DIETARY ELECTROLYTES AND CYCLOSPORINE TOXICITY
Experimental studies in spontaneously hypertensive rats

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Yliopistopaino 2008
To my family

Soli Deo gloria
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This thesis is based on the following original publications referred to in the text by their Roman numerals.


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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ATII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AZA</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane of the glomerulus</td>
</tr>
<tr>
<td>([\mathrm{Ca}^{2+}]_i)</td>
<td>Ionised intracellular calcium</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporine-A</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelin-derived hyperpolarising factor</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>Ionised magnesium</td>
</tr>
<tr>
<td>MMF</td>
<td>Mycophenolate mofetil</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetyl-[beta]-D-glucosaminidase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>P</td>
<td>Phosphorus</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PGI(_2)</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PT</td>
<td>Proximal tubule</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>RVR</td>
<td>Renal vascular resistance</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley rat</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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<tr>
<td>WKY</td>
<td>Wistar-Kyoto rat</td>
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ABSTRACT

Cyclosporine-A (CsA) is a cornerstone in organ transplantation as an effective immunosuppressant. Hypertension and nephrotoxicity are common side-effects of CsA. A challenge in experimental studies has been the lack of an animal model in which hypertension and nephrotoxicity concurrently occur.

A direct correlation between hypertension and cardiovascular risks is well established. Epidemiological data demonstrate a positive association between sodium (Na) intake and blood pressure. Additionally, there is evidence suggesting a correlation between low dietary magnesium (Mg) and potassium (K) intake and high blood pressure.

The present study was designed to develop an experimental model to study concomitantly presenting CsA-induced hypertension and nephrotoxicity.

As this showed to be a good new model to study CsA toxicity, we further investigated the effect of different dietary modifications such as Mg and/or K supplementation on prevention of the toxicity. Isradipine, a calcium-channel blocker, is often used in the treatment of hypertension. The possible additive effect of combination of Mg, which acts as an endogenous calcium antagonist, and isradipine was also investigated.

We used 8-week-old spontaneously hypertensive rats (SHR) and their hypertension-resistant genetic controls, Wistar-Kyoto rats (WKY). The “normal”, low-sodium rat chow (Na 0.3%, K 0.8% and Mg 0.2%) served as a control chow. In the high-sodium diet, the Na content was 2.6%, in the high-potassium the K content was 2.4% and in the high-magnesium the Mg content was 0.6% of the dry weight. The dose of CsA was 5 mg/kg/day s.c. Isradipine was added to the chow to produce an average daily dose of approximately 20 mg/kg of body weight. In this experimental model, accelerated hypertension developed and metabolic and renal structural changes associated with CsA toxicity were demonstrated. LVH also developed.

High dietary Na alone or CsA with the low-Na diet produced a minor increase in blood pressure and slight disturbance in renal function. By contrast CsA during a high-Na diet caused a pronounced rise in blood pressure and marked nephrotoxicity seen as decreased creatinine clearance, increased levels of serum creatinine and urea, and increased urinary protein excretion. Loss of Mg into urine caused Mg deficiency in tissues. Renal dopaminergic deficiency and paradoxal activation of RAAS were detected. In a histological evaluation severe thicken-
ing of the media of the afferent arterioles, fibrinoid necrosis of the arteriolar wall, and at worst haemorrhagic necrosis of the glomerular tuft were seen. In the epicardial arteries of the heart, a marked luminal narrowing, intimal and medial proliferation and a pronounced increase of connective tissue were detected. In the myocardium, vast scarring was seen. Mg supplementation inhibited the increase of the blood pressure, development of LVH, proteinuria and activation of the RAAS. The glomerular injury was also prevented. Both K and Mg alone and the two in combination, prevented the increase in blood pressure. Isradipine and Mg had rather equal antihypertensive effects, and the combination of these did not give any further advantage. Mg supplementation, alone and in combination with K, prevented LVH whereas K alone did not. Isradipine protected better than Mg from LVH, but the combination of isradipine and Mg was the most effective. Isradipine did not prevent from Mg loss.

We demonstrated that the combination of high dietary Na and CsA accelerates the development of hypertension and nephrotoxicity. We also developed an animal model in which both hypertension and nephrotoxicity concomitantly appeared as a result of a high Na intake in SHR receiving even a low dose of CsA. The nephrotoxicity was both functional and structural, also myocardial infarctions were detected. The development of hypertension and organ toxicity could be prevented by dietary Mg. The beneficial effect of Mg was enhanced by combining it with isradipine and to some extent with K.
1 INTRODUCTION

Before the advent of dialysis, patients with terminal renal failure had a poor prognosis. Pioneer surgeons attempted to save their patients’ lives with kidney transplantation, but the patients died soon after the transplantation. The first renal transplantation with long-term success was performed in 1954 in the USA between identical twins (for review see Huhtamies and Relander 1997).

Although the development of techniques in vascular surgery would have made successful renal transplantation technically possible in the beginning of the 20th century, the unawareness of the concept and mechanisms of rejection made all attempts desperate.

In Finland the first kidney transplantations were performed in 1964 and 1965. Unfortunately, the patients died soon after the operations (for review see Huhtamies and Relander 1997). The first kidney transplantation with long-term success was performed in Finland in 1966 from father to son (Huhtamies and Relander 1997).

Before the invention of true immunosuppressive agents, total body irradiation was used in the late 1950’s to prevent rejection, but it appeared to be too dangerous in comparison to its benefits. In the early 1960’s the antimetabolites, namely thiopurine, azathioprine and 6-mercaptopurine, improved the long-term outcome of kidney transplantations and the combination of corticosteroids and antimetabolites further improved the outcome. The patient and allograft survival rates remarkably improved after the introduction of cyclosporine A (CsA) in 1978 (Calne et al. 1978).

The concurrent developments in dialysis substantially improved the results of kidney transplantations as using effective renal replacement therapy the patients were in a better condition before transplantation and in case of acute rejection they could be dialysed. Also the development of anaesthetics whose elimination was independent of renal function and improved intra- and postoperative monitoring of patients have had an important role in improving transplant graft function.

Kidney transplantation was established as a routine treatment for end-stage renal disease in the 1970’s and 1980’s. The improved results of kidney transplantations encouraged to perform transplantations of the heart, lung and liver.

Cyclosporine is more effective and safer than the previously used immunosuppressants,
corticosteroids and azathioprine. In addition to its use in prevention of rejection after organ transplantation, CsA is also used at lower doses to treat autoimmune diseases.

The main side-effects of CsA-therapy, i.e. hypertension and nephrotoxicity, did not become evident in the preclinical trials. Soon after clinical experience with CsA had been gained, nephrotoxicity was reported (Calne et al. 1978). When the use of CsA became more common, hypertension was considered another serious side-effect.

In the prevention of rejection of the transplanted kidney, the use of CsA is crucial, but it is paradoxical that this effective drug is nephrotoxic itself. Heart transplantation patients with a well-functioning new heart may develop renal insufficiency caused by CsA and then need a renal transplant. Additionally, after transplantation, mostly because of the immunosuppressive drugs, hypertension has been documented in 30% to 100% of the patients (Textor et al. 1994). Hypertension, if not treated, is a major risk factor for LVH, stroke, coronary heart disease and renal insufficiency (Collins et al. 1990, MacMahon et al. 1990).

Many attempts have been made to develop an animal model which would correspond to the side-effects of CsA seen in man but they have failed because the animals have not developed concurrent hypertension and nephrotoxicity. An explanation for this phenomenon may have been the difference in dietary Na intake between rats and humans. The chow of the laboratory rats has low Na content whereas the Western human diet contains significantly more Na. High Na intake is indeed a well known risk factor for hypertension. Furthermore, an imbalance between high dietary Na and low dietary Mg and/or K intake seems to increase the risk of hypertension. Additionally, CsA increases excretion of Mg into urine thus leading to Mg deficiency.

The present study was designed to develop an experimental animal model to study the concomitantly presenting, common and clinically important side-effects of CsA-therapy, hypertension and nephrotoxicity. The Mg status of the animals was also assessed, because CsA-induced hypomagnesaemia is supposed to have a significant role in the development of CsA toxicity. Dietary modifications, i.e. adding Na, Mg and K were performed. We also compared the effects of Mg and isradipine, a widely used calcium antagonist, in the treatment of post-transplant hypertension.
2 REVIEW OF THE LITERATURE

2.1 Hypertension and the risk of cardiovascular and renal disease

High blood pressure is an independent risk of myocardial infarction, heart failure, stroke and renal disease (Chobanian et al. 2003). In addition, morbidity in and mortality from cardiovascular diseases increase with rising blood pressure without a clear threshold. Family heritage and individual life-style are the two main factors contributing to increased blood pressure. The major alterable risk factors for hypertension are obesity, high dietary intake of Na, excessive consumption of alcohol and low physical activity (The Finnish Current Care Guidelines, Finnish Hypertension Society 2005). The modifications of life-style are extremely important in order to both decrease the risk factors and to enhance efficacy of antihypertensive drugs (Chobanian, et al. 2003). Recommendations of the Finnish Hypertension Society concerning changes in lifestyle are listed in Table 1.

Table 1. Recommendations by the Finnish Hypertension Society

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Recommendation</th>
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<tbody>
<tr>
<td>Intake of sodium</td>
<td>&lt; 2 g / d</td>
</tr>
<tr>
<td>Intake of potassium</td>
<td>Women &gt; 3 g / d, men &gt; 3.5 g / d</td>
</tr>
<tr>
<td>Obesity</td>
<td>If overweight (BMI &gt; 25) reduction of weight by 5–10%</td>
</tr>
<tr>
<td>Physical activity</td>
<td>At least ≥ 3 times / week</td>
</tr>
<tr>
<td>Alcohol consumption, g / week (doses / week)</td>
<td>Women &lt; 160 g (&lt;14), men &lt; 240 g (&lt; 21)</td>
</tr>
<tr>
<td>Smoking</td>
<td>Cessation of smoking</td>
</tr>
</tbody>
</table>

The first-line drugs used in the treatment of uncomplicated essential hypertension are angiotensin-converting enzyme (ACE) inhibitors, angiotensin-receptor II antagonists, Ca channel blockers, diuretics and β-blockers. On average, they are equally effective in lowering elevated blood pressure (Finnish Hypertension Society 2005).
2.2 Long-term regulation of blood pressure

Blood pressure is mainly determined by peripheral vascular resistance and cardiac output. The control of blood pressure can be divided into rapid, intermediate and long-term regulation. The rapid regulation which occurs within seconds is mediated by the autonomic nervous system, mainly the sympathetic nerves.

The intermediate regulation becomes effective within 20 minutes and lasts for several hours. The main regulatory mechanisms are: 1) the renin-angiotensin vasoconstrictor system 2) stress-relaxation of the vasculature and 3) diffusion of fluid between the capillaries and the surrounding tissues leading to readjustment of the blood volume (Guyton and Hall 2000).

The long-term regulation of blood pressure is determined by water and salt balance, depending on both the intake and excretion of salt and fluid.

A key regulator of blood pressure is the balance between contraction and relaxation of the vascular smooth muscle cells (VSMC) in the arteries and arterioles. Vasoconstriction and vasodilation are mediated by an increase or decrease in intracellular calcium [Ca^{2+}] and/or by altering the sensitivity of the contractile muscle system to [Ca^{2+}]. Circulating and local hormones, mediators secreted by the sympathetic nerves and the vascular endothelium with its various functions control the tone of VSMC.

In addition to its role as a mechanical barrier, the vascular endothelium acts as a source of numerous potent mediators of the blood pressure. In an intact endothelium the balance between vasoconstriction and vasodilation tends to shift towards vasodilation (Fig 1). The en-

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Figure 1. Some factors affecting the balance between vasoconstriction and vasodilatation in blood vessels. In normal circumstances vasodilatation prevails (Born et al 1998).
dothelium releases vasodilators, such as nitric oxide (NO), prostacyclin (PGI₂) and endothelin-derived hyperpolarising factor (EDHF). However, vasoconstrictors are released by the endothelium, too. These include angiotensin II (ATII), endothelin (ET), thromboxane A₂ and some prostaglandins. Endothelium derived factors promote (ET and AT II) or inhibit (NO and PGI₂) proliferation and hypertrophy of the arterial VSMC.

2.3 Regulation of sodium excretion

In normally functioning kidneys increased arterial pressure increases Na and water excretion. Under normal conditions water and Na balance can be maintained because of the pressure-natriuresis mechanism (Fig 2). This so-called pressure natriuresis is believed to play a central role in the long-term regulation of the body fluid volumes and the arterial pressure (Coleman et al. 1972, Cowley 1992). If the intake of Na is high, the blood pressure will rise. This prevents from accumulation of Na. The hypotensive effect of salt restriction in essential hypertension was first described by Ambard & Beaujard in 1904 (for review see de Wardener 1990). It has been shown in a Na-loading test that significantly more hypertensive subjects than normotensive subjects were Na-sensitive. The degree of plasma renin activity (low, normal or high) did not predict the

Figure 2. Pressure-natriuresis curve and factors shifting the curve. The original data have been adapted from Guyton et al (1972).
response to Na (Weinberger et al. 1986). Other studies in severely Na-sensitive patients have re-
vealed that the rise in blood pressure occurring after an acute infusion of Na results in a slower
rate of urinary Na excretion (Fujita, et al. 1980, Hollenberg 1980). Tuomilehto et al. showed in
an epidemiological study that a high Na intake predicted mortality and risk for coronary heart
disease independently of other risk factors such as hypertension (Tuomilehto et al. 2001).

2.4 Renal physiology

Kidney is the main regulator of the fluid and electrolyte balance, and it also takes part in the
elimination/excretion of drugs. Structural overview of the nephron is shown in Fig 3. Furthermore,
the kidneys regulate the blood pressure through RAAS by producing renin. The secre-
tion of renin is affected by renal blood flow (RBF) which is essential for glomerular filtration.
The renal vessels are sympathetically innervated. RAAS also affects RBF. Renal autoregulation
maintains mean arterial blood pressure within the range of 80 to 180 mmHg, when renal func-
tion is intact.

Figure 3. Structural overview of the nephron. (adapted from O’Callaghan 2006)
2.5 Dietary electrolytes and hypertension

2.5.1 Sodium

The total sodium content of extracellular fluid is the main determinant of the extracellular volume. Sodium participates in the regulation of the acid-base balance and in the maintenance of the membrane potential of the cells. The human body contains about four moles (92 g) of sodium, approximately 40% of which is found in the skeleton. The highest salt intakes are usually associated with a diet high in processed foods (because of the high amount of dietary sodium added in processing) and the lowest intakes are associated with diets emphasising fresh fruits, vegetables and legumes (The National Findiet 2007 Survey).

The main dietary sources of sodium are foods and beverages containing sodium chloride. Grain and bakery products are the most significant source of added sodium, constituting 35% of the intake in Finland. In men meat products provide another 30%. Dairy products are responsible for another 10% of sodium and vegetables, fruits and berries, and products made of them are responsible for another 10% (The National Findiet 2007 Survey).

Sodium intake varies greatly between different cultures. The Intersalt study, in which more than 10,000 people from 32 cultures participated, revealed that the sodium intake, when estimated by 24-h urinary excretion of sodium, ranged from 0.2 mmol (4.6 mg) to 242 mmol (5.6 g) in different populations (Intersalt Cooperative Research Group 1988).

Considering the extensive variation in the patterns of physical activity and climatic exposure, a safe minimum intake of sodium is estimated to be 0.6 g / day (Finnish Nutrition Recommendations 2005). In Finland, the current recommendation for sodium intake is <2 g/d (The Finnish Hypertension Society 2005). In common diets these recommendations are considerably exceeded, even in the absence of sodium chloride (The National Findiet 2007 Survey).

2.5.1.1 Physiology of sodium

Sodium is mainly absorbed in the small intestine by active transport. The excretion of sodium is equal to sodium intake under steady-state condition, and the kidneys maintain the total body sodium at a constant level. Of the Na filtered in the glomeruli, only 1% is excreted in to the urine and 99% reabsorbed, mainly in the proximal tubule (Fig 4).

2.5.1.2 Sodium and hypertension

Although most studies have emphasised the harmful effects of a high dietary Na intake via the increase in the blood pressure, it has recently become evident that excess dietary Na also has other harmful effects on the cardiovascular system. There is a correlation between the urinary excretion of Na and left ventricular mass, and this is independent of a change in blood pressure (Du Cailar et al. 1992). A high intake of Na increases the cardiac mass in both SHR and normotensive controls, WKY rats, although the blood pressure in the WKY rat does not increase (Frohlich et al. 1993).

2.5.2 Potassium

Potassium is the principal intracellular cation. It participates in the regulation of the electrical excitability of nerve and muscle cells and the acid-base balance of the body. The human body contains about four moles (156 g) of K, of which 95% is exchangeable. The richest sources of K are fruits, vegetables and berries. In Finland women receive 32% and men 26% of their intake of K from these. Grain and dairy products are both responsible for 20% of daily intake. Beverages, mainly coffee, are the source of another 15% (Findiet 2007 Survey).
The daily K intake in adults usually ranges from 50 to 100 mmol (2–3.8 g) depending on food selection. In Finland the intake ranges from 3.2–4.2 g (Findiet 2007 Survey). The current Finnish recommendation for K intake is 3.5 g/day for men and 3.1 g/day for women (Finnish Hypertension Society 2005).

2.5.2.1 Physiology of potassium

Potassium is actively absorbed by the intestinal mucosa and more than 90% of ingested potassium is absorbed. Approximately 90% of the ingested K ends up in the urine. Potassium is freely filtered in the glomeruli and nearly completely reabsorbed in the proximal tubules (Fig 4). The distal convoluted tubule and the collecting duct secrete K into the urine (Guyton and Hall 2000).

The maintenance of K balance depends mainly on kidney function. The Na+/K+ATP-ase which is present in all cells maintains the extracellular concentration of K constant. Aldosterone mainly regulates the urinary secretion of K. In the distal tubules aldosterone stimulates the secretion of K and re-absorption of Na thus enhancing exchange between luminal Na and intracellular K (Guyton and Hall 2000).

A moderate increase in the dietary intake of K does not usually cause toxic effects; patients with renal insufficiency may make an exception. Intake of K rarely causes hyperkalaemia in normal conditions, but hyperkalaemia may occur as a consequence of massive K release following cell lysis or strenuous exercise (Guyton and Hall 2000).

2.5.2.2 Potassium and hypertension

Addison was the first to suggest in 1928 that K might lower the blood pressure. Since then numerous experimental, epidemiological and clinical studies have been performed. Nearly all of them show an inverse correlation between K intake and hypertension both in normotensive and hypertensive subjects (Whelton et al. 1997, He and MacGregor 1999). Increased intake of K may also prevent from strokes independently of its effects on the blood pressure both in epidemiological (Khaw and Barrett-Connor 1987, Cappuccio and MacGregor 1991, Ascherio et al. 1998) and experimental (Tobian 1986) studies.

The blood-pressure-lowering mechanisms of K seem to be multifactorial. This effect of K has been evident in salt-sensitive hypertension both in humans and animals (Karppanen 1991, Tobian 1997). This could be related to potassium-induced natriuresis and to decrease of the volume load (Mervaala et al. 1992). Other proposed mechanisms are reduced sympathetic activity (Fujita and Sato 1992), decreased pressor response (Campbell and Schmitz 1978), and improvement of endothelial function (Mervaala et al. 1994, Tolvanen et al. 1998).
2.5.3 Magnesium

2.5.3.1 Physiology of magnesium
Magnesium is the fourth most abundant cation in the body and the second most plentiful cation in the intracellular fluid. Mg serves as a cofactor for about 300 cellular enzymes many of which are involved in energy metabolism. Mg also participates in protein and nucleic acid synthesis within the cell. In the cardiovascular system Mg regulates contractile proteins and modulates the transmembrane transport of Ca, Na and K (for review see Saris et al. 2000).

The adult body contains approximately one mole (21–28g) of Mg which is distributed approximately equally between the soft tissues and the skeleton. In plasma Mg can be found in three fractions: an ultrafiltrable fraction of ionised Mg (70–80%), complex-bound Mg (1–2%) and a protein-bound non-ultrafiltrable fraction (20–30%) (Lewenstam 1993). Only about one per cent of the total body Mg is ionised and physiologically active. The distribution of extracellular Mg is shown in Fig 5.

![Diagram](image)

Figure 5. Diagram demonstrating mechanisms of magnesium homeostasis in humans. Extracellular (serum) magnesium concentrations are regulated through gastrointestinal absorption, renal excretion and exchange from bony compartments. Serum magnesium comprises three major fractions of magnesium, 1) protein-bound magnesium, 2) complexed magnesium and 3) ionised magnesium (Mg\(^{2+}\)). Extracellular Mg\(^{2+}\) is freely exchanged with intracellular Mg\(^{2+}\) (adapted from Touyz 2004).
In Finland the mean daily intake of magnesium is about 400 mg in men and 320 mg in women (Findiet 2007 Survey). About one third of daily intake of Mg becomes from grain- and bakery products; the rye bread is the most important source (Findiet 2007 Survey).

About 30% of dietary Mg is absorbed in the small intestine, but if the intake is decreased absorption can be increased. Magnesium is absorbed actively through the intestinal mucosa. In gastrointestinal disorders such as intestinal malabsorption, steatorrhoea and chronic pancreatic insufficiency the intestinal absorption may be disturbed.

Approximately 75% of the total plasma Mg is filtered through the glomerular membrane. 15% is reabsorbed in the proximal tubules, mostly in the thick ascending loop of Henle. In normal conditions, only 5% of the filtered Mg is excreted into the urine (Fig 4). Hypercalcaemia, hypercalciuria, excessive Na intake and several drugs, especially diuretics, cisplatin, CsA and gentamicin cause Mg loss into the urine (Guyton and Hall 2000).

The understanding of the endocrine factors that control Mg homeostasis is incomplete (Saris et al. 2000). The kidney, gastrointestinal tract and bone closely regulate the cellular availability of Mg, the kidney being the most important organ responsible for the regulation of body Mg (Quamme 1986).

The essential role of Mg in modulating transport functions and receptors, signal transduction, energy metabolism, enzyme activities and nucleic acid and protein synthesis makes Mg deficit a potential health hazard (Saris et al. 2000).

Mg depletion can roughly be divided into four categories: gastrointestinal abnormalities associated with malabsorption or excessive fluid and electrolyte losses, renal dysfunction with defects in Mg reabsorption, general malnutrition and alcoholism, and iatrogenic causes such as use of drugs that cause urinary Mg wasting. Anorexia, nausea, vomiting, lethargy and weakness are typical early symptoms of Mg deficiency. In severe deficiency paraesthesiae, muscular cramps, irritability, decreased attention span and mental confusion can be seen (Guyton and Hall 2000).

There is no clear evidence that high oral intake of Mg in healthy persons would be harmful except for diarrhoea which is more or less an unpleasant symptom. Impaired renal function in association with high intake of Mg may cause hypermagnesaemia.

2.5.3.2 Magnesium and hypertension

Hypertension and low magnesium intake have been linked together. Epidemiological and experimental studies support the role of Mg deficiency in the pathogenesis of hypertension (Touyz et al. 1992, Resnick et al. 1997, Kawano et al. 1998, Jee et al. 2002). The NHANES-study (Cappuccio et al. 1985, Hajjar et al. 2001) demonstrates reverse relationships between dietary Mg intake as well as serum Mg concentration and blood pressure. However, the evidence from epidemiological studies is inconsistent.

There is increased evidence to suggest that dietary Mg deficiency plays an important role in the pathogenesis of ischaemic heart disease, sudden cardiac arrhythmia, sudden
cardiac death, pre-eclampsia/eclampsia and hypertension (Arsenian 1993, Altura and Altura 1995).

The exact mechanism of the molecular contractile action of Mg is not known, but Mg probably influences ionised calcium [Ca<sup>2+</sup>] which is a major determinant of vascular smooth muscle cell (VSMC) contraction. In VSMC Mg inhibits transmembrane calcium transport and calcium entry, decreases the contractile action of vasoactive agents, or intracellularly as a calcium antagonist modulates the vasoconstrictive action of [Ca<sup>2+</sup>] (Iseri and French 1984, McHugh and Beech 1996, Nakajima et al. 1997, Yoshimura et al. 1997).

In the VSMC, the Ca influx leading to muscle contractions occurs through voltage-operated and receptor-operated channels. Mg can antagonise the entry of vascular Ca (Altura and Altura 1991). The Mg-induced vasodilatation seems to be in vitro partly mediated by both endothelium-dependent and -independent mechanisms (Laurant and Berthelot 1994).

2.6 Immunosuppressive therapy after organ transplantation

The human body identifies its own cells through major histocompatibility complex (MHC I) human leukocyte antigen (HLA class I) molecules which are expressed practically by all cells. When a foreign organ is transplanted, the immune system of the body reacts against foreign MHC I molecules. This reaction is referred to as rejection.

After organ transplantation life-long immunosuppression is necessary to prevent from and to treat rejection. The main target of immunosuppressive therapy is to inhibit the lymphocyte-mediated specific immune response. In addition to organ transplantation, immunosuppressive treatment is used also in autoimmune diseases such as rheumatoid arthritis, psoriasis, glomerulonephritis, vasculitis, ulcerative colitis and Crohn’s disease. Because immunosuppressive treatment attenuates natural immunity, opportunistic infections and the risk of malignancies are therefore side-effects of most of these drugs.

After organ transplantation maintenance of immunosuppression is achieved with combination of immunosuppressive agents. Combination therapy aims to minimise the side-effects of each drug used (Denton et al. 1999). Calcineurin inhibitors, CsA and tacrolimus are cornerstones in immunosuppressive regimens and they are combined with corticosteroids and antiproliferative agents such as azathioprine or mycophenolate mofetil (Krensky et al. 2006). Mechanism of action and side-effects of the most commonly used immunosuppressive drugs are listed in Table 2.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Side-effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine-A</td>
<td>Calcineurin inhibition, IL-2 production inhibition</td>
<td>Hypertension, nephrotoxicity, dyslipidaemia, hyperglycaemia, increased risk of opportunistic infections and malignancies</td>
<td>Schreiber and Crabtree 1992</td>
</tr>
<tr>
<td>Tacrolimus (FK506)</td>
<td>Calcineurin inhibition, IL-2 production inhibition</td>
<td>Hypertension, nephrotoxicity, dyslipidaemia, hyperglycaemia, increased risk of opportunistic infections and malignancies</td>
<td>Schreiber and Crabtree 1992</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>Inhibition of transcription of cytokine genes</td>
<td>Growth retardation, hypertension, osteopenia, poor wound healing, increased risk of infections, cataracts, hyperglycaemia, obesity, dyslipidaemia</td>
<td>Krensky et al. 2006</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Inhibition of purine synthesis, DNA and RNA synthesis</td>
<td>Bone marrow suppression</td>
<td>Chan et al. 1987</td>
</tr>
<tr>
<td>Mykophenolate mofetil (MMF)</td>
<td>Active metabolite of MMF inhibits denovo pathway of purine synthesis (selective to B- and T-lymphocytes)</td>
<td>Leucopenia, diarrhoea, vomiting, increased risk of CMV sepsis</td>
<td>Fulton and Markham 1996</td>
</tr>
<tr>
<td>Sirolimus (Rapamycin)</td>
<td>Inhibition of IL-2 induced cell cycle progression</td>
<td>Hyperlipidemia, anaemia, leucopenia, thrombocytopenia, fever, hypokalaemia or hyperkalaemia and gastrointestinal disorders</td>
<td>Kuo et al.1992; Murgia et al. 1996, Hong and Kahan 2000</td>
</tr>
<tr>
<td>Muronabi-CD3 (OKT3) (monoclonal antibody)</td>
<td>Diminishes the number of CD-3 positive lymphocytes</td>
<td>“Cytokine release syndrome” (high fever, headache, tremor, nausea or vomiting, diarrhoea, abdominal pain, malaise, muscle and joint pains and generalised weakness)</td>
<td>Hong and Kahan 1999</td>
</tr>
<tr>
<td>Daclizumab, Basiliximab</td>
<td>Inhibits IL-2 -mediated T-cell activation</td>
<td>Anaphylactic reactions, lymphoproliferative disorders, opportunistic infections</td>
<td>Hong and Kahan 1999</td>
</tr>
</tbody>
</table>
2.6.1 Cyclosporine-A

2.6.1.1 Short history

The comprehension of the significance of being able to avoid undesirable reactions of the immune system after organ transplantation began to grow in the early 1960’s and the need to develop effective medications emerged. New effective agents were searched from nature as well. Soil samples were randomly collected and screened. In 1969 Hans Peter Frey brought a soil sample from a holiday trip in Norway (Svarstad et al. 2000). In routine tests a fungus, *Tolypocladium inflatum*, was extracted. It proved to inhibit the growth of other fungi and after exhaustive chemical analyses of the active substance, CsA was identified (Borel 2002).

CsA revolutionarily improved the short-term results of organ transplantation by significantly reducing rejection. In animal trials preceding the clinical use of CsA, its main side-effects, nephrotoxicity and hypertension, did not occur. So it was a surprise, when after a short period of clinical use Calne et al. noted nephrotoxicity in renal transplantation patients after CsA therapy (Calne et al. 1978). At first it was speculated that the noted nephrotoxicity would more likely be due to rejection (Starzl et al. 1980), but nephrotoxicity as a side-effect of CsA was soon confirmed in other transplant patients (Klintmalm et al. 1981, Nizze et al. 1988). When the indications of CsA use were widened to autoimmune diseases such as rheumatoid arthritis, psoriasis and uveitis, nephrotoxicity and hypertension were also seen in these patients (Palestine et al. 1986, Magina et al. 2005). At that time the doses of CsA were higher than those used today. Nowadays the combination of immunosuppressants with different mechanisms of action enables use of a lower dose of each drug and so milder side-effects. Active research is going on to develop new immunosuppressants with still fewer side-effects.

2.6.1.2 Pharmacokinetics and pharmacodynamics of CsA

Cyclosporine A is a cyclic endecapeptide. It is the first T-lymphocyte selective drug to inhibit lymphocyte function without concomitant myelosuppression. CsA is a calcineurin inhibitor. In a T-lymphocyte, CsA binds to cyclophilin resulting in subsequent interaction with calcineurin to block the activity of this phosphatase. Calcineurin-catalysed dephosphorylation is required for induction of a number of cytokine genes including that for interleukin-2 (IL-2), a prototypic T-cell growth and differentiation factor (Krensky et al. 2006) (Fig 6).

CsA is mainly metabolised in the liver by CYP3A (Krensky et al. 2006). More than twenty metabolites have been identified but all of them have both reduced biological activity and toxicity than the parent drug. Despite the fact that CsA has improved the results after organ transplantations in a revolutionary manner, its clinical use is complicated because of varying bioavailability, a narrow therapeutic window and complex interactions with other drugs (for review see Krensky et al. 2006).
In clinical use, CsA can be administered intravenously or orally. In rats, with the dose of 5–10 mg of CsA, subcutaneous dosing has proved to be the most reproducible and steady plasma levels are gained with little variation over a 24-h period (Wassef et al. 1985).

After solitary organ transplantations, calcineurin inhibitors are often combined to other immunosuppressive drugs such as corticosteroids and antiproliferative agents such as azathioprine or mycophenolate mofetil.

In organ transplantation the initial dose of CsA is often fairly high for 1–2 weeks, after which the dose is gradually decreased to the maintenance dose. The protocols for immunosuppressive drug administration vary between centres. In autoimmune disease, the doses are usually lower and the use of CsA is often temporary or intermittent.
2.6.1.3 CsA toxicity

2.6.1.3.1 Hypertension

CsA is associated with an increased incidence and severity of hypertension. The arterial blood pressure rises within days to weeks after the beginning of CsA administration (Sturrock et al. 1995). Even after a single dose of CsA in healthy volunteers the blood pressure will rise (Hansen et al. 1997). Lack of circadian rhythm characterises CsA-induced hypertension (Textor et al. 1994, Taler et al. 1995). The absence or even reversal of the normal nocturnal fall in the blood pressure in hypertensive patients predisposes them to rapid development of hypertensive end-organ injury including left ventricular hypertrophy, retinal and intracranial haemorrhage and even stroke (Textor et al. 1994). CsA does not cause hypertension only in patients receiving CsA after transplantation but also when CsA is used for the treatment of autoimmune diseases (for review see Porter et al. 1990). However, other factors may also increase the blood pressure; the underlying disease, rejection of the transplanted organ and concomitant medication. The combination of CsA and corticosteroids further increases the risk of hypertension.

The natural course of post transplant hypertension is not known because patients are monitored carefully, and in the case of hypertension, they are early treated with anti-hypertensive medication.

Prevalence

Patients receiving heart or bone marrow transplantation are often normotensive before transplantation; the underlying disease, terminating at transplantation, is not particularly associated with hypertension. In these patients the prevalence of pre-transplant hypertension varies between 5–10% (for review see Taler et al. 1999). After cardiac transplantation and the onset of CsA therapy, the prevalence of hypertension has been reported to be as high as 70–100% and after bone marrow transplantation 33–60% (Emery et al. 1986, Taler et al. 1999). Patients in need for a liver transplant suffer from liver insufficiency, vasodilatation and hypotension which after liver transplantation changes to CsA-induced vasoconstriction. Their blood pressure commonly increases by 40–50 mmHg over several weeks; first normotension is achieved but then hypertension often develops (Textor 1993).

Renal transplant patients are especially challenging because approximately half of them are hypertensive before transplantation. After transplantation up to 90% become hypertensive if treated with CsA (Taler et al. 1999). On the other hand, complications of transplantation such as rejection and renal artery stenosis can also increase hypertension and nephrotoxicity.
Pathophysiological characteristics

Increased sympathetic activity
The activation of the sympathetic nervous system has been proposed as one of the mechanisms of CsA toxicity although the evidence is contradictory. In one study cardiac transplant recipients as well as patients suffering from myasthenia gravis showed an elevated blood pressure and increased sympathetic activation related to CsA treatment (Scherrer et al. 1990). However, later studies have not confirmed these findings (Kaye et al. 1993, Stein et al. 1995, Carvalho et al. 1999). In experimental studies, the results are controversial, too. Renal denervation or adrenergic blocking medications inhibit CsA-induced sympathetic nerve action on the kidney (Murray and Paller 1986). A number of experimental studies have shown that even after renal denervation renal function decreases (for review, see de Mattos et al. 1996).

Renin-angiotensin system activation
The renin-angiotensin-aldosterone system (RAAS) is important in regulating the blood pressure, body volume homeostasis and homeostasis of Na. Factors that substantially decrease the blood pressure or decrease peripheral resistance activate release of renin from the kidneys. Renin, as an enzyme, in turn activates a pathway that results in formation of angiotensin II. AT II is an extremely powerful vasoconstrictor. The cardiovascular effects of angiotensin II are predominantly mediated via angiotensin II type 1 (AT_1) receptors. These effects include vasoconstriction, the production of endothelin and superoxide, sympathetic activation, aldosterone release, Na and water retention and cellular growth. In an in vitro study Avdonin et al. showed that in human VSMC CsA increased the number of AT_1 receptors (Avdonin et al 1999)

The effects of CsA on RAAS in man are contradictory, increased, normal and even low plasma renin activity has been reported (Myers et al. 1988) (Bantle et al. 1987) (Mason et al. 1991). It has been speculated that CsA induces the activation of local RAAS. This is not necessarily seen as a change in plasma renin activity (for review, see Mason et al. 1991, Young et al. 1995). The activation of RAAS induced by CsA is a consistent finding in rats (Mason et al. 1991).

Nitric oxide
Nitric oxide (NO) is a highly reactive gas with a short half-life. NO is synthesised within the endothelial cells from arginine by the enzyme NO synthetase (NOS). NO, earlier called endothelium-derived relaxing factor, (EDRF) (Furchgott and Zawadzki 1980) is a potent endogenous vasodilator (Ignarro et al. 1981).

Normal endothelium constantly releases low levels of NO and additional NO is released in response to physiological and pathological stimuli. NO is present in all endothelial tissue from large arteries to the smallest capillary networks.

CsA may prevent normal vascular relaxation by interfering with the L-arginine-NO path-
way (Bloom et al. 1995). When L-arginine, the precursor of NO, was given to young cardiac transplantation patients receiving CsA, a transient decrease in blood pressure was detected (Gomez et al. 2001). In renal transplant patients, L-arginine increased renal plasma flow (RPF), glomerular filtration rate (GFR) and natriuresis (Andres et al. 1997). In CsA treated rats L-arginine inhibited a rise in the mean arterial pressure and a decrease of nitrate/nitrite and cGMP in urine (Oriji and Keiser 1998). Experimentally CsA can impair either NO production (Vaziri et al. 1998) or release (Sudhir et al. 1994).

Oxidative stress (ROS formation)
It has been proposed that CsA causes hypoxia and thus free radical formation in the kidney (Zhong et al. 1998). Recent studies suggest that the formation of reactive oxygen species (ROS) may play an important role in the pathogenesis of CsA toxicity (Nishiyama et al. 2003, Louhelainen et al. 2006). Buetler has speculated that in studies in which CsA has been used in therapeutic doses alterations in Ca²⁺ homeostasis leading to smooth muscle cell contraction can be detected. On the other hand the free radical formation seems to need 10–100 times greater concentration of CsA than that toxic to humans. However, local generation of free radicals can occur, but current techniques of free radical formation may be too insensitive to detect it (Buetler et al. 2000)

Endothelin
Endothelial cells mainly synthesise endothelin-1 (ET-1). It is a potent vasoconstrictor and induces fibrosis and vascular as well as cardiac hypertrophy.
ET-1 levels have shown to increase after CsA-administration in renal transplant patients (Cauduro et al. 2004). CsA induces ET-1 synthesis in cultured human endothelial cells (Bunchman and Brookshire 1991). Animal studies suggest that ET-1 may participate in the development of hypertension during CsA treatment (Bartholomeusz et al. 1996).

Other suggested mechanisms
Other mechanisms regulating the vascular tone or renal function have also been studied as possible participants in CsA-induced hypertension. The cardiovascular effects may be related to reduced renal production of prostacyclin (Textor et al. 1992) or dopamine (Pestana et al. 1995), increased production of thromboxane (Benigni et al. 1988) and leukotriene (Butterly et al. 2000). Hypertension may exist without concurrent depression of glomerular filtration rate (Bennett and Porter 1988). It has also been reported that oxidative stress and the platelet activating factor (PAF) might also participate in CsA-induced hypertension.

The mechanisms by which CsA induces hypertension and nephrotoxicity mainly result from the vasoconstriction secondary to endothelial disorders (for review, see Rodicio 2000) and seem to be multifactorial.
2.6.1.3.2 Nephrotoxicity

CsA-induced nephrotoxicity covers a wide spectrum from mild reversible renal insufficiency to end-stage renal disease. CsA causes acute functional and chronic structural nephrotoxicity. CsA-induced renal side-effects can also be classified as tubular or vascular (Mason 1990). Recipients of non-renal organ transplants with chronic renal failure have been shown to have a mortality risk 4.55 times higher than their counterparts with normal renal function (Ojo et al. 2003).

Functional

Vasoconstriction

Functional changes are more common, dose-dependent and may be reversible after drug withdrawal. They are related to an imbalance between vasodilatation and vasoconstriction and/or tubular toxicity. Vasoconstriction occurs in the afferent arteriole, but also in the adjacent small arteries including the glomerular tuft (for review, see Campistol and Sacks 2000). Intrarenal vasoconstriction and increased renal vascular resistance (RVR) lead to a decrease in renal blood flow (RBF) and the glomerular filtration rate (GFR). The vasoconstriction is thought to maintain low-grade intrarenal ischaemia, which is supposed to lead to cycles of injury and repair in regions vulnerable to hypoxia (for review, see Rezzani 2004).

Even a single oral dose of CsA (10 mg/kg) to healthy volunteers decreased the GFR (Hansen et al. 1997). In the rat, renal vasoconstriction has been shown by Murray et al. who gave CsA to conscious rats; RVR increased and RBF decreased (Murray et al. 1985). The exact mechanism of renal vasospasm is not completely understood but practically the same pathophysiological mechanisms as in CsA-induced hypertension have been widely investigated; sympathetic activation, activation/inhibition of RAAS, decreased production of vasodilatory agents and/or increased production of vasoconstrictive agents (for review, see Campistol and Sacks 2000).

Tubular functional

In tubular dysfunction solute transport alterations such as a decrease in Mg reabsorption, a decrease in secretion of K, protons (H\(^+\)), uric acid and increased reabsorption of sodium are seen after administration of CsA (for reviews, see Mason 1990, Olyaei et al. 1999).

Structural

Vasoconstriction and direct endothelial cell damage may lead to necrosis of an endothelial cell and then to hyalinosis (for review, see Rezzani 2004). Hyalinisation may obliterate the arteries/arterioles and further decrease the renal and glomerular blood flow. Obliterated arteries/arterioles may increase the renal vascular resistance and cause chronic ischaemia in the renal tubuli. This may partly cause structural tubulointerstitial lesions induced by CsA. Occlusion
or obliteration of the afferent arteriole can also lead to glomerular collapse followed later by glomerulosclerosis (for review, see Mason 1990). Functional and histological changes may often be dissociated and do not necessarily occur chronologically (Davies et al. 2000).

Tubuli
Structural changes more rarely occur, they are usually irreversible and serious once established. The classical morphological lesions related to CsA toxicity in the tubuli include dilatation, calcification, atrophy and isometric vacuolisation and necrosis (Mihatsch et al. 1988).

Direct tubular toxicity may occur independently of haemodynamic changes (Carvalho da Costa 2003). Signs of tubulointestinal injury without evidence of vascular effects in kidney transplant patients have been reported (Benigni et al. 1999).

Arterioles
Vascular changes are mostly confined to the afferent arteriole. The damage to the endothelial cells and smooth muscle cells may lead to occlusion and the obliteration of the artery. Collapse and ultimately sclerosis of the glomerulus may develop (for review, see Mason 1990). Vasoconstriction may also lead to tubular lesions and interstitial scars (Mihatsch et al. 1995). In a short time after the initiation of treatment, CsA may give rise to arteriolar vacuolisation and even necrosis of the endothelial cells can follow. Later fibrin and platelet thrombi cover the bare basement membrane. Simultaneously, plasma protein insudate in the vascular wall forms nodular protein deposits which narrow the vascular lumen. This process is often called hyalinosis (for review, see Davies et al. 2000). Also intimal thickening and narrowing of the vascular lumen due to deposition of amorphous materials rich in acid proteoglycans have been described as a second form of CsA-induced arteriolopathy (for review, see Davies et al. 2000).

Glomeruli
CsA induced glomerular changes have been considered a consequence of arteriolar lesions in the glomerulus. Small glomeruli and wrinkling of the glomerular basement membrane (GBM) represent minor changes (Morozumi 2004). The mesangium may be destroyed and a thickening of GBM can often be seen. More severe changes include focal segmental sclerosis, fibrin thrombi inside the capillary tuft and collapse of the glomerulus and global glomerular sclerosis (Lewis et al. 1994). The obliteration of the afferent arteriole results in localised ischaemia with glomerular collapse followed by glomerulosclerosis (for review, see Mason 1990).

Interstitium
In the renal cortex, irregular foci of striped fibrosis can be seen. Tubular atrophy is often found adjacent to fibrosis. Striped fibrosis, oedema and scarring coexist with inflammation. Local ischaemia with patchy or striped fibrosis occurs.
It has been emphasised that in a kidney allograft biopsy a finding of interstitial fibrosis without arteriolar changes does not confirm the diagnosis of CsA nephrotoxicity (Bennett et al. 1994). In renal allografts several other factors in addition to CsA can produce or contribute to fibrosis; ischaemia, hypertension, increased ureteral pressure, glomerular diseases and rejection (for review, see Racusen et al. 2002). Renal histological changes can in experimental animals be minimal, non-specific or even absent although renal dysfunction is remarkable (Mihatsch et al. 1988).

Hypertension and nephrotoxicity are often found in the same patient, but they do not always develop at the same rate. In the study by Falkenhain et al. histological changes in the kidney and an increase of the blood pressure did not correlate (Falkenhain et al. 1996). Several risk factors are mentioned: functional impairment pre-treatment, high CsA-dose and excessive blood concentrations of CsA, age, hypertension and co-medication with other nephrotoxic drugs (for review, see Vercauteren et al. 1998). Direct tubular toxicity may also occur independently of haemodynamic changes (Carvalho da Costa et al. 2003). Evidence of tubulointestinal injury independently of vascular effects in kidney transplant patients has been reported (Benigni et al. 1999).

**Nephrotoxicity after renal transplantation**

CsA was used clinically for the first time by Calne in 1978 and functional nephrotoxicity was detected in all of his seven patients (Calne et al. 1978). The detection of CsA toxicity in a renal allograft is challenging. Histological changes may also be due to rejection, ischaemia of the allograft, infection and de novo glomerular disease or other causes.

In a longitudinal study renal histology was investigated 1 and 2 weeks, and 1, 3, 6, and 12 months after renal transplantation, and then annually for ten years (Nankivell et al. 2004). All patients (n=98) had diabetes and were treated with CsA. Functional nephrotoxicity was defined as an acute increase in serum creatinine by more than 25% above the baseline, and acute rejection was excluded by biopsy. The most sensitive indicator of CsA toxicity was arteriolar hyalinosis, which could be predicted by functional CsA toxicity and the CsA dose. A threshold CsA dose more than 5 mg/kg/d predicted worsening of arteriolar hyalinosis on consecutive histology. After ten years of treatment striped fibrosis, progressive arteriolar hyalinosis and ischaemic glomerulosclerosis were practically universal.

In another study, 10 out of 22 patients had irregular foci of or stripes of tubular atrophy accompanied by fibrosis. In four patients focal segmental sclerosis and ischaemic collapse were documented (Benigni et al. 1999).

**Nephrotoxicity after liver transplantation**

An early report by Klintmalm (Klintmalm et al. 1981) showed increased creatinine levels in 50% (n=12) of orthotopic liver transplantation (OLTx) patients within 2–3 weeks after OLTx.
The CsA dose was relatively high (16±3 mg/kg/d), and with the decrease of the dose to 9±2 mg/kg/d the patients’ kidney function returned to normal. The majority of liver transplantation patients receiving CsA develop some degree of renal insufficiency. In a long-term follow-up 80% of liver recipients had mild or moderate renal insufficiency (serum creatinine > 125 μmol/l) (n=136, median follow-up 7.75 years, range 5–14.5 years). In another study 4.9% had chronic renal failure (serum creatinine > 220 μmol/l) and 5.4% had end-stage renal disease (ESRD) (n=834, follow-up six months) (Gonwa et al. 2001).

The incidence of severe chronic renal failure and end-stage renal disease in patients who survived one year or more was 4% and 1.9%, respectively (Fisher et al. 1998). Chronic renal failure is an important cause of morbidity and is associated with a high mortality (Fisher et al. 1998).

In a recent study Pillebout and co-workers have underlined that OLTx recipients with chronic renal failure should not be regarded as suffering from calcineurin-inhibitor toxicity without thorough investigations including renal histology (Pillebout et al. 2005). Renal histological lesions on average 4.8±0.7 years (range 0.5–11.6 years) after OLTx in 26 patients suffering from renal failure revealed the aetiology of renal involvement to be multifactorial; in addition to CNI-induced changes also lesions related to e.g. diabetes were detected.

**Nephrotoxicity after heart and lung transplantation**

Goldstein et al. reported that 6.5% of 296 heart transplant recipients developed ESRD requiring chronic haemodialysis (Goldstein et al. 1997). Myers and co-workers were the first to report CsA-induced nephrotoxicity in other than kidney transplant patients (Myers et al. 1984). Both functional and structural changes were detected. Of the 32 heart transplant recipients treated for more than one year with CsA, ESRD developed in two patients. Five CsA-treated patients in whom GFR was moderately or severely depressed underwent renal biopsy. Focal and sometimes segmental sclerosis was detected and patchy tubular atrophy and degenerative tubular changes seen.

Nizze et al. studied 41 kidneys at autopsy of heart transplant patients who had received CsA (Nizze et al. 1988). In most patients, the dose had been ≤10 mg/kg/d, and the mean survival time after transplantation had been 84±215 days. The striped or focal fibrosis was always accompanied by tubular atrophy. Normal renal structure occurred adjacent to regions containing interstitial fibrosis and atrophic tubules. CsA-arteriolopathy was always focal and only a small number of arterioles had been affected. It was often found near the vascular pole of the glomerulus.

In heart transplant patients given CsA (5±2 mg/kg/d) for more than two years, 30% of glomeruli showed global and 10% segmental sclerosis, when a renal biopsy was performed and tubular atrophy and striped or sometimes diffuse interstitial fibrosis was found. CsA arteriolopathy was again detected (Bertani et al. 1991).
In a post-mortem histological study in heart and liver (n=13 and 3, respectively) transplantation patients 73% had interstitial fibrosis and tubular atrophy and a majority had fibroelastic hyperplasia of the arteries. Renal cortical infarctions were detected in four heart transplant recipients (Falkenhain et al. 1996).

Patients developing ESRD after cardiac transplantation are three times more likely to die than those who do not need haemodialysis (Goldstein et al. 1997).

**Nephrotoxicity after bone marrow transplantation**

In a retrospective analysis of bone marrow transplant recipients morphological evidence of CsA-associated nephropathy was identified in 67% of renal biopsy (n=12) or autopsy (n=12) specimens (Dieterle et al. 1990). In another post-mortem study of the kidneys of bone marrow transplant recipients (n=100) fibrin and/or platelet thrombi in arterioles/glomeruli, glomerular collapse, tubular atrophy, interstitial fibrosis and CsA-arteriolopathy were more common in the CsA group than in the control group (Nizze et al. 1988).

Although uncommon and generally observed in bone marrow transplant recipients only, acute nephrotoxicity can manifest as de novo or recurrent haemolytic uraemia syndrome (for review, see Olyaei et al. 1999).

**Nephrotoxicity in patients with autoimmune disease**

Functional nephrotoxicity was evaluated in a meta-analysis (Vercauteren et al. 1998). Controlled, randomised trials with a treatment period of two months or more were included. The mean dose of CsA was 4.8 mg/kg/d and the mean duration of the treatment 6.4 months. Nephrotoxicity was specified as an increase of the serum creatinine level by 50% or more above the baseline at least once during the study period. In the CsA-treated group, 21.5% (n=576) showed such a rise in serum creatinine in comparison with 1.3% in the control group.

The most frequent lesions in patients with autoimmune disease are interstitial fibrosis, tubular atrophy and hyalinosis (Mihatsch et al. 1994). When pre- and post-treatment renal biopsies have been taken, de novo interstitial fibrosis and tubular atrophy have been detected in 40–65% of patients after one-year treatment with CsA using a daily dose ≤5 mg/kg (Vercauteren et al. 1998). The main weakness in many clinical studies is the absence of renal histology. There are few reports on renal structure and function having been studied concurrently.

In conclusion, CsA-induced hypertension and nephrotoxicity are mainly caused by the vasoconstriction secondary to endothelial disorders (for review, see Rodicio 2000).

**Magnesium loss**

Thompson et al. were the first who reported the association between CsA-therapy and hypomagnesaemia and Mg loss to urine in bone marrow transplant patients (Thompson 1984,
June et al. 1985). They suggested that hypertension and hypomagnesaemia in CsA-treated patients might be linked together (June et al. 1986).

Hypomagnesaemia has been proposed to be both the cause and consequence in CsA nephrotoxicity both in experimental models and in humans. It has been assumed that normal renal tubular function may protect from Mg depletion and Mg deficiency, but CsA may interfere with the capacity to reduce Mg excretion when the intake of Mg is low (Rob et al. 1994). On the other hand, low serum concentrations of Mg²⁺ in renal transplant patients have been reported (Mazzaferro et al. 2002). It has been also suggested that renal failure after transplantation plays an antagonistic role to CsA-induced Mg wasting (Mazzaferro et al. 2002).

The development of hypomagnesaemia and inappropriately increased urinary excretion of Mg can be noted during the second or third week after renal transplantation in CsA treated but not in azathioprine treated patients (Barton et al. 1987). In Wistar rats, with doses of CsA of both 10 mg and 20 mg/kg/d p.o., hypomagnesaemia developed in 7 days when measured as plasma Mg concentration. Despite significantly lower plasma Mg concentrations, the excretion of magnesium to urine was higher in the CsA groups (Wong and Dirks 1988).

In some animal studies the investigators, however, have achieved contradictory results. Although CsA induced urinary Mg wasting and low serum Mg concentrations were observed, Mg content was increased in muscle, liver and kidney but not in bone after 3 or 4 weeks of treatment with CsA (Barton et al. 1989, Nozue et al. 1993). As an explanation for the difference from the general pattern, the authors suggested a shift of Mg from plasma to tissue compartments. Others have not confirmed these results. Rob et al. 1996 suggested that increased Mg in tissues could be due to a methodological cause; in many studies atomic absorption spectrophotometry is used, but Nozue et al. used a colorimetric technique for Mg measurement (Rob et al. 1996). The finding that CsA did not cause any change of the Mg content of the femur has been criticised because the intake of magnesium was 5-fold of the normal requirement and probably high enough to exceed urinary loss (Rob et al. 1996).

2.6.1.3.3 Experimental models for CsA nephrotoxicity

Normotensive rats on normal laboratory diet

Renal functional and structural toxicity of CsA have been tried to produce experimentally either by using high to very high doses of CsA or by combining some other noxa with the CsA treatment. When CsA was given at the dose of 15 mg/kg/day i.m. for 4 weeks to unilaterally nephrectomised rats in which the remaining kidney had been subjected to 45 min of warm ischaemia, diminished GRF was reduced by the nearly a half (Provoost et al. 1986). When normotensive rat strains received CsA at doses of 5–100mg/kg via various routes of administration (p.o., i.v., s.c. or i.p.,) and were treated for 5–91 days (Mihatsch et al. 1986), tubular lesions could be seen when the dose of CsA was more than 10 mg/kg/d. No glomerular or vascular lesions
were noted. In another long-term study, after 5 months treatment with a high dose of CsA (40 mg/kg/48hr) some increase in mesangial matrix and perivascular changes could be seen, but no arteriolar or arterial changes were detected (Bertani et al. 1987). Potentially nephrotoxic drugs (gentamicin, amphotericin B or ketoconazole) given with CsA (20 mg/kg/d) did not cause any more pathological changes than those mild changes seen with CsA alone (Ryffel et al. 1986).

Normotensive rats on low sodium diet
In a rat model, in which normotensive uninephrectomised rats were exposed to extreme Na depletion before and during CsA administration, GFR was reduced. Histologically, striped tubulointestinal fibrosis and hyaline arteriolopathy, were induced in the kidneys (Shihab et al. 2003). However, no glomerular changes were detected. In this model hypertension did not develop, and the rats became rather hypotensive (Elzinga et al. 1993). Many studies on the mechanisms of CsA-induced nephrotoxicity have been conducted by using this model. Rats in a laboratory environment normally receive a diet, in which the sodium content is quite low. Still lower sodium content may alter the mechanisms of blood pressure regulation and the homeostatic mechanisms in hypertension and hypotension are probably different. Some studies of CsA toxicity using this model are summarised in table 3.
### Effects of CsA on blood pressure and renal histology on low dietary sodium in experimental studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sodium diet (% of the dry weight of the chow)</th>
<th>Dose of CsA (mg/kg/d)</th>
<th>Duration (d)</th>
<th>Change in SBP</th>
<th>Tubuli and interstitium</th>
<th>Arteriole</th>
<th>CsA concentration (mg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sprague-Dawley</td>
<td>0.05%</td>
<td>2.5 s.c.</td>
<td>21</td>
<td>↔</td>
<td>Mild proximal tubular collapse, isometric microvacuolisation. Intratubular calcification</td>
<td>↔</td>
<td>702±39</td>
<td>Gardner et al. 1996</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>0.05%</td>
<td>7.5 s.c.</td>
<td>21</td>
<td>↔</td>
<td>Proximal tubular collapse, isometric microvacuolisation. Intratubular calcification</td>
<td>↔</td>
<td>1849±131</td>
<td></td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>0.05%</td>
<td>15 s.c.</td>
<td>35</td>
<td>↔</td>
<td>Tubulo-interstitial fibrosis, prominent tubular calcification, tubular atrophy</td>
<td>VSMC of afferent arteriole were replaced by a PAS-positive material</td>
<td>5050±470</td>
<td>Franceschini et al. 1998</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>0.05%</td>
<td>15 s.c.</td>
<td>28</td>
<td>↔</td>
<td>Tubular dilatation, atrophy, thickening of tubular basement membrane (&lt;10% of tubuli injured), mild striped interstitial fibrosis</td>
<td></td>
<td>5250±410</td>
<td>Asai et al. 2002</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>0.05%</td>
<td>7.5 s.c.</td>
<td>28</td>
<td>↔</td>
<td>Tubulo-interstitial fibrosis in 26%-36% of tubuli</td>
<td>Arteriolar hyalinosis in 31%-45% of afferent arterioli</td>
<td>2422±214</td>
<td>Shihab et al. 2003</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>0.05%</td>
<td>15 i.p.</td>
<td>↔</td>
<td></td>
<td>Cellular vacuolisation, tubular swelling, necrosis and atrophy in &lt;25% of tubuli, interstitial fibrosis</td>
<td>Arteriolar hyalinosis, hyaline depositions within afferent arteriole causing medial thickening</td>
<td></td>
<td>Capasso et al. 2008</td>
</tr>
</tbody>
</table>

SBP change in systolic blood pressure, s.c. subcutaneously, i.p. intraperitoneally
SHRs are first normotensive the for 6–8 weeks and then they develop labile hypertension, which becomes fixed after 12 weeks. Some studies of CsA toxicity using this model are summarised in table 4.

### Effects of CsA on blood pressure and renal histology, the influence of initial age, CsA dose and duration of the treatment in SHRs

<table>
<thead>
<tr>
<th>Strain</th>
<th>Initial age (weeks)</th>
<th>Dose of CsA (mg/kg/d)</th>
<th>Duration (days)</th>
<th>Change in SBP</th>
<th>Changes in histology</th>
<th>CsA concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>5</td>
<td>20 p.o.</td>
<td>56</td>
<td>↑</td>
<td>mild arteriolopathy (1.6/4) n.s.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>10</td>
<td>20 p.o.</td>
<td>98</td>
<td>++</td>
<td>very mild arteriolathy, n.s.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>20 p.o.</td>
<td>28</td>
<td>++</td>
<td>moderate arteriolathy 2.8/4 *</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>25</td>
<td>20 p.o.</td>
<td>28</td>
<td>++</td>
<td>mild arteriolopathy, n.s.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>30</td>
<td>20 p.o.</td>
<td>28</td>
<td>++</td>
<td>very mild arteriolopathy, n.s.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>↑</td>
<td>PAS+ deposits in periglomerular arteriolar wall</td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>8</td>
<td>25 p.o.</td>
<td>56</td>
<td>++</td>
<td>no vascular, glomerular or tubular changes 332±11 ng/ml</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>5</td>
<td>25 p.o.</td>
<td>28</td>
<td>++</td>
<td>no vascular, glomerular or tubular changes 261±79 ng/ml</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>10</td>
<td>25 p.o.</td>
<td>56</td>
<td>++</td>
<td>no vascular, glomerular or tubular changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>20</td>
<td>25 p.o.</td>
<td>28</td>
<td>++</td>
<td>moderate arterioglomerular changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>50</td>
<td>25 p.o.</td>
<td>28</td>
<td>++</td>
<td>moderate arteriolopathy changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>100</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>PAS+ deposits in periglomerular arteriolar wall</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>no vascular, glomerular or tubular changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>PAS+ deposits in periglomerular arteriolar wall</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>no vascular, glomerular or tubular changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>PAS+ deposits in periglomerular arteriolar wall</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>no vascular, glomerular or tubular changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>PAS+ deposits in periglomerular arteriolar wall</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>no vascular, glomerular or tubular changes</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>PAS+ deposits in periglomerular arteriolar wall</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>15</td>
<td>25 p.o.</td>
<td>28</td>
<td>+</td>
<td>no vascular, glomerular or tubular changes</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- *: Significant difference
- n.s.: Not significant
- PAS+: Periodic acid–Schiff

**References:**
- Ryffel et al. 1986
- Nahman et al. 1988
3 AIMS OF THE STUDY

The adverse effects of CsA such as magnesium wasting, hypertension and nephrotoxicity are clinically well known. There has been a lack of animal models in which concomitant nephrotoxicity and hypertension would occur.

The general aim of the study was:

to develop an animal model in which the rise of blood pressure and associated functional and renal structural changes associated with CsA toxicity would be demonstrated.

The specific aims of the study were to:

I. examine the effect of concomitant high dietary sodium and CsA in the spontaneously hypertensive rat (SHR) on blood pressure and renal function and structure.

II. characterise more specifically the pathogenesis of hypertension and nephrotoxicity in CsA-treated SHR on high dietary sodium.

III. investigate more specifically possible structural renal and cardiac changes in CsA-treated SHR on high dietary sodium.

IV. examine whether dietary restriction of sodium and/or dietary supplementation of magnesium or potassium could protect against CsA-induced renal and cardiac damage in CsA-treated SHR.

V. investigate the effect of isradipine on CsA toxicity and to compare isradipine with magnesium which acts as an endogenous calcium antagonist. The possible additive effect of combination of magnesium and isradipine was also of interest.
### 4 MATERIALS AND METHODS

#### 4.1 Experimental animals

Male spontaneously hypertensive rats (SHR) were purchased from Harlan (Sprague Dawley, Indianapolis, IN, USA). The procedures and protocols of the study were in accordance with our institutional guidelines and were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland. In the beginning of the study, the blood pressure- and body weight-matched SHR were divided into groups to receive different diet and drug regimens. They had free access to the chow and tap water at all times. The rats were weighed daily during the experiments.

#### 4.2 Diets and drug treatments

The experimental diets were based on a commercial standard low-mineral rat chow (R36, Finnewos Aqua) (Na 0.3%, Mg 0.2%, K 0.8%, Ca 1.0%, P 0.75% of the dry weight of the chow) into which sodium, magnesium and potassium were added. In high Na diet the Na content was 2.6%. In study V also a low-Na (Na 0.08%) diet was used. In high K diet the K content was 2.4% and in high Mg, the Mg content was 0.6% of the dry weight. The exact compositions of the diets in each study are given in the original publications I–V. A summary of the experimental design of the studies is presented in Table 5.

#### 4.2.1 Cyclosporine-A

CsA (Sandimmun® infusion concentrate 50 mg/ml, Sandoz Ltd, Basel, Switzerland) was diluted in a lipid solution (Intralipid®, Kabi Pharmacia, Stockholm, Sweden) to produce a 25-mg/ml solution that was administered once a day at a daily dose of 5 mg/kg s.c. The control rats received the same volume of the vehicle.
Table 5. Summary of the experimental design of the studies

<table>
<thead>
<tr>
<th></th>
<th>Strain</th>
<th>Groups</th>
<th>Diet (% of the dry weight of the chow)</th>
<th>CsA</th>
<th>Variables studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I</td>
<td>SHR</td>
<td>Control group</td>
<td>C</td>
<td>0.3</td>
<td>Systolic blood pressure, heart rate, LVH index, body weight gain, factors reflecting renal function (serum creatinine, creatinine clearance, serum urea, plasma renin activity, serum aldosterone, 24-hour protein excretion).</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>High-sodium group</td>
<td>Na</td>
<td>2.6</td>
<td>Ca, Mg, P.</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>Low-sodium and CsA group</td>
<td>CsA</td>
<td>0.3</td>
<td>24-hour food and water intake, urine volume and urinary excretion rates of Na, K, Mg, Ca and P.</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>High-sodium and CsA group</td>
<td>CsA+Na</td>
<td>2.6</td>
<td>Mg level of bone.</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>High-sodium, high-magnesium and CsA group</td>
<td>CsA+Na+Mg</td>
<td>2.6</td>
<td>Na, K, Mg, Ca, P levels of kidney and heart.</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>Low-sodium, high-magnesium and CsA group</td>
<td>CsA+Mg</td>
<td>0.3</td>
<td>Tissue concentrations of CsA (heart, kidney, liver, striated muscle) and whole blood.</td>
</tr>
<tr>
<td>Study II</td>
<td>SHR</td>
<td>As in Study I</td>
<td>As in Study I</td>
<td></td>
<td>The difference of blood pressure at the beginning and at the end of the study. Glomerular damage index (DI). Correlations between DI, changes in blood pressure, serum creatinine, proteinuria and the concentration of Ca. CsA-concentration in the kidney tissue</td>
</tr>
<tr>
<td>Strain</td>
<td>Groups</td>
<td>Diet (% of the dry weight of the chow)</td>
<td>CsA</td>
<td>Variables studied</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------</td>
<td>----------------------------------------</td>
<td>-----</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na</td>
<td>K</td>
<td>Mg</td>
<td></td>
</tr>
<tr>
<td>Study III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium and CsA group</td>
<td>C</td>
<td>0.8</td>
<td>0.2</td>
<td>CsA</td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium, high-potassium and CsA group</td>
<td>K</td>
<td>2.4</td>
<td>0.2</td>
<td>CsA</td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium, high-magnesium and CsA group</td>
<td>Mg</td>
<td>0.8</td>
<td>0.6</td>
<td>CsA</td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium, high-potassium, high-magnesium and CsA group</td>
<td>K+Mg</td>
<td>2.4</td>
<td>0.6</td>
<td>CsA</td>
</tr>
<tr>
<td>SHR</td>
<td>Low-sodium group</td>
<td>SHRs</td>
<td>0.3</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>SHR</td>
<td>Low-sodium and CsA group</td>
<td>CsA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>Low-sodium group</td>
<td>WKY</td>
<td>0.3</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>WKY</td>
<td>Low-sodium group</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WKY</td>
<td>Low-sodium and CsA group</td>
<td>CsA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>High-sodium group</td>
<td>Na</td>
<td>0.8</td>
<td>0.2</td>
<td>CsA</td>
</tr>
<tr>
<td>WKY</td>
<td>High-sodium group And CsA group</td>
<td>CsA+Na</td>
<td>2.6</td>
<td>0.2</td>
<td>CsA</td>
</tr>
</tbody>
</table>

Epicardial arteries, fibrotic scar tissue in the heart and glomerular changes from 0 to 3 were evaluated. Cardiac and renal damage indices were evaluated.
<table>
<thead>
<tr>
<th>Study</th>
<th>Strain</th>
<th>Groups</th>
<th>Diet (% of the dry weight of the chow)</th>
<th>CsA</th>
<th>Variables studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>SHR</td>
<td>High-sodium and CsA group</td>
<td>CsA+Na</td>
<td></td>
<td>Systolic blood pressure, heart rate, LVH index, body weight gain. Ionised serum Mg$^{2+}$ concentration, plasma pH, bone Mg concentration, Factors reflecting renal function (serum creatinine, creatinine clearance, serum urea, plasma renin activity, serum aldosterone, 24-hour protein excretion, urinary NAG excretion, urinary dopamine excretion, urinary noradrenaline excretion). 24-hour food and water intake, urine volume and urinary excretion rates of Na, K, Mg, Ca and P. CsA concentrations in whole blood, kidney and heart. Renal histology.</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>High-sodium, high-potassium and CsA group</td>
<td>CsA+Na+K</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>High-sodium high-magnesium and CsA group</td>
<td>CsA+Na+Mg</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>High-sodium, high-potassium, high-magnesium and CsA group</td>
<td>CsA+Na+K+Mg</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>Low-sodium group</td>
<td>C</td>
<td>0.3</td>
<td>Systolic blood pressure, heart rate, LVH index, body weight gain, ionised plasma Mg$^{2+}$ concentration, plasma pH, renal damage score, 24-hour protein excretion, urinary dopamine excretion, urinary noradrenaline excretion.</td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>Low-sodium and CsA group</td>
<td>CsA</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>High-sodium group</td>
<td>Na</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WKY</td>
<td>High-sodium and CsA group</td>
<td>CsA+Na</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Groups</td>
<td>Diet (% of the dry weight of the chow)</td>
<td>CsA</td>
<td>Variables studied</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------</td>
<td>----------------------------------------</td>
<td>-----------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Study V SHR</td>
<td>High-sodium and CsA group</td>
<td>C</td>
<td>0.2</td>
<td>Systolic blood pressure, heart rate, LVH index, body weight gain, plasma concentrations of Na, K, Ca, Mg²⁺ and Ca. Factors reflecting renal function (creatinine clearance, 24-hour protein excretion). Urine volume and osmolality. Whole blood CsA-concentration.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High-sodium and CsA group</td>
<td>CsA+Na</td>
<td>2.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High-sodium, isradipine and CsA group</td>
<td>CsA+isra</td>
<td>0.8</td>
<td>Renal ED-1 positive cells, myocardial ANP mRNA.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium, high-magnesium and CsA group</td>
<td>CsA+Mg</td>
<td>0.6</td>
<td>Epicardial arteries and myocardium were evaluated from 0 to 3. Renal histology (glomerular damage index, intrarenal arteries, tubuli and cortical interstitium were scored from 0 to 3.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium, isradipine, high-magnesium and CsA group</td>
<td>CsA+isra+Mg</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>Very low-sodium group</td>
<td>CL</td>
<td>0.08</td>
<td>Systolic blood pressure, heart rate, LVH index, body weight gain. Factors reflecting renal function (creatinine clearance, plasma renin activity, serum aldosterone, 24-hour protein excretion). Urine volume and urinary excretion rates of various minerals (Na, K, Mg, Ca and P). Whole blood CsA-concentration. Renal ED-1 positive cells, myocardial ANP mRNA. Epicardial arteries and myocardium were evaluated from 0 to 3. Renal histology (glomerular damage index, intrarenal arteries, tubuli and cortical interstitium were scored from 0 to 3.</td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>Very low-sodium group</td>
<td>CsAL</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium and CsA group</td>
<td>CsA</td>
<td>2.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>Very low-sodium, CsA and isradipine group</td>
<td>CsAisra</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR</td>
<td>High-sodium and isradipine group</td>
<td>CsAisra</td>
<td>2.44</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SHR, spontaneously hypertensive rat, WKY, Wistar-Kyoto rat.
4.2.2 Isradipine

Isradipine was added to the chow (350 mg isradipine per kg of dry weight) to produce an average daily dose of approximately 20 mg/kg.

4.3 Measurement of systolic blood pressure and heart rate

Systolic blood pressure and heart rate were measured with a tail-cuff blood pressure analyser (Apollo-2AB Blood Pressure Analyser, model 179–2AB, IITC Life Science, USA). The analogue signals of systolic blood pressure and heart rate were automatically converted to digital values by an online microprocessor. Before the measurements, the rats were warmed for 10–15 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 readings from three to five measurements.

4.4 Metabolic studies and sample preparation

In the beginning of the study, the rats were housed individually in metabolic cages for a 24-hour period, and they had free access to tap water and chow. Food and water intake and urine volumes were measured, and urine samples were frozen until the analyses. This was repeated in the second, fourth and sixth week of the study.

4.5 Tissue preparation for morphology and histological scoring

Sagittal central pieces of the left ventricle of the heart and the hilar area of the kidney were cut and fixed with 4% buffered paraformaldehyde at room temperature. Tissues were dehydrated in gradual alcohols and embedded in paraffin. Sections of 2–3 μm were cut using a Leitz microtome (Leitz 1512; Leitz, Wetzlar, Germany). The sections were deparaffinised and rehydrated prior to being stained with haematoxylin-eosin and Masson trichrome. The tissues were examined without knowledge of the identity of the rats.
4.5.1 Histological scoring

4.5.1.1 Kidney
Interstitial, tubular, glomerular and vascular changes were quantitated using scores from 0 to 3 defined as:

Score 0. Normal arterioglomerular unit with open capillary lumens and normal afferent arteriole. No tubular atrophy, interstitial inflammation or interstitial fibrosis. Media slender in large vessels.


Score 3. Necrotic arterioglomerular units with medial necrosis of the arteriolar wall. Diffuse tubular atrophy with many hyaline casts, striped fibrosis, and sometimes medial necrosis of large vessels.

To emphasise the degree of changes in the kidneys, a renal damage index (RDI or DI) was used. RDI was calculated by the assessment of 100 consecutive arterioglomerular units in each kidney and the number of affected glomeruli in each score group (e.g. ax0 + bx1 + cx2 + dx3; a + b + c + d = 100 glomeruli; score 0–3 = the degree of the change.

4.5.1.2 Heart
Three representative epicardial arteries were evaluated and scored from 0–3.

0 epicardial artery wall slender, normal
1 normal intima: media slightly thickened or normal; slight increase in connective tissue around the epicardial artery
2 pronounced thickening of arterial wall, especially in media; marked increase of connective tissue around the epicardial artery
3 pronounced thickening of intima and media, clear narrowing of the lumen; excessive scar-like increase of connective tissue around the epicardial artery
Myocardium was scored as follows:
0 normal
1 patchy increase of slender connective tissue bundles
2 patchy increase of thick connective tissue bundles partly coalescing
3 coalescing, vast areas of connective tissue

4.6 Biochemical and hormonal determinations

Plasma renin activity (PRA) was determined by a radioimmunoassay of angiotensin I (Medix Angiotensin I test; Medix Biochemica, Kauniainen, Finland). Serum aldosterone was determined by a solid-phase radioimmunoassay specific for aldosterone (Coat-A-Count Aldosterone, Diagnostic Products Corp., Los Angeles, CA, USA). Ionised magnesium and ionised calcium were measured directly with selective electrode for Mg and Ca from an undiluted sample (Microlyte 6 KONE Instruments, Espoo, Finland) according to the protocol of the manufacturer. As an anticoagulant, Ca-titrated lithium-heparin was used. Blood counts including thrombocytes were determined using routine clinical laboratory methodology. Serum cholesterol was determined using an enzymatic colorimetric method (CHOD-PAP, Hitachi 917; Roche Diagnostics, Mannheim, Germany).

Whole-blood, renal, myocardial, hepatic, and striated muscle tissue CsA concentrations were determined by fluorescence polarization immunoassay (Abbott TDX cyclosporine monoclonal whole-blood method, Abbott Laboratories, USA) using a monoclonal antibody specific for the parent molecule. Before CsA determinations, tissue samples (≈100 mg) were weighed, minced, and homogenized in buffer (10 mmol/L PBS, 50 mmol/L Tris-HCl, 0.5% Triton). The volume of the homogenate was adjusted with buffer to give a final tissue amount of 100 g/l.

Twenty-four-hour urine samples were collected into polypropylene tubes containing 1.0 ml 6 M HCl as the preservative and stored at -80°C until assayed. Total protein concentration of urine was determined by the method of Lowry et al (Lowry OH et al. 1951) after precipitation with 10% trichloroacetic acid. Urine creatinine was analysed with an enzymatic analyser (Kone Specific; Kone Co., Espoo, Finland). Electrolytes were determined using a Baird PS-4 inductively coupled plasma emission spectrometer (Baird Co., Bedford, MA, USA). Urinary noradrenaline and dopamine were analysed by high-performance liquid chromatography (HPLC) with electrochemical detection as described elsewhere (Tuomainen, Männistö 1997). The activity of urinary N-acetyl-[beta]-D-glucosaminidase (NAG) was determined by using 3-cresolsulfonphtalein-N-acetyl-[beta]-glucosamine as the substrate (Noto A et al. 1983). The method was adapted to a Cobas Fara II random access analyser (F. Hoffman-La Roche, Basel, Switzerland). Kinetic follow-up of the reaction was used instead of determining end point absorbance.
4.6.1 Electrolytes (tissues)

The concentrations of the elements Na, K, P, Mg, and Ca in urine, heart, kidney and bone were determined by using a Baird PS-4 inductively coupled plasma emission spectrometer (Baird Co) as described in detail elsewhere (Laakso, Tikkanen 1991).

4.7 Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's test. Data for multiple observations over time were analysed using two-way ANOVA with repeated measures for overall treatment effect, and Tukey's test was used for multiple pairwise comparisons of treatment groups at different times. Linear regression lines were calculated by the least-squares method. Differences between means of $P < .05$ were considered significant. The data were analysed with SYSTAT statistical software (SYSTAT Inc). The results are expressed as mean ± SEM.
5 RESULTS

The combination of high dietary Na and CsA in SHRs induced concomitant acceleration of hypertension, LVH and both functional and morphological nephrotoxicity as well as cardiac histological changes. Proteinuria, increased serum creatinine and increased excretion of NAG indicated renal insufficiency. Reduced tissue concentrations of Mg reflected Mg deficiency.

5.1 Blood pressure

In all studies, CsA induced a marked increase in blood pressure in SHRs on high dietary Na when compared to any other group. During the moderately low-Na diet in SHRs, CsA induced a slight, but significant rise in blood pressure in both absence and in presence of Mg (Study I). The high-Na diet alone also produced an increase in blood pressure in SHRs (Study I). CsA induced a significant increase in the blood pressure in WKY rats on the high-Na diet.

Potassium and magnesium, alone and in combination, were equally effective in preventing the increase of blood pressure during the high-Na diet and CsA (Study IV). When the effects of isradipine and magnesium were compared, the preventive effect was equal, but the combination of isradipine and magnesium did not give any further benefit (Study V).

5.2 Left ventricular hypertrophy

The combination of CsA and high dietary Na increased the LVH index when LVH was estimated as left ventricle wet weight-to-body weight-ratio (Studies I and III). During the moderately low-Na diet, CsA did not affect the LVH index (Study I). Supplementation of Mg, alone and in combination with K, prevented an increase in LVH index, whereas K alone did not (Study III). When isradipine and Mg were compared, isradipine was more effective in protection against the development of LVH, but the combination of isradipine with Mg was the most effective (Study V). The high-Na diet caused LVH in normotensive WKYs, both in the presence and absence of CsA treatment.
5.3 Renal function

The high-Na diet alone or CsA treatment during the moderately low-Na diet either in the absence or in the presence of Mg supplementation did not significantly affect renal function when measured with serum creatinine, creatinine clearance, serum urea, PRA, serum aldosterone, and 24-h urinary protein excretion in SHR (Study I).

Creatinine clearance was decreased, serum creatinine and serum urea concentrations were increased, and PRA, serum aldosterone concentration, and 24-hour urinary protein excretion increased 5–7-fold by CsA treatment during the high-Na diet (Study I). Also, urinary NAG excretion, indicating tubular damage, increased during the six study weeks (Study IV). K decreased proteinuria by 25% but did not markedly affect serum creatinine, creatinine clearance or urinary NAG excretion in CsA-treated SHRs on the high-Na diet. On the high-Na diet, Mg prevented from CsA-induced activation of the RAAS when measured with PRA (study I and IV) and aldosterone (study I and IV), and also CsA-induced increases in serum urea concentration and 24-hour urinary protein excretion (Study I and IV). Mg did not markedly affect urinary NAG excretion in the CsA-treated SHRs on the high-Na diet (Study IV).

Isradipine decreased proteinuria by 75%, Mg by 47% and the combination of isradipine and Mg by 76%. Both isradipine and Mg preserved protein urinary excretion and creatinine clearance at the same level as the control group receiving high dietary Na (Study V).

5.4 Histology

With high dietary Na CsA induced marked renal and cardiac histological changes. The glomerular damage index (GDI) was significantly higher in the CsA-treated high-Na diet group than in the other groups in all studies (Studies II, III and V). Tubular atrophy and dilatation with interstitial fibrosis were noted (Study V). Intrarenal arteries had proliferative changes in the intimal and medial layers (V). Infiltration of ED-1 positive mononuclear cells was found in the perivascular areas (Study V). In the heart, luminal narrowing of the coronary arteries and left ventricular scarring were obvious in the SHRs on high dietary Na and CsA (Studies III and V). Both Mg and isradipine prevented as well from glomerular damage as tubular, arteriolar and interstitial changes. Isradipine completely protected against histological changes (Studies II, III and V). K alone was less effective than Mg in the prevention of renal histological changes (Study III). Mg alone but not K protected against cardiac damage, but the combination of both was the most effective (Study III).

CsA only caused minor histopathological changes in SHRs receiving the low-Na diet (Study
III). When normotensive WKYs were given CsA on high dietary Na, changes in the coronary arteries and increased glomerular damage index were observed (Study III).

### 5.5 Urinary catecholamine excretion (Study IV)

Urinary dopamine excretion was approximately 50% lower in CsA-treated SHRs on a high-Na diet than in the Mg supplementation treatment groups. K supplementation had no effect on urinary dopamine excretion. In WKY rats CsA with high dietary Na, urinary dopamine excretion was lower than in rats receiving CsA with low dietary Na. There were no differences in urinary excretion of noradrenaline between the groups.

### 5.6 Electrolyte concentrations

After the six-week-treatment, CsA caused Mg wasting from the bone during the high-Na diet, whereas during Mg supplementation the bone Mg concentration of the CsA-treated SHR was significantly increased (Study I). The dietary level of Na alone did not have effect on bone Mg level.

CsA increased the urinary excretion of P during both the moderately low-Na and high-Na diets. Mg supplementation prevented CsA-induced phosphaturia (Study I). The urinary excretion of Ca was markedly increased during the high-Na diet irrespective of the dietary Mg level (Study I). Supplementation of K with high dietary Na did not influence serum Mg$^{2+}$ or bone Mg concentrations (Study IV). Supplementation of P did not either have an effect on excretion of Mg, Ca or P into the urine, but decreased urinary excretion of K. (Study IV).

In CsA-treated SHRs on a high-Na diet, plasma Mg$^{2+}$ as well as bone Mg concentrations were the lowest, although the differences did not quite reach statistical significance. Supplementation of Mg was associated with 50% and 16% increases in plasma Mg$^{2+}$ and bone Mg concentrations, respectively.

Renal concentration of K was slightly increased and concentration of Ca was markedly increased by CsA during the high-Na diet (Study I). Renal Mg concentration was slightly higher in the CsA group on the high-Na diet than in the CsA group on the moderately low-Na diet (I).

The CsA-induced rises in renal Ca and K contents during the high-Na diet were blocked by Mg supplementation. High Na and CsA produced myocardial Mg depletion and accumulation of Ca, which were completely blocked by Mg supplementation (Study I).
5.7 CsA concentrations

Dietary Na did not have an effect on the CsA concentrations in whole blood, kidney, liver, heart or striated muscle (study I). The CsA concentrations in whole blood, kidney, and heart averaged 730 ± 52 ng/ml, 127 ± 6 ng/g and 71 ± 4 ng/g, respectively. Modifications of dietary electrolyte composition did not influence the concentrations of CsA in whole blood, kidney, or heart (Study IV). The main results are summarised in table 6.
Table 6 Summary of main results of the studies

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Group names as in table 5. SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; SBP, systolic blood pressure; Na, sodium; Mg, magnesium; K, potassium; Ca, calcium; CsA, cyclosporine-A; LVH-index expressed as the ratio of left ventricular wet weight to body weight.

↑ or ↓ p<0.05 in comparison with the control group. ↑↑ p<0.05 in comparison with all the other groups.

* No difference between Mg-supplementation groups.
6 DISCUSSION

For the last decades CsA has been a cornerstone in organ transplantation as an effective immunosuppressant. However, hypertension and nephrotoxicity hamper the improvement in graft and patient survival. The side-effects of CsA have been under intensive investigation. A marked problem in experimental studies has been the lack of an animal model in which hypertension and nephrotoxicity concurrently occur.

With regard to CsA toxicity, a direct correlation between hypertension and cardiovascular risks is well established. Similarly, epidemiological data demonstrate a positive correlation between the intake of Na and blood pressure. There is also evidence suggesting a relationship between low dietary Mg and high blood pressure. Hypomagnesaemia induced by CsA, if not treated, plays an important role in the development of experimental hypertension.

We demonstrated that the combination of high dietary Na and CsA accelerates the development of hypertension and nephrotoxicity. We developed an animal model, in which both hypertension and nephrotoxicity concomitantly appeared as a result of high Na intake in SHR also receiving a low dose of CsA. The development of hypertension and organ toxicity was prevented by dietary Mg. The beneficial effect Mg was enhanced by combining it with isradipine and to some extent K.

6.1 Methodological aspects

6.1.1 Experimental animals

Okamoto and Aoki (1963) developed a model of experimental hypertension in which hypertension and its complications develop with age (Yamori and Swales 1994). The spontaneously hypertensive rat (SHR) was developed from the normotensive strain and has since then been widely used as a hypertensive model. Wistar-Kyoto (WKY) is generally used as the normotensive control. They are descendants of the same strain as SHRs but the strains were not bred simultaneously (Johnson 1992, Yamori and Swales 1994). SHR and WKY strains have been separated from the original colony and each other for at least 40 years, so some
genetic differences are thought to be due to genetic drift (Johnson 1992, Yamori and Swales 1994).

Hypertension both in humans and SHR is polygenic in origin and influenced by different environmental factors (Yamori and Swales 1994). Still, majority of the knowledge on genetic hypertension is the result of the research on SHRs (Zicha and Kunes 1999).

The dietary intake of Na has a dose-dependent effect on blood pressure in the SHR (Louis et al 1971) as also seen in our study. Interestingly, we demonstrated that a diet high in Na produced a slight increase (+16 mmHg) in the blood pressure of also in WKYs suggesting that this normotensive rat strain is to some extent salt-sensitive (Study IV).

The theory that the kidneys have a key role in essential hypertension has gained further support. It is known, that SHRs will become normotensive if their kidneys are removed and they get transplanted kidneys from normotensive rats. This observation has also gained clinical support. Curtis et al. reported of six patients who had essential hypertension because of nephrosclerosis and when their own kidneys were removed and new kidneys were transplanted, these patients became normotensive (Curtis et al. 2000).

Because our hypothesis was that CsA-induced hypertension and nephrotoxicity could be salt-sensitive, we chose the SHR as the experimental animal. We also had previous experience on the effects of high-dietary sodium, resembling that of the human diet, on SHR (Mervaala et al. 1992).

6.1.2 Histology

After discovery of CsA-induced nephrotoxicity in humans, it was difficult to distinguish between the histological changes caused by CsA toxicity and organ rejection. Because the drug toxicity could not be observed in animal models receiving CsA with a dose similar to that needed to prevent rejection clinically, it made the task even more complicated. CsA-induced histological changes are typical, but not specific.

In our model, CsA-induced changes occur as patchy ones; a totally destroyed arterio-glomerular unit can be surrounded by normal ones. Thus, we developed a scoring system, which considers 100 glomeruli of each kidney slide, and the degree of the changes is emphasised. We first assessed the overall expression of the kidney grading it from 0 to 3, but we found this too imprecise. (Study II) We also regarded interstitial fibrosis too mild a change to be mentioned. A basis for this was the experience from biopsy and autopsy samples of transplanted kidneys of humans treated with CsA. In the subsequent study we further developed our assessment technique so that we at different sessions evaluated separately arterio-glomerular units, tubuli and interstitium (Studies II and V). In Fig 7 the histological changes are clearly seen.
There are few studies on medullar histology after CsA treatment. In a study of Rosen et al. 1990 thick ascending limb cell atrophy with concomitant fibroblastic proliferation and collagen formation was detected. These changes were present in the inner stripe of the outer medulla and the medullar ray. Rosen and co-workers used Sprague-Dawley rats, gave furosemide and rats were on a salt-depleted diet. CsA-dose was 12.5 mg/kg/d s.c. They concluded, that CsA causes chronic injury to putative zones of hypoxia.

Medullar histology is often ignored when assessing renal histology. We noticed changes in the collecting duct and also the interstitium of the medulla. The characterisation and importance of these changes needs further investigation.
6.1.3 Possible mechanisms of the protective effect of magnesium and isradipine

Because of the essential role of Mg as a required cofactor of over 300 enzyme systems, Mg deficiency is a potential health hazard (Saris et al. 2000). Magnesium has been called “nature’s physiological calcium channel blocker” (Iseri and Frenh 1984). During Mg depletion intracellular Ca rises. Since Ca plays an important role in skeletal and smooth muscle contraction, a state of Mg depletion may result in muscle cramps, hypertension and coronary and cerebral vasospasms. Reduction of the extracellular Mg has been shown to induce contractions of isolated rat arteries as well as of isolated perfused rat mesenteric arterioles (Altura and Altura 1978).

Several mechanisms of the relaxant action of Mg on vascular smooth muscle have been proposed. Mg2+ decreases intracellular Ca2+ (D’Angelo et al. 1992) by alteration of membrane permeability, and/or by blocking of the voltage-dependent L- or T-type Ca2+ channels. Inhibitory effects of Mg2+ on receptor-mediated Ca2+ influx have also been reported (Wallnöfer et al. 1989). In VSMC the contractile agonists, such as endothelin, induce Ca2+ release from the internal cellular stores and influx of Ca2+ through the receptor-operated Ca2+ channels or through the voltage-dependent Ca2+ channels. The receptor-activated Ca2+ entry is partly mediated by Ca2+ permeable non-selective cation channels, which is resistant to organic Ca2+ antagonist such as nifedipine (van Renterghem et al. 1988). Extracellular Mg2+ has been shown to inhibit receptor-mediated non-selective cation-channels in VSMCs, probably via direct blockade of the channels, which may contribute to the vasodilating effects of Mg2+ (Nakajima et al. 1997). This may explain the additive effect of dihydropyridine-type calcium L-channel blocker isradipine and Mg in the prevention of CsA-induced vasoconstriction.

6.1.4 Hypertension, left ventricular hypertrophy and cardiac histology

In the present study the effects of a high intake of Na and CsA treatment were examined in SHR during the development phase of hypertension. In agreement with previous experimental findings (Aoki 1972 et al., Tobian 1991, Mervaala et al. 1992), a high intake of Na alone increased the blood pressure in the SHR. The combination of CsA and high intake of Na caused a further increase in the blood pressure together with a marked increase in LVH. An important finding was that CsA caused significant coronary artery disease and cardiac infarctions. We also examined the effects of CsA in WKY rats on a high-Na diet. Interestingly, CsA induced vascular damage in the coronary arteries, although the morphological changes were less prominent than in SHRs. To our knowledge, this is the first report describing the detrimental interaction between cyclosporine A and a high sodium diet in normotensive rats.
In our studies in the SHR, the adverse effects of CsA with high dietary Na clearly exceeded those produced by CsA when the intake of CsA was moderately low. Our finding is in accordance with a previous clinical study by Curtis and co-workers. They suggested already in 1988, that hypertension in CsA-treated patients is Na-dependent (Curtis et al. 1988). In this short-term study Na restriction had evident antihypertensive effects in transplant patients treated with CsA.

There are few studies in which the intake or excretion of Na has been studied in hypertensive patients receiving CsA. Magina et al. treated patients with psoriasis with CsA (3 mg/kg/d) during low and high dietary Na content. With high dietary Na the patients’ blood pressure increased (Magina et al. 2005).

It is generally accepted that dietary Na restriction is essential in the treatment of hypertension. It may per se lower the blood pressure in salt sensitive individuals and a low enough intake of dietary Na may also enhance the effect of an antihypertensive medication. Our experimental study further supports the idea that it is feasible to pay attention to reduction of the salt load in patients receiving CsA.

### 6.1.5 Nephrotoxicity

Cyclosporine causes a pronounced vasoconstriction of the preglomerular (afferent) arteriole. CsA has been shown to exert direct toxic effects on bovine vascular cells in culture, and this damage leads to release of several vasoactive agents (Zoja et al. 1986). Vasoconstriction and endothelial cell damage may result in necrosis of smooth muscular cells and hyalinisation the vessel wall. Hyalinisation can obliterate the afferent arteriole (for review see Campistol and Sacks 2000). Renal blood flow and glomerular filtration rate decrease and blood creatinine levels increase. These events are seen in our model. Obliteration of arteries may increase the renal vascular resistance and cause chronic ischaemia and this in turn lead to tubulointestinal lesions (Ong and Fine 1994). CsA can also have a direct toxic effect on the proximal tubular cells and this in turn can stimulate a local inflammatory response seen as macrophage infiltration (for review see Campistol and Sacks). In our model increased NAG indicated proximal tubular injury, and also perivascular monocyte/macrophage infiltration could be detected. Some investigators have speculated, that tubulointerstitial lesions may develop independently of vascular lesions through different mechanisms (Benigni et al. 1999).

In our model the vascular lesions dominated, tubular lesions were remarkable, and if the experimental time would have been longer, the interstitial fibrosis would might have become more prominent. On the other hand, a longer experiment time would have increased the mortality of the rats, because untreated SHRs on high dietary Na became so hypertensive and end-organ damage was so evident.
Dopamine plays an important role in the regulation of renal Na excretion. The activation of peripheral dopamine receptors influences cardiovascular and renal function e.g. by decreasing vascular resistance and facilitating Na and water excretion (Hussain and Lokhandwala 2003). In various forms of hypertension deficiencies in renal dopamine synthesis and/or secretion have been reported. On a high dietary Na intake, urinary dopamine excretion is lower in salt-sensitive hypertensive subjects than in normal subjects (Shikuma et al. 1986). Defective dopaminergic action also in the kidneys in hypertensive animal models has been reported.

An important finding of the present study was that, in SHRs on the high-Na diet, CsA-induced hypertension and nephrotoxicity are associated with renal dopaminergic deficiency. To our knowledge, the present study provides first evidence that the renoprotective effect of Mg, alone and in combination with K, is associated with prevention of CsA-induced renal dopaminergic deficiency.

High dietary intake of Na and CsA induced simultaneously with hypertension marked nephrotoxicity which was seen as increased serum creatinine, decreased creatinine clearance and proteinuria. Changes in renal glomeruli and tubuli were found in histological examination. Our findings clearly contradict the conclusions by Elzinga et al. and Gerkens et al. that restriction of Na would potentiate CsA-induced nephrotoxicity, whereas a high intake of Na might even protect against renal damage caused by CsA treatment (Elzinga et al. 1993, Gerkens et al. 1984). In both of these studies Na depletion had been produced by a salt-free diet. In addition Elzinga et al. used a uninephrectomised model and gave furosemide. This caused a decrease in GFR, cortical fibrosis and extensive atrophy of mTAL. This model describes more salt-deficiency than salt restriction. A salt-depleted experimental model has been widely used in studying CsA-toxicity. Interstitial fibrosis is a prominent finding in a low-salt model. Hypertension does not develop and neither do glomerular nor vascular changes. In addition, relatively high CsA doses (15 mg/kg/d) are needed to produce nephrotoxicity.

The morphological changes found in the kidneys of CsA-treated SHR on the high-Na diet closely resembled those observed in malignant hypertension and support the notion that arterial hypertension plays an important role in the pathogenesis of kidney damage in our model.

6.1.6 Effect of magnesium and potassium on CsA-induced hypertension and nephrotoxicity

Accumulating evidence suggests that dietary Mg deficiency plays an important role in the pathogenesis of hypertension and ischaemic heart disease (Arsenian 1993, Altura and Altura 1995). Vascular effects of decreased extracellular Mg concentrations are increased vascular tone, (Altura et al. 1984), endothelial permeability and production of vasoactive agents and
cytokines, increased oxidative stress (Weglicki et al. 1996) and enhanced vascular reactivity to vasoconstrictor agents. Mg also acts as a weak Ca channel blocker (Altura and Altura 1985). Additionally, CsA induces Mg wasting both experimentally and clinically (Barton et al. 1989, June et al. 1985).

In our study CsA caused some Mg loss in SHR on a low-Na diet, but with high dietary Na CsA significantly decreased Mg levels in the heart and bone.

Dietary magnesium effectively prevented from CsA-induced hypertension, LVH, functional and structural nephrotoxicity and from cardiac histopathological changes. Magnesium supplementation also corrected magnesium depletion as measured from bone and heart.

Potassium has a blood pressure-lowering effect in humans (Cappuccio and MacGregor 1991). Tobian and co-workers (Tobian et al. 1985) showed that the supplementation of K protected against Na-induced lesions in renal tubules, arteries and glomeruli. It has been suggested that the renoprotective effect of K is partly mediated by a pressure-independent mechanism. It is noteworthy that K alone only offered slight protection against cardiac or renal histopathological changes caused by CsA, but the combination of K and Mg was effective in our study. The mechanism of additive protection by Mg and K remains open.

Both K and Mg supplementation showed beneficial effects against CsA-induced hypertension and nephrotoxicity in SHR. The protective effect of Mg exceeds that of K in the prevention of LVH, proteinuria and serum creatinine increase.

6.1.7 Clinical implications and future aspects

Magnesium depletion induced by CsA-treatment seems to play an important role in hypertension and nephrotoxicity. Magnesium supplementation significantly prevented these side-effects in the rat and did not have disadvantages. However, magnesium supplementation can cause diarrhoea, and this may limit use of Mg in some patients.

On the basis of the experimental studies described in this thesis, clinical studies on the effects of sodium restriction and magnesium supplementation in patients on CsA therapy would be warranted.
7 SUMMARY AND CONCLUSIONS

1 Hypertension and associated renal functional and structural changes caused by CsA toxicity could be demonstrated in an animal model using SHRs. Adding NaCl concentration in the rat diet to level often found also in Western type food items, and CsA treatment at dosage to produce clinically relevant blood drug concentration, produced side-effects typical for organ transplant patients receiving CsA chronically.

2 High sodium alone or CsA with low dietary sodium increased the blood pressure slightly, with no significant disturbance in renal function. High sodium and CsA together produced a marked rise in the blood pressure, Creatinine clearance was decreased and serum creatinine and urea as well as 24-hour urinary protein excretion were increased.

3 High dietary sodium and CsA induced magnesium loss into urine and caused magnesium deficiency in tissues as well as a fall in serum ionised magnesium concentration Mg²⁺. Renal dopaminergic deficiency and paradoxal activation of RAAS was detected.

4 Histologically arteriolar, glomerular, tubular and interstitial damage in the kidneys was detected and epicardial arterial damage and myocardial infarctions in the heart. These histopathological changes correlated with pathophysiological phenomena such as hypertension, LVH and proteinuria and renal insufficiency.

5 Magnesium supplementation inhibited the increase in blood pressure, development of LVH, proteinuria and activation of RAAS. The renal and cardial injury was also prevented. Magnesium alone and in combination with potassium prevented from the increase in blood pressure and LVH.

6 Magnesium alone was effective in prevention of the toxicity of high sodium and CsA. The combination of Mg and K was still more effective but K alone had little effect.

7 Isradipine and magnesium had equal antihypertensive effects and their combination did not give any further benefit. Isradipine protected better than magnesium against LVH. Isradipine did not prevent from CsA-induced magnesium deficiency, which may lead to the harmful phenomena associated with chronic Mg depletion.

In conclusion, an experimental animal model for CsA-induced concomitant hypertension and nephrotoxicity, both functional and structural, was developed when dietary sodium level was
raised to a level similar to typical human sodium intake. Magnesium supplementation effectively prevented from hypertension and nephrotoxicity. Isradipine was slightly more effective, but with isradipine treatment magnesium deficiency was not improved.
This study was carried out at the Institute of Biomedicine, Pharmacology, University of Helsinki, from year 1995 until 2008.

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