. 0.08% Na level in the diet); #, $p<0.05$ compared with control strain kept on the same diet (Study I).

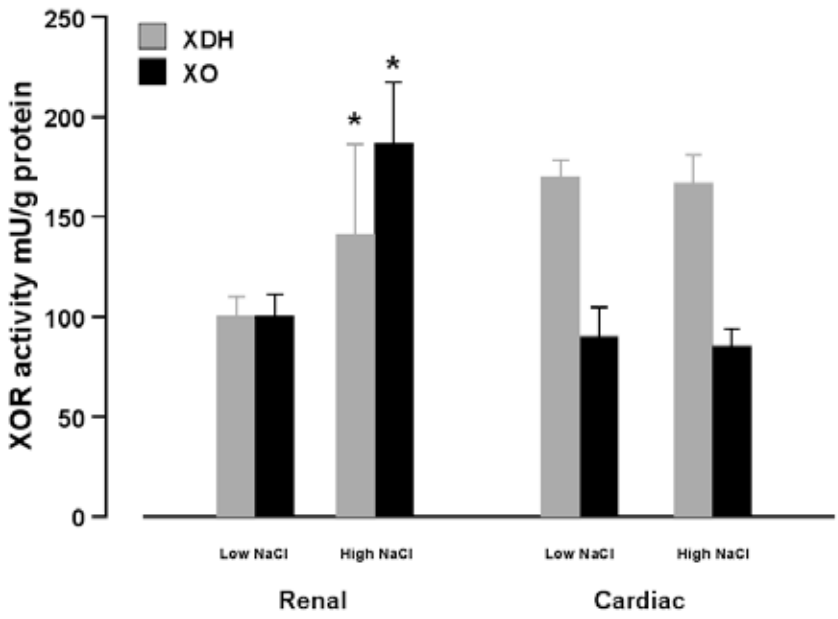

Figure 8. Renal and cardiac XDH and XO activities of SHRs kept on low (0.2% NaCl w/w) and high (6.2% NaCl w/dry w) NaCl containing diets for six weeks. *, $p<0.05$ compared with low NaCl group. N=7 in both groups (Laakso, Pere, Lapatto, previously unpublished data.)

Spontaneously hypertensive rats (SHRs): SHRs kept for eight weeks on a low NaCl diet (0.20% NaCl w/dry w in the diet) had markedly higher kidney XOR activities than WKY rats (Table 1). Increased NaCl intake (6.5% NaCl w/w) produced increased kidney XDH and XOR in SHRs. Increasing dietary NaCl intake from 0.20% to 6.50% elevated systolic blood pressure and relative kidney weight in SHRs but not in WKY rats (Study III).

In a separate unpublished (Laakso, Pere, Lapatto) experiment SHRs were kept 6 weeks on low (0.20% w/w NaCl) and high (6.2% w/w NaCl) NaCl diets. The tissue samples were stored only a few weeks prior to analysis. This additional study was carried out to investigate whether cardiac XOR activity is influenced by dietary salt and resulting hypertension. While renal XOR activities were increased with higher NaCl intake, cardiac XOR did not show any response (Figure 8).

In addition to XOR another hypoxia-regulated enzyme G6PDH was determined for comparison. Renal G6PDH activities were increased in the salt-sensitive Dahl SS/Jr rats kept on highest NaCl intake level (6.50% w/w NaCl), whereas in salt-resistant Dahl SR/Jr rats any significant effect after variation of NaCl intake levels was not found (Table 5).

Table 5. Renal activities of selected enzymes of salt-sensitive (Dahl SS/Jr) and salt-resistant (Dahl SR/Jr) rats (Study I) kept on different NaCl intake levels (Panel 6B) NS, not significant; * p< 0.05. (unpublished data relating to Study I).

NaCl% (w/w diet)	**N**	**G6PDH** (mU/g)	**LDH** (mU/g)	**ALDH** (mU/g)
Dahl SR/Jr				
0.20	4	8.47 ± 0.56	0.47 ± 0.05	2.74 ± 0.80
6.50	4	9.11 ± 0.15	0.43 ± 0.03	3.45 ± 1.08
Dahl SS/Jr				
0.05	3	9.34 ± 0.38	0.45 ± 0.02	3.32 ± 0.96
3.25	5	9.25 ± 0.25	0.44 ± 0.02	2.50 ± 0.09
6.50	6	15.50 ± 1.71*	0.55 ± 0.03	4.20 ± 0.75
ANOVA				
Dietary NaCl		p<0.001	NS	NS
Strain		p<0.01	NS	NS

5.3 INHIBITION OF NITRIC OXIDE SYNTHASE (STUDY II)

NOS inhibition by L-NAME upregulated the renal XO, XDH and XOR activities from 10% up to 36% depending on the amount of NaCl in the diet (Table 6). Increases in renal XDH following different levels of NaCl were dose-dependent in SHRs both in the presence and in the absence of the L-NAME treatment. No dose-related effects were found between the level of NaCl intake and renal XO activity (Study II).

Long-term L-NAME treatment increased SBP markedly, approximately by 50 mm Hg. Dietary salt did not produce any significant or dose-response effect on blood pressure on groups treated with or without L-NAME (Study II).

L-NAME slightly increased renal G6PDH activities in groups kept on high NaCl diet (Table 6), whereas ALDH and LDH activities remained the same, or tended to be lower in L-NAME treated groups (previously unpublished results).

NOS inhibition increased circulating arginine levels at low NaCl intake levels (Figure 10). At 1.1% of NaCl in the diet NOS inhibition did not have any effect. However, at the high level (6.2%), NOS inhibition decreased circulating arginine levels (previously unpublished results).

Table 6. Renal activity of XO, XDH and G6PDH in SHRs kept on different NaCl diet with or without NOS inhibitor L-NAME (Study II). *, p<0.05.(Study II)

NaCl% (w/w diet)	**N**	**XO** (mU/g)	**XDH** (mU/g)	**G6PDH** (mU/g)
Controls				
0.2	8	23 ± 1	49± 3	15.8 ± 0.3
1.1	8	24 ± 1	52 ± 4	15.3 ± 0.4
6.2	8	24 ± 1	55 ± 3	15.3 ± 0.4
L-NAME				
0.2	7	27 ± 2	56 ± 3	16.9 ± 0.6
1.1	7	28 ± 2	61 ± 6	17.5 ± 0.5 *
6.2	9	32 ± 2*	75 ±5 *	17.8 ± 0.6 *
ANOVA				
Dietary NaCl		NS	p<0.05	NS
L-NAME		p<0.0001	p<0.0001	p<0.001

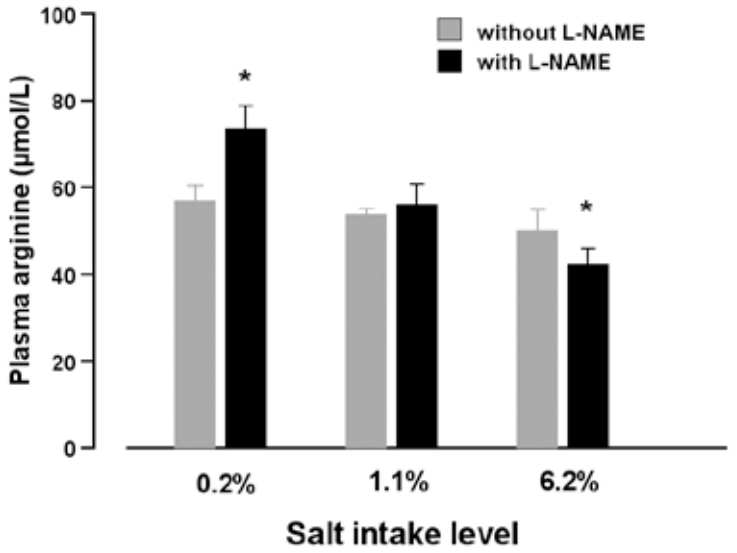

Figure 10. Effect of unspecific NOS inhibition with L-NAME on circulating arginine levels in SHRs, kept three weeks on different salt intake levels (N=7–9 per group). The percentages refer to percents of NaCl in the diet (w/w) *, p<0.05 vs control group receiving the same amount of NaCl (Laakso, Vaskonen, unpublished data).

5.4 Increased nitric oxide availability (Study II)

The NaCl induced SBP was attenuated by IS-5-MN administration (Study II). In spite of a decrease in blood pressure, and the relatively long duration of the experiment (eight weeks), renal XO, XDH or XOR activities did not markedly change in the groups treated with IS-5-MN. Different NaCl intakes did not affect renal XOR activity. IS-5-MN treatment was found to alleviate NaCl-induced increase in SBP, but it did not produce any further decrease in SBP in the animals kept on the low NaCl diet. Renal G6PDH activities ranged from 16.3 to 19.3 mU/g, with no significant differences or tendencies. Renal ALDH ranged from 4.6 to 5.3 mU/g in low and high NaCl groups without IS-5-MN, respectively. The low NaCl group with IS-5-MN treatment had mean renal ALDH activity 6.0 ± 1.5 mU/g, whereas the group receiving IS-5-MN treatment with high NaCl diet had renal activity 4.3 ± 0.5 mU/g. None of the differences in these enzyme activities including LDH were statistically significant.

5.5 XOR inhibition during development of hypertension (Study III)

Allopurinol (eight weeks, 10–21 mg/kg bw per day) did not alleviate hypertension. SHR groups with allopurinol tended to have lower blood pressure than groups without allopurinol ($p<0.05$ in ANOVA). WKY rats kept on the high NaCl diet with allopurinol had approximately 10 mm Hg lower SBP levels compared with WKY rats on the same diet, but without allopurinol ($p<0.05$).

The weight gain of SHRs kept on high NaCl was lower than SHRs on low NaCl diet despite of the similar food intake rate. In order to obtain unbiased relative renal and cardiac weight data, regression equations beween body weight and cardiac weight and between body weight and renal weight, were first established using all age groups of SHRs kept on low NaCl diet. Renal and cardiac weights were then calculated for all SHRs as residuals with respect to the corresponding regression line. Residuals are depicted in Figure 9 and indicate that the allopurinol treatment prevented NaCl-induced renal and cardiac hypertrophy ($p<0.05$).

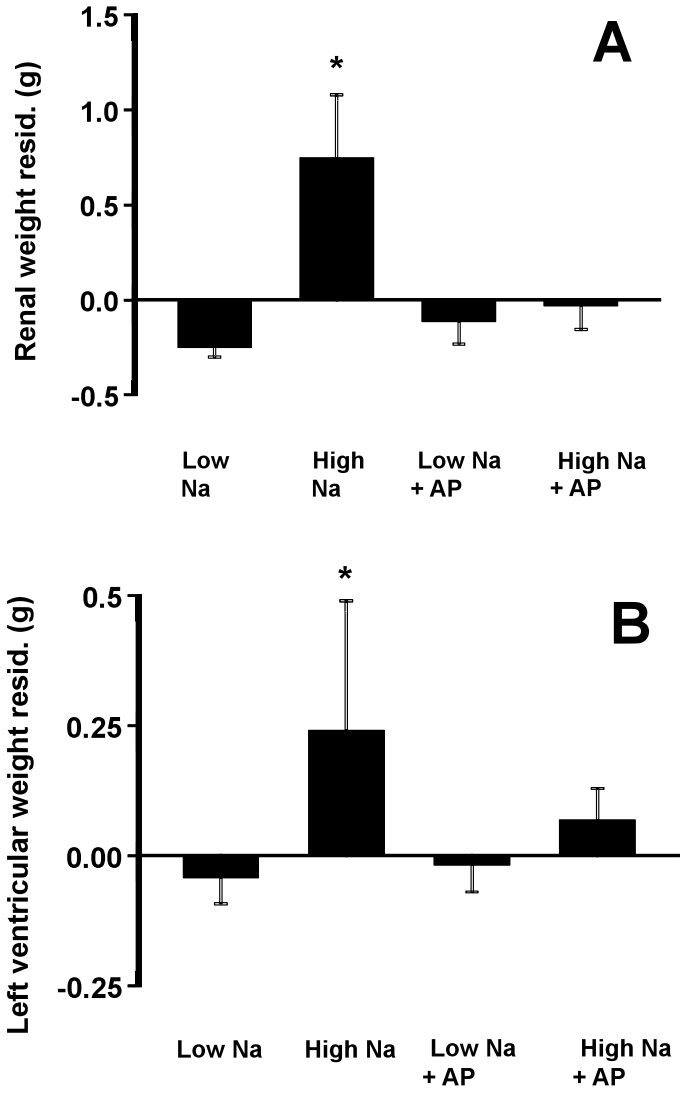

Figure 9. Differences of organ weights of 13-week old SHRs kept on low (0.3% w/w) or high (6% w/w) NaCl diet with or without allopurinol (AP) in comparison with organ weight development of SHRs kept on the low NaCl diet. A highly significant linear correlation between body weight and renal ($r=0.85$, $p<0.001$) and left-ventricular weight ($r=0.87$, $p<0.001$) were obtained by regression analysis using SHR data from all age groups kept on low NaCl diet. The difference in organ weight residuals between the observed value of renal weight or left ventricular weight, and the corresponding expected values obtained from the regression equations were then used to produce the graph. *, $p<0.05$ (Study III). Reproduced with permission.

5.6 ZUCKER FA/FA MODEL (STUDY IV)

Initially, Zucker fa/fa rats had significantly higher body weight (mean 547 g) than the lean Zucker rats (mean 390 g). During the 10 month experiment Zucker fa/fa gained substantially more weight than the lean control rats (mean value of 770–814 g for obese Zucker fa/fa groups compared with mean of 550 g in the lean controls). Weight gain in Zucker fa/fa was similar regardless of diet.

Obese Zucker rats had nearly twice as high renal XDH and XO activities than lean Zucker rats (Figure 11). In spite of this, renal XDH mRNA levels did not show strain-related differences. In the liver the differences in XDH and XO activities were much lower. Also hepatic mRNA levels did not differ between lean and obese rats on the control diet.

Ang II receptor blockage by valsartan was not found to have any significant effects on weight gain, plasma glucose or cholesterol levels, whereas plasma triglyceride levels were significantly decreased. SBP was increased in obese Zucker fa/fa rats during the experiment reaching 149.1 ± 5.0 mm Hg in the controls. In the valsartan treated group SBP was slowly changed throughout the course of the experiment and was only towards the end significantly lower than in the obese control group. Liver weight (normalised with the length of tibia) was higher in obese animals than in lean animals. Surprisingly, the valsartan group had the largest livers.

Valsartan decreased SBP by approximately by 20 mm Hg. Hepatic XOR was markedly decreased by chronic valsartan administration from 144 mU/g to 38 mU/g. The mRNA levels remained unchanged in the liver and in the kidney. The effect of valsartan on renal enzyme activities was less pronounced, approximately 15%, but the difference was marked between the lean and obese groups (Figure 11). No change was observed in the XO/XDH ratio.

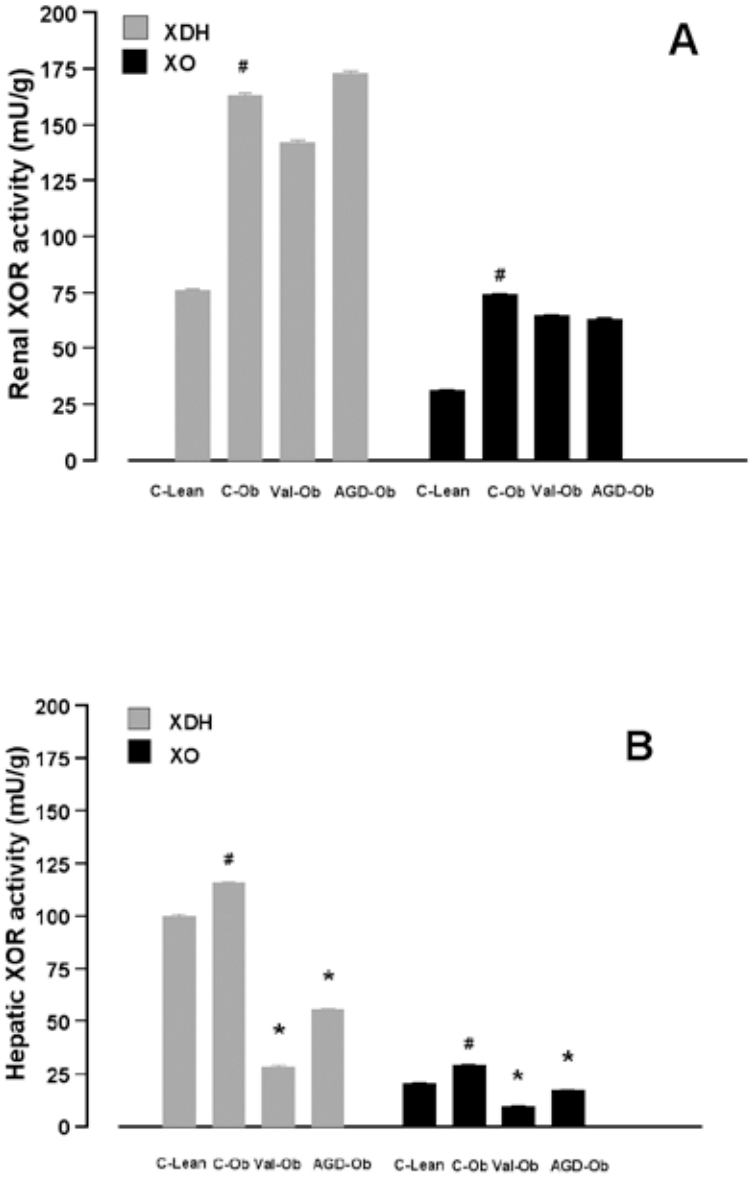

Figure 11. Renal (panel A) and hepatic (panel B) XDH, XO and XOR activities of lean and obese Zucker rats kept 10 months on standard diet with or without valsartan and on the atherogenic diet containing 6% w/w NaCl and butter. #, $p<0.05$ between lean and obese substrains; *, $p<0.05$ in comparison with obese controls. #, $p<0.05$ strain-related difference in rats kept on control diet. C-Lean, lean Zucker rats on control diet; C-Ob, obese Zucker fa/fa group on control diet; Val-Ob, obese Zucker fa/fa on control diet with valsartan and ADG-Ob, obese Zucker fa/fa on atherogenic diet.

5.7 EFFECTS OF CYCLOSPORINE

Effects of CsA on renal XDH and XO are presented. As depicted in Figure 12, CsA substantially increased renal XO activity, whereas XDH activity remained unchanged. XOR activities were lower in CsA experiment in comparison with other SHR studies presented, mean value for total XOR activity in the control rats kept on low NaCl diet was 16.6 mU/g.

Results relating to the effects of CsA on renal function and blood pressure have been previously published separately (Mervaala *et al.*, 1997) and will be referred to only in the discussion part of the present study.

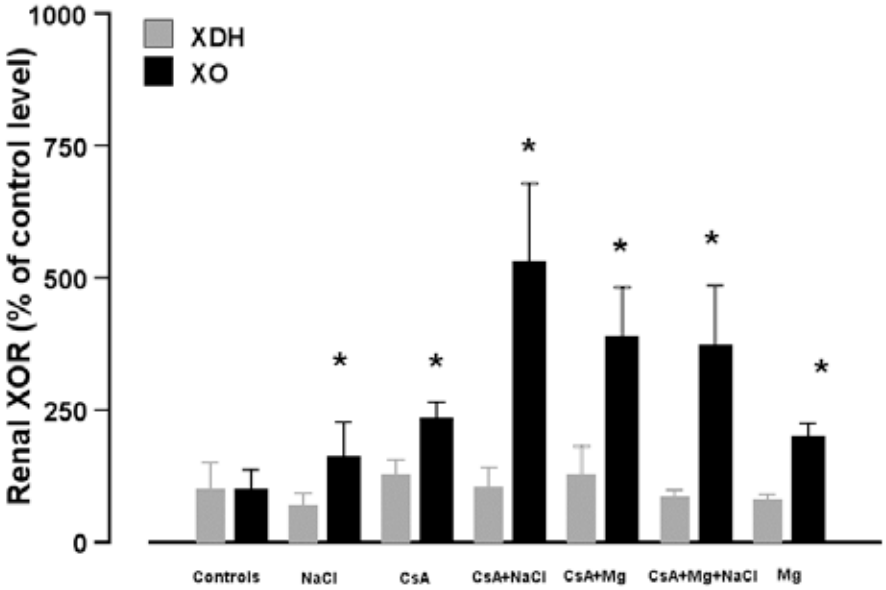

Figure 12. Effects of nephrotoxic doses of cyclosporine (CsA) on renal XDH and XO in SHRs. Controls were kept on standard laboratory chow. NaCl refers to a diet containing 6.6% NaCl (w/w) and Mg to a diet containing the same amount of NaCl (6.6% w/w) with the addition of 0.8% Mg^{2+} (as $MgCl_2$). Each group consisted of 7–8 rats. These data show that the activity of potentially ROS generating XO isoform was markedly upregulated in CsA induced nephropathy and furthermore modified by dietary NaCl, while renal XDH remained unchanged. *, $p<0.05$ in comparison with respective control. Unpublished data (Laakso, Pere, Mervaala and Lapatto). The experimental setting with functional and histopathological data has been previously published in detail (Mervaala *et al.*, 1997).

5.8 SUMMARY OF FINDINGS

The main findings of the studies are presented in Table 7. Strain related upregulation of renal XDH and XO and specific mRNA was found in one obese rat strain and two rat strains having a predisposition to hypertension. Only slight strain-related changes were found in the heart and in the liver. Increased dietary NaCl induced hypertension and resulted in renal and cardiac hypertrophy in hypertension-prone rats. This was also observed to a much lesser extent in hypertension resistant rats. In salt-sensitive Dahl SS/Jr XOR induction with dietary NaCl was accompanied with upregulation of renal G6PDH activity. LDH remained unchanged. Concomitantly with increases in blood pressure, there was an upregulation of renal XDH and XO activities, and specific mRNA. Allopurinol completely inhibited XOR activity in vivo. XOR inhibition was not accompanied with any decrease in blood pressure levels in salt-sensitive SHRs, whereas in WKY controls a slight, but significant decrease was noted. In spite of the lack of effects on blood pressure, allopurinol prevented development of cardiac and renal hypertrophy.

NOS inhibition induced malignant hypertension, LVH, and renal hypertrophy. In the kidney, XOR activity was upregulated in L-NAME treated rats. In this relatively short experiment with L-NAME treated rats, increasing dietary salt did not aggravate hypertension. Increased levels of dietary NaCl did not induce renal XOR activity. NaCl loading decreased circulating arginine levels in L-NAME treated rats. Renal G6PDH was slightly increased. NO donor IS-5-MN alleviated hypertension without any effects on renal XOR.

Nephrotoxic CsA doses resulted in substantial upregulation of the XO isoform only. XO activity was increased further by increasing dietary NaCl intake.

At the end of the 10 month experiment, XOR activities were increased in kidneys and liver of obese Zucker fa/fa rats compared with lean controls. The atherogenic diet did not aggravate obesity or increase blood pressure. Administration of Ang II receptor blocker valsartan, markedly decreased hepatic XOR activity, and to a lesser extent, also renal XOR. In contrast to hypertension-prone rat, in obese Zucker fa/fa rat the changes in XOR activity were derived from post-translational modification, since specific mRNA remained unchanged.

Table 7. Summary of the findings related to XOR induction in the present series of studies

Model	Experimental Setting	Main finding	Reference
Dahl SS Jr /Dahl SR Jr	Dose response study with NaCl daily doses ranging from deficient (0.05% NaCl w/w in the diet) to excessive (6% NaCl w/w in the diet). Duration 8 weeks.	Strain-related difference in XOR activity. XOR induction with increasing salt intake in hypertension-prone, salt-sensitive Dahl SS/Jr strain rats. Down-regulation of XOR by NaCl deficient diet.	STUDY I
SHR/WKY	NOS inhibition by L-NAME, NaCl intake (0.2-6.2% w/w). Duration 3 weeks.	XOR induction by L-NAME. Aggravation of salt-induced hypertension.	STUDY II
SHR/WKY	NO surplus by IS-5-MN, low (0.7%) and high (6.0%w/w) NaCl intake. Duration 8 weeks.	No effects on XOR. Modest alleviation of NaCl-induced BP levels.	STUDY II
SHR/WKY	XOR inhibition by allopurinol, observations during development of hypertension, low (0.2% w/w) and high NaCl intake (6.5% w/w). Duration 3-8 weeks depending on group.	Strain-related differences in XOR activity and specific mRNA levels. NaCl induced XOR upregulation (activity and expression) in the SHR kidney but not in the heart and to much lesser degree in the liver. No upregulation in WKY rats. XOR inhibition did not alleviate hypertension, but prevented development of renal and left ventricular hypertrophy.	STUDY III
Obese (fa/fa) and lean Zucker rats	Atherogenic (high fat) diet with 6.0% w/w NaCl. Low NaCl diet with or without Valsartan. Duration 10 months.	Strain-related difference in renal and hepatic XOR activity, but not in specific mRNA levels. An increase of dietary salt and saturated fat intake did not induce renal XOR or SBP. Valsartan administration lowered SBP, reduced hepatic XOR substantially, and renal XOR modestly.	STUDY IV
SHR/WKY	Nephrotoxic CsA-doses with salt loading (high NaCl diet 6.6% w/w). Duration 6 weeks.	Substantial CsA-induced upregulation of XO isoform activity in rats kept on high salt diet. No change in XDH.	Previously unpublished data

6 Discussion

6.1 METHODOLOGICAL ASPECTS

6.1.1 *Animal models of essential hypertension and metabolic syndrome*

While hypertension involves complex genetic and environmental factors in human populations, well-defined animal models in controlled experimental settings provide a possibility for experimentation and have greatly increased the understanding of underlying factors in cardiovascular diseases. Several genetic models of hypertension have been generated. However, none of the models reflects all of the mechanisms underlying essential hypertension or metabolic syndrome.

Inbred rat strains, such as SHR, have been developed by selective mating of normotensive WKY rats for 20 generations or more in order to obtain the hypertensive phenotype. SHRs begin to generate hypertension at approximately five weeks of age. The selection procedure did not involve dietary manipulation. SHR is by far the most used experimental rodent model corresponding to approximately 30% of publications found with the keywords 'rat, hypertension'. Another inbred strain, hypertension-prone Dahl SS/Jr and its control, salt-resistant Dahl SR/Jr has been established by selection on the basis of differences in blood pressure after challenging with a diet containing 8% (w/w) NaCl. In these models the hypertension trait results from a number of genes. Breeding protocols have produced several rat strains, in which blood pressure increases in a reproducible manner. The models show complex genetic background and in that resemble human essential hypertension (Rapp, 2000).

The molecular basis of essential hypertension is polygenic and has remained unresolved. The inbred hypertension- and obesity-prone rat models have different genetic determinants, which interact with dietary factors. Particularly Dahl SS rats and SHRs have been used to identify quantitative trait loci (QTL), i.e. areas of DNA in chromosomes, which associate with the phenotype (Aneas *et al.*, 2009, Mattson *et al.*, 2008). The hypertensive phenotype comprises of relevant features, such as salt-sensitivity, spontaneous hypertension, insulin resistance, proteinuria etc. Once a promising loci has been found, the respective chromosome (or the area of DNA) can be transferred to a recipient strain to produce chromosomic substituent strains (consomic strains). The identified QTLs have been divided in to loci, which are associated with salt-sensitivity, to loci which affect the phenotype regardless of the salt intake or have influence only at a 'normal' salt intake level (Aneas *et al.*, 2009).

There are also surgical models, such as the Goldblatt model, in which, following the removal of one of the kidneys, the renal artery of the remaining kidney is constricted. Kidney removal is not a prerequisite, and there are several variations of the model. These invasive models mimic renal disease, and have been used in short-term mechanistic studies (Pinto *et al.* 1998).

Numerous pharmacological models exist, such as the DOCA-NaCl model, described earlier, as well as models based on the inhibition of NO production (Ribeiro *et al.*, 1992) or inhibition of urate elimination (Mazzali *et al.*, 2002). Several genetically modified rats have been developed, which specifically target one or a few factors involved in the development of hypertension. Double transgenic rat expressing the human renin and angiotensinogen gene, described by Ganten *et al.* (1992) is one of such approaches. As compared with inbred rodent models of essential hypertension, both pharmacological and transgenic models have features relating to secondary forms of hypertension.

The obese Zucker fa/fa rat is a prediabetic model of metabolic syndrome, which has a predisposition for development of mild hypertension and develops at a lower rate than in SHRs or Dahl SS/Jr rats. The Zucker fa/fa rat is originated from a spontaneous mutation (Zucker, 1965), which was later localized in the leptin receptor gene. The phenotype is characterized by hyperphagia, hyperlipidemia and mild glucose intolerance (Speakman *et al.*, 2008). While the obese Zucker rat has been demonstrated to have only one mutation resulting in obesity, there are also several polygenic models of metabolic syndrome, such as Goto-Kagizaki rat. Evidence has accrued that obesity is a complex and heterogenic state.

The salt-sensitivity of obese Zucker fa/fa rat has been a controversial subject. According to Reddy and Kotchen (1992) Zucker rats are salt-sensitive in comparison with genetically close lean controls, whereas Pawloski *et al.* (1992) demonstrate that obese Zucker rats are normotensive regardless of the NaCl content in the diet. In these models, salt-sensitivity has been associated with hyperglycemia (Carlson *et al.*, 2000). In the present studies serum glucose levels of obese Zucker fa/fa rats did not differ from lean Zucker rats and the rats were not salt-sensitive (Study IV).

In the rat and in most other mammals urate is metabolized by uricase in the liver to allantoin, while the kidneys do not express the enzyme (Watanabe *et al.* 2002). None of the species generally used to model hypertension are uricotelic. On the other hand the situation allows to study local effects of urate production. For studies on XOR, generation of urate is only one of features, which may relate to cardiovascular phenomena.

6.1.2 *Dietary modifications*

The NaCl intake in present studies varied were typically from 0.2 to 6.6% (w/w) in the diet. Such levels are commonly used in rodent models of hypertension.

Typically, a basic commercial rodent diet contains 0.275 g of Na^+ / 100 g of the diet, i.e. in relation to energy content of the diet 0.22 g/MJ as Na^+, i.e. 0.54 g NaCl /MJ. This level is relatively close to the current recommendation policy for intake level for non-obese humans, 6–7 g/d, i.e. approximately 0.5 g/MJ. In relation to energy intake, the low intake of NaCl in the present rodent studies was close to dietary recommendations for humans by WHO (Nishida *et al.*, 2004).

The lowest dietary level of NaCl investigated (Study I) was 0.05% (w/w) and clearly deficient, since weight gain in both salt sensitive and resistant rats was substantially decreased, whereas 0.20% (w/w) dietary NaCl resulted in the approximately same weight gain as from higher NaCl levels. Modifying the diet with NaCl did not influence palatability as judged from food intake levels (data not shown).

6.1.3 *Biochemical methods*

Several methods can be used to determine XO and XDH activity. Some of the methods use artificial substrates and the others rely upon endogenous substrates. Whereas highly sophisticated studies have been published describing mechanisms of XOR catalyzed reactions, no systematic studies are available describing the substrate preferences of all XOR isoforms.

In the Studies I, II and III, the conversion of pterin to a fluorescent product isoxanthopterin was used. In the Study IV radiolabeled xanthine was used as a substrate and the formation of radioactive urate was determined with a radiodetector after HPLC separation. As indicated in Section 2.9 supra, XOR proteins can have different activities. XOR participates in pterin metabolism and both reactions are physiological.

Some of the published assays use artificial substrates, such as 2,6- dichlorophenolindophenol, which can be determined by UV spectroscopy. Determination of fluorescent product, resorufin, which is formed by XO catalyzed O_2^- generation from Amplex Red, has also been used. Furthermore, discoloration of methylene blue to determine total XOR has been employed. Intercalibration studies of the various methods have been scarce.

6.2 NaCl-induced blood pressure and renal and cardiac hypertrophy

In salt-sensitive rats, intake levels at or exceeding 3.25% NaCl (w/w) in the diet resulted in sustained increase of systolic blood pressure levels reaching 200 mmHg or more. Such malignant hypertension results in target organ changes and damage, including left ventricular hypertrophy and renal enlargement as observed in Studies I and II, an effect, which is well-established in the literature (Mervaala *et al.*, 1992 and Mervaala *et al.*, 1994b).

Histological examinations were not carried out in the present set of studies. Previous reports have shown that NaCl -induced renal growth is due mainly to renal hypertrophia in Dahl SS/Jr rats (McCormick *et al.*, 1989).

In the Study I, a significant correlation was found between relative kidney weight and renal XOR activity in Dahl SS/Jr but not in Dahl SR/Jr rats. In pathological renal enlargement, the diffusion distances for oxygen may be increased and limit oxygen availability in the most susceptible areas.

An atherogenic diet, containing a high NaCl level and butter, increased blood pressure only modestly in obese Zucker fa/fa rats in comparison with rats kept on control diet (Study IV). Liver weights (adjusted to tibia length) were higher in obese Zucker fa/fa rats in comparison with the lean controls.

6.3 STRAIN-RELATED DIFFERENCES IN RENAL XOR ACTIVITY

The findings (Studies I and II) of increased kidney XOR activities in two different rat models of hypertension, namely the Dahl salt-sensitive model and the SHR model, may reflect the intrinsic metabolic differences between these strains and associate with the hypertensive trait.

In agreement with our findings, Delano *et al.* (2006) found upregulated XOR and NAD(P)H oxidase levels in SHR in several cell types including leucocytes, arterial, and venous endothelium, by using specific antibodies and horseradish peroxidase staining.

Salt-sensitivity of obese Zucker fa/fa rats has been a controversial subject (Fujiwara *et al.*, 1999). In contrast to SHRs, only the XOR activities were increased in Zucker fa/fa rats on the control diet, whereas specific XDH mRNA levels remained unaltered. In obese Zucker fa/fa rats (Study IV), also hepatic XOR activity was elevated to some extent and again, without effects on XDH expression.

6.4 NaCl INTAKE AND RENAL XOR ACTIVITY

A dose-dependent induction of XOR was found in response to increases in salt intake in salt-sensitive rats in contrast to salt-resistant rat strains. Literature searches demonstrate that Study I is the first report on the effects of dietary salt on renal XOR activities. Two salt-sensitive rat strains Dahl SS/Jr and SHR and their respective genetic controls Dahl SR/Jr and WKY were used. Furthermore, the effects were seen only in the kidney of hypertension-prone salt-sensitive rat strains, and not in the heart.

In both Dahl SS/Jr and Dahl SR/Jr control strain, renal XOR activities were markedly decreased when the rats were kept on NaCl deficient diet, and did not show any effect on blood pressure compared with low NaCl intake levels (0.08% w/w Na^+). This result indicates that renal XOR activity is, at least at low NaCl intake levels, associated with renal sodium and possibly with chloride handling.

In humans, high circulating urate levels have been proposed as one of the means to maintain blood pressure during salt restriction. This ability of high urate levels has been claimed as one of the evolutionary benefits obtained in humans and great apes, when the ability to degrade urate was silenced due to mutations in the uricase gene. This hypothesis was put forward by Johnson *et al.* (2008), when it was found that inhibition of uricase by oxonic acid in rats results in the development of salt-sensitive hypertension. In the

present study, rats fed with deficient NaCl levels resulted in growth retardation, and did not upregulate XOR, but on the contrary XOR activity was substantially decreased in both salt-sensitive and salt-resistant rats as a response to NaCl restriction. The result indicates that XOR activity is not regulated as to increase urate production during NaCl restriction, but rather, to decrease it.

There are not many studies addressing the effects of dietary salt intake on XOR activity or expression. Lenda and Boegehold (2002) reported that both XOR and NADPH oxidase activities and expression were unchanged in skeletal muscle microcirculation of Spraque-Dawley rats kept for 4–5 weeks on a diet containing either low or high amounts of NaCl. In spite of the lack of response in enzyme activities, the authors found an increase in ROS generation in the microvessels, which was inhitable by oxopurinol and DPI.

In the present studies renal XOR activities correlated with renal weight to body weight ratios, which may indicate that renal XOR induction is a consequence of hypertension and subsequent renal damage. Statistically, kidney XDH and XOR activities revealed a close correlation with systolic blood pressure in both the Dahl SS/Jr and SHR.

Systolic blood pressure of Dahl SS/Jr rats kept on standard NaCl diet (0.20% w/w) did not differ from the group kept on the NaCl deficient diet. Renal XOR activity was decreased substantially in the NaCl deficient group, indicating that blood pressure is not the only factor contributing to changes in renal XOR activity.

In obese Zucker rats (Study IV), blocking the angiotensin receptor with valsartan decreased both renal XOR activity as well as XOR expression at the mRNA level, which indicates that XOR regulation is related to angiotensin II levels or its effects on blood pressure, or both.

6.5 NITRIC OXIDE AND XOR

6.5.1 *Inhibition of nitric oxide synthesis*

In the present study (Study II), unselective NOS inhibition by L-NAME was found to produce a modest upregulation of renal XOR activity. In this experimental setting, increasing the intake of NaCl increased renal XOR activity in L-NAME treated SHRs. Inhibition of NOS activity by L-NAME has been previously reported to increase the oxygen cost of NaCl transport in Wistar rats (Deng *et al.*, 2005). Unselective NOS inhibition has demonstrated to increase oxygen consumption by several mechanisms including an increase in mitochondrial metabolism. A relatively high daily L-NAME dose was used, 20 mg/kg, which was reported to produce not only malignant hypertension but also cardiac infarcts in all test animals (Vaskonen *et al.*, 1997).

In the present study, NOS inhibition increased SBP regardless of the NaCl intake level. Administration of a low dose of L-NAME, which is not sufficient to increase blood pressure, has been shown to render salt-resistant animals to salt-sensitive (Salazar *et al.*,

1993). Increased level of dietary salt aggravates the target organ damage caused by L-NAME treatment leading to high occurrence of stroke (Vaskonen *et al.*, 1997). In the present study, NaCl-induced changes, with or without NOS inhibition, in renal XOR activity were rather small, which may relate to the relatively short duration (three weeks) of the experiment.

The present study also indicated that circulating arginine levels are decreased along with increasing NaCl-intake in the L-NAME treated SHRs, but not in the controls. Kitiyakara *et al.* (2001) also found higher circulating arginine levels in Sprague-Dawley rats kept on low NaCl intake. Arginine is synthesized endogenously mainly in renal tubules (Lim *et al.*, 2007). While arginine may be spared by NOS inhibition, the present study may also indicate that there is a concomitant upregulation of arginase (EC 3.5.3.1) acitivity. In cell cultures and also *in vitro*, L-NAME has been shown to inhibit arginase (Reisser *et al.*, 2002). Recent studies suggest that the mitochondrial arginase isoform II regulates NO production in the endothelium (Lim *et al.*, 2007). Arginase inhibition has been shown to alleviate development of hypertension in SHRs but not hypertension-induced cardiac hypertrophy (Bagnost *et al.*, 2008).

Together, the results from Study II and the current literature indicate that NOS inhibition by L-NAME may modify NO production by several effects on arginine metabolism and, furthermore, these effects are modified by dietary salt intake. The L-NAME treated SHR is a high-renin model of hypertension (Vaskonen *et al.*, 1997). In addition to its guanylate cyclase mediated vasodilatator effects, NO is a powerful regulator of cellular oxygen consumption (Victor *et al.*, 2007). NO deficiency may impair oxygen distribution and subsequently result in XOR induction.

6.5.2 *Treatment with a nitric oxide donor*

The nitric oxide donor, IS-5-MN, significantly reduced salt-induced hypertension. The rats treated with IS-5-MN were still hypertensive and sensitive to dietary NaCl. IS-5-MN treatment did not have any effects on renal XOR activity. These results are in line with previous studies indicating that endothelial NO production is upregulated in SHR before and after the onset of hypertension (Vaziri *et al.*, 1998).

Organic nitrates are important antianginal vasodilatating drugs introduced more than one hundred years ago. Prolonged therapy with organic nitrates is associated with loss of efficacy. These drugs can be considered as prodrugs, since many of their effects have been attributed to release of NO. Chen *et al.* (2002) have shown that aldehyde dehydrogenases (ALDH1 and more notably the mitochondrial isoform, ALDH2) metabolize glycerol trinitrate (GTN) to nitrate, whereas IS-5-MN and its dinitrated congener IS-5-DN, are not at all metabolized by ALDH. Both cytosolic (ALDH1) and mitochondrial ALDH2 convert GTN to nitrite (Beretta *et al.*, 2008), which can then be converted to NO by XOR. In the present study, dietary NaCl increased total ALDH activity and in theory would

therefore not reduce the efficacy of reactive organic nitrates, such as GTN. Furthermore, the present study also indicates that NO derived from IS-5-MN does not downregulate ALDH in vivo. The enzyme which activates IS-5-MN has not been identified.

6.6 RENAL XOR INDUCTION BY CYCLOSPORINE

CsA is an immunosuppressive drug used in organ transplantation. CsA use is plagued by its acute and chronic nephrotoxic effects. Dietary NaCl has been reported to aggravate CsA induced nephrotoxicity in rats (Pere *et al.*, 2000). CsA nephrotoxicity is associated with fibrosis, hypertension, renal vasoconstriction, subsequent renal hypoxia, as well as increased angiotensin II and aldosterone activity resulting in potentially irreversible renal dysfunction (Bobadilla and Gamba, 2007).

Results relating to effects of CsA on renal function and SBP have been previously published separately (Mervaala *et al.*, 1997), where CsA produced malignant hypertension, which was aggravated by increase in dietary NaCl. The combined CsA/high NaCl diet was accompanied with up to a sevenfold increase in the amount of protein excreted in urine. Magnesium supplementation decreased blood pressure levels and proteinuria in groups treated with CsA and kept on high NaCl diet. It is also noteworthy that the same CsA treated rats had twice as high plasma renin activity than controls kept on the low NaCl diet (Mervaala *et al.*, 1997).

In the present study, CsA was found to induce the activity of renal XO while renal XDH remained unchanged. XO induction was alleviated by magnesium supplementation and exacerbated by increased NaCl intake. These results parallel with previously published CsA induced renal dysfunction and histological changes (Mervaala *et al.*, 1997).

Studies on Wistar-Kyoto rats have reported that allopurinol markedly alleviates CsA induced hypertension (Kobelt *et al.*, 2002). In their study Kobelt and coworkers (2002) used an oral CsA dose at 8 mg/kg body weight/d and allopurinol at 50 mg/kg body weight/d. Furthermore, the authors found that CsA decreased renal plasma flow, which was markedly improved by allopurinol. CsA has been reported by Mazzali *et al.* (2001b) to increase uric acid levels in rats. Increasing urate levels using oxonic acid to inhibit uricase exacerbates CsA nephrotoxicity (Mazzali *et al.*, 2001b). These observations are in line with the present data, which indicate that the activity of the urate producing enzyme XO is upregulated by high NaCl intake and that CsA further increases the enzyme activity.

$MgCl_2$ has been found to alleviate CsA toxicity ((Mervaala *et al.*, 1997), and in the present study it decreased NaCl and CsA induced XO activity. Used alone $MgCl_2$ was found to induce XO in a similar fashion as NaCl, an effect, which may relate to independent influence of chloride ion as discussed supra in Section 2.3.1. $MgCl_2$ supplementation has been considered beneficial in experimental studies on the harmful effects of dietary NaCl (Mervaala *et al.*, 1992).

It is possible that XO induction by CsA is the result from direct cellular toxic effects of CsA. It is interesting to note that in rat pulmonary endothelial cell cultures, tobacco smoke condensate has been reported to induce XOR (Kayyali *et al.*, 2003), indicating that XOR induction could be part of a pre-programmed general cellular response to toxic compounds. Many hepatotoxic compounds, such as halothane and ethanol, has been reported to increase the release of XOR from the liver into the circulation (Pacher *et al.* 2006).

The present study demonstrated that CsA is able to upregulate only the XO isoform. XO has been associated with ROS production and subsequent tissue damage in I/R (McCord, 1985). In Fischer rats, a few days after kidney transplantation, renal XOR activity has been reported to substantially increase in allografts, up to levels 15 times higher than in controls, whereas in syngrafts no change was observed (Sun *et al.*, 2004). The study indicates that XOR may also have a role relating to the survival of the transplant. Present data on induction of XO by CsA justify further studies on feasibility of XOR inhibition to improve allograft survival and to avoid CsA toxicity.

6.7 XOR and metabolic syndrome

In the present studies XOR activity was not only found to be upregulated in hypertension-prone rats, but also in rats with a predisposition to obesity, i.e. Zucker fa/fa rats. Administration of valsartan, an AT_1 receptor blocking agent, did not lower body weight and did not have any effect on circulating cholesterol levels. Valsartan is not an agonist of the nuclear receptor, peroxisome proliferator-activated receptor-γ (PPARγ). In this respect it differs from telmisartan (Sugimoto *et al.* 2006) Recently, Cheung *et al.* (2007) reported that XOR +/- knock-out mouse had 50% reduction in adipose mass. In the same study it was also found that XOR is a regulator of PPARγ in 3T3-Li adipose cell cultures.

In the present study, chronic administration of valsartan decreased hepatic XOR activity substantially, but did not have any effects on XOR at mRNA level. In the kidney valsartan had a less pronounced effect on renal XOR activity and showed no effects on specific mRNA levels. In spite of the downregulation of XOR activity any reduction in weight gain was not found in the valsartan treated group. Interestingly, valsartan reduced hepatic and renal XOR activities in obese Zucker rats, pointing towards that hepatic XOR is regulated by Ang II. There is evidence that urate in turn can regulate intracellular Ang II levels (Corry *et al.*, 2008) revealing a possibility of positive feed-back loop.

There is evidence indicating that hepatic effects of Ang II receptor blockers are mediated through upregulation of $Ang_{1–7}$ rather than inhibition of Ang II receptor AT_1 (for review, see Lubel *et al.*, 2008). With respect to metabolic syndrome, there has been more interest on cardiovascular than hepatic effects of Ang II receptor blockers.

6.8 INHIBITION OF XOR ACTIVITY

Allopurinol is an established urate lowering agent and a specific inhibitor of XOR (reviewed by Pacher *et al.*, 2006). XOR and AO convert allopurinol to the even more potent XOR inhibitor oxypurinol. Allopurinol is not an inhibitor of aldehyde oxidase and has a short half-life, 2.5 hours, in contrast to 15 hours of oxypurinol. It does, however, have greater bioavailability than oxypurinol and as such was therefore chosen for the present experiments. While both compounds inhibit urate production, there is evidence indicating that the inhibited enzyme may still transfer electron to oxygen and thus produce ROS (Myamoto *et al.* 1996). This may partly explain some of the controversial results.

To avoid toxic side-effects and nonspecific effects of the drug, the lowest possible allopurinol dose level was chosen, but at a level that still completely inhibited XOR activity.

In the present study Allopurinol did not have any significant effects on blood pressure. In contrast to the present findings, a randomized, double-blind, placebo-controlled clinical study with allopurinol has recently been carried out by Feig and Soletsky (2008) who found that allopurinol can decrease blood pressure in humans.

Allopurinol has later been shown to decrease blood pressure, if urate degradation by the uricase enzyme has been inhibited by oxonic acid (Mazzali *et al.*, 2001a).

Most, but not all experimental studies using allopurinol or oxopurinol, have had a positive outcome with regard to cardiac parameters. It is not known whether these effects can be attributed to effects of allopurinol on XOR or to effects on other pathways.

On the other hand, a recent study with the Dahl SS/Jr strain demonstrated, in line with this study, that allopurinol did not alleviate the salt-induced development of hypertension. In agreement with this result allopurinol was reported not to improve renal function (Tian *et al.*, 2008).

6.9 XDH TO XO CONVERSION

The conversion from XDH to XO has been proposed to be important in the pathogenesis of reperfusion induced tissue injury. It has been postulated that during prolonged ischemia, XDH to XO conversion is increased. At the time when circulation and oxygen availability is re-established, XO produces harmful reactive oxygen species resulting in organ damage (McCord and Roy, 1982; McCord, 1985).

In the present work, the Dahl SS/Jr rats had higher XO/XOR ratios compared with the SHRs. The data have been derived from separate experiments, which may partly explain the difference. Sample handling may also affect XO/XOR ratios. Since the experiments were independently carried out direct comparison of the results was not possible.

CsA nephrotoxicity was discussed separately in the Section 6.6, but in this case a marked XDH to XO conversion took place, which presumable was protease mediated.

6.10 HYPOXIA AND RENAL XOR

As hypoxia is a sufficient, but not unique signal for XOR induction, the finding of increased renal XOR in hypertensive rats is in line with the hypothesis of hypertension associated renal hypoxia. The first direct demonstration of renal hypoxia in hypertensive rat was made by Welch *et al.* (2001). Oxygen content in arterial and venous renal blood was measured in WKY rats and SHRs with an oximeter. The authors found an oxygen tension (pO_2) of 40–50 mm Hg in the renal cortex, whereas in the medulla pO_2 was only 10mm Hg. In the cortex, pO_2 was found to be 10mm Hg lower in the SHRs than in WKY rats. The authors were also able to estimate that in relation to a given amount of Na^+ transport, the corresponding renal oxygen cost is approximately 50% higher in hypertensive SHRs than in normotensive WKY rats. Li and co-workers (2003) have confirmed the previous findings of decreased oxygen levels in the SHR kidney by MRI techniques. Recent experimental studies have provided evidence that the lower renal oxygen tension in SHRs precedes the development of hypertension (Welch, 2006).

In cultures of human bronchial epithelial cells, normoxic oxygen levels have been reported to markedly suppress XOR activity. Linder *et al.* (2003) found that extremely low oxygen levels (0.5–3%) increased XOR activity by 300–800%. In cultured endothelial cells, low oxygen levels, comparable to those occurring clinically in modest arterial hypoxia (i.e. 10% O2) increased XOR levels threefold (Kelley *et al.*, 2006). In both studies, suppression of protein synthesis did not alter XOR activity. Contrary to cell culture studies, the present *in vivo* study (Study III) on SHRs demonstrated that not only XOR activities, but also renal XDH mRNA levels were increased. Hypoxia-induced synthesis of XOR protein has been found in cell lines of endothelial origin (Terada *et al.*, 1997).

The activities of several enzymes, regulated by hypoxia and redox homeostasis, were studied. HIF-1α mediates upregulation of many enzymes involved in glycolysis. HIF-1αα does not regulate XOR (Linder *et al.*, 2003). LDH is an enzyme which usually shows one of the highest responses to hypoxia (Marti *et al.*, 1994). G6PDH catalyzes the first and rate-limiting step in pentose phosphate shunt, which generates reducing equivalents such as NAD(P)H, used for example by glutathione reductase to regenerate GSH from its oxidized form GSSG. GSH may then be used by glutathione peroxidase to degrade H_2O_2 into H_2O and O_2. As the pentose phosphate pathway spares oxygen, it is an important source of reducing equivalents, when oxygen tension is low (Bartrons *et al.*, 2007).

The response of these enzymes in the present studies was variable. In Study II, NOS inhibition by L-NAME produced a small, but significant decrease in renal LDH, and a small increase in G6PDH activities in SHRs. In contrast to SHR data, in Dahl salt-sensitive SS/Jr rats, renal G6PDH was significantly induced (up to 67%) at highest NaCl intake level (6.5% w/w), whereas, in salt-resistant Dahl rats, no response was obtained.

It is interesting to note that upregulation of G6PDH is associated with increased cell growth in cultures of fibroblasts (Tian *et al.*, 1998). IS-5-MN did not exert any effect on renal G6PDH activities. These data point towards that NaCl-induced changes in glucose metabolism occurs in the Dahl SS/Jr kidney, while in SHR the response is blunted.

In contrast to SHR and Dahl models, increasing NaCl intake did not induce renal XOR in obese Zucker fa/fa rats. In this model dietary NaCl produced only modest increase in SBP. The Ang II receptor blocker, valsartan, resulted in an alleviation of blood pressure and only a slight decrease in renal XOR activity in obese Zucker fa/fa rats kept on low NaCl intake. These results point towards that Ang II upregulates XOR activity, but the effect is tissue specific.

6.11 CLINICAL IMPORTANCE

As stated earlier, XOR has been detected in the human liver and the intestine. The low or undetectable cardiac activities decreased scientific interest in XOR inhibition as a means to treat cardiac dysfunction, but the interest in XOR has not totally declined. In a more recent electron spin resonance based studies by Spiekermann and co-workers (2003) demonstrated that a XO isoform is localized in human endothelium, from where it can be liberated with a bolus of heparin. The authors also found upregulation of XOR activity in the coronary arteries of patients with coronary disease along with another ROS producing enzyme, NAD(P)H oxidase.

While studies on rodent models have revealed some positive effects of XOR inhibition (Table 3), results from clinical studies have been controversial (Table 4). It can also be noted that even in studies with a positive outcome, it is not known, whether the effects can be attributed to XOR inhibition or to other possible effects of allopurinol and/or oxypurinol. Nearly all clinical studies so far have been based on surrogate endpoints and involved a small number of patients. In a recently reported OPT-CHF multicenter study, 405 patients with NYHA class III–IV congestive heart failure were recruited (Hare *et al.*, 2008). In the study, oxypurinol was added to the best conventional therapy in a double blind setting. The clinical outcome was not improved in unselected patients, but statistically significant benefits in terms of mortality and need for emergency treatments or hospitalization were found in a subgroup selected on the basis of high urate levels. It was also noted that the use of oxypurinol in such a group is not without risk. Subgroup analysis revealed that the group experiencing harmful consequences was found to consist of patients, whose serum urate levels failed to decrease in response to oxypurinol therapy.

Whether XOR inhibition confers any advantage to allograft survival and possibilities to prevent or alleviate renal pathologies during immunosuppression is also controversial. In combination with an experimental study showing alleviation of CsA-induced renal dysfunction by allopurinol (Kobelt *et al.*, 2002), the present findings of CsA induced XOR

upregulation indicates that XO is involved in CsA-induced renal effects. Human XOR activities have been studied after lung and heart transplantation involving CsA treatments (Kinnula *et al.*, 1997). Any XOR activity or induction in the lung, heart or plasma was not detected by analysing the activity using radiolabeled ^{14}C-xanthine as a substrate. The methods were fully functional as hepatic and intestinal XOR activities were significant.

The present experimental studies indicate that renal XOR is upregulated in hypertension and metabolic syndrome and also that dietary factors and antihypertensive drugs influence renal XOR. Inhibition of XOR did not decrease SBP. In salt-sensitive rats both renal and cardiac enlargement was prevented by allopurinol treatment. These effects were found in rats after chronic allopurinol treatment, which was started prior to development of hypertension. More experimental studies are needed to clarify these issues.

Further experimental studies would be needed to clarify the role of XOR in left ventricular hypertrophy, which is clinically one of the serious outcomes associated with essential hypertension.

6.12 FUTURE DIRECTIONS

Present studies showed that XOR inhibition does not have an effect on blood pressure, but is able to prevent cardiac and renal hypertrophy. Although the mechanisms are unclear, the latter finding may provide therapeutic possibilities. At present there are no specific inhibitors for XO and XDH or useful *in vivo* inhitors of XDH to XO conversion. Such compounds could be helpful to clarify the physiological and pathological role of different XOR catalyzed reactions by separating the generation of ROS from other catalytic activities. Such inhibitors could be tested for prevention of CsA induced toxicity during organ transplantation. They could also be used to clarify the physiological and pathophysiological roles, if any, of nitrate and nitrite reduction.

7 Summary

Experimental studies were carried out to investigate the role of XOR in hypertension and metabolic syndrome. Renal and cardiac XDH and XO activity and expression of XDH mRNA was studied in hypertension-prone rats during the development of NaCl-induced hypertension. A specific XOR inhibitor, allopurinol was used to clarify whether XOR has any causal role in the development of hypertension. Pharmacological modifications of NO production and availability were used to study interrelationships between NO and XOR. Renal XOR was studied during CsA induced immunosuppression, as NaCl has been previously shown to aggravate CsA nephrotoxicity, resulting in malignant hypertension. Interrelationships between RAS and XOR in the kidney and the liver of obese rats were studied by inhibiting RAS with valsartan, an angiotensin II receptor blocker. With reference to the reviewed literature, XOR may have competing roles generating the powerful antioxidant urate on one hand and ROS and possibly NO on the other.

The main outcomes are:

• Renal XOR is a marker of salt-sensitivity in rodent models of hypertension. All studied hypertension-prone, salt-sensitive rat strains had higher renal activity of XDH and XO than their genetically close normotensive control strains.

• Dietary NaCl dose-dependently induced renal XDH and XO activities in hypertension-prone, salt-sensitive rat strains with increasing intake of NaCl, whereas cardiac and hepatic XOR activity remained unchanged. Increased intake of NaCl did not alter renal XOR activities in salt-resistant strains. Renal XOR activity correlated with blood pressure levels and with cardiac and renal hypertrophy.

While renal XOR activity may be increased by several potential mechanisms, including angiotensin II, XOR upregulation is likely to result from salt-induced renal hypoxia in hypertension-prone rats. Upregulation of renal G6PDH in Dahl SS/Jr salt-sensitive rats also points towards NaCl-induced renal hypoxia.

In the salt-sensitive rats, upregulation of renal XOR was accompanied with increased mRNA levels indicating that higher XOR activity in the salt-sensitive kidney derives from local protein translation rather than from binding of circulating XOR enzyme or post-translational modifications alone.

• Decreasing NO production by NOS inhibition with L-NAME induced malignant hypertension and upregulated renal XDH and XO activities. Since L-NAME is known to result in marked hemodynamic changes in renal circulation, its effect on renal XOR activity may also, at least partially, be attributed to changes in oxygen availability.

NO surplus, derived from IS-5-MN administration, markedly alleviated salt-induced hypertension, but had only negligible effects on renal XOR activity indicating that *in vivo* XOR is not inhibited by NO.

• Induction of renal XDH and XO in SHRs do not precede the development of hypertension, but progress concomitantly with an increase in SBP indicating that XOR does not exert any major causal role in the development of hypertension. The results also provide evidence that physiological levels of circulating urate do not mediate the development of salt-induced hypertension in spontaneously hypertensive rats, since inhibition of urate producing enzyme, XOR, did not prevent development of hypertension. As allopurinol is known to inhibit also XOR catalyzed NO generation, the result indicates that XOR mediated nitrate recycling to NO is not involved in long-term regulation of blood pressure.

• Chronic XOR inhibition abolished salt-induced renal and cardiac hypertrophy, pointing towards a pathophysiological role of XOR in NaCl-induced target organ dysfunction. Allopurinol mediated alleviation of cardiac and renal hypertrophy occured in spite of salt-induced high systolic blood pressure.

• Nephrotoxic doses of the fungal immunosuppressive peptide, CsA, resulted in marked XO induction in the SHR kidney. Furthermore, CsA administration coupled with a high NaCl intake upregulated XO. The induction of the XO isoform with no effects on XDH resembles an ischemia-reperfusion setting described in the literature, and suggests the possibility that XO induced ROS production is involved in CsA induced renal damage.

• The study using Zucker fa/fa rats indicates that XOR upregulation is associated not only with the hypertension trait, but also with the predisposition to obesity and metabolic syndrome. The Zucker fa/fa strain was not salt-sensitive. In this model renal XOR is not regulated by Ang II. Blocking Ang II receptors downregulated hepatic XOR levels indicating that Ang II regulates hepatic purine metabolism.

8 Conclusion

Upregulation of renal XOR is associated with the hypertensive trait. Furthermore, increasing NaCl intake increases renal XOR activity. In metabolic syndrome XOR is upregulated in the liver and in the kidney. Ang II is a potential regulator of hepatic XOR activity, since the angiotensin receptor blocker downregulated XOR. While increased NO levels did not inhibit XOR in vivo, decreased NO production upregulated renal XOR. The ROS producing XOR isoform XO, likely participates in CsA-induced nephrotoxicity and hypertension. XOR does not have any causal role in the development of hypertension. Renal and left-ventricular hypertrophy are harmful consequences of increased NaCl intake, which can be alleviated or even prevented in experimental models of hypertension by chronic administration of a specific XOR inhibitor.

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