CYCLOSPORINE A-INDUCED HYPERTENSION AND NEPHROTOXICITY IN SPONTANEOUSLY HYPERTENSIVE RATS ON HIGH-SODIUM DIET

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Academic Dissertation

To be presented, with the permission of the Medical Faculty of the University of Helsinki, for public examination in the small auditorium of the Department of Pharmacology and Toxicology, University of Helsinki, Siltavuorenpenger 10 A, on December 21, at 12 noon.

Helsinki 2000

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ISBN 952-91-2936-X

ISBN 952-91-2937-8 (PDF version http://ethesis.helsinki.fi)

ISBN 952-91-2938-6 (html version http://ethesis.helsinki.fi)

Helsinki 2000, Yliopistopaino

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by Roman numerals I–IV:

I Mervaala E, Lassila M, Vaskonen T, Krogerus L, Lähteenmäki T, Vapaatalo H, Karppanen H (1999). Effects of ACE inhibition on cyclosporine A-induced hypertension and nephrotoxicity in spontaneously hypertensive rats on a high-sodium diet. Blood Pressure 8: 49–56.

II Lassila M, Finckenberg P, Pere A-K, Krogerus L, Ahonen J, Vapaatalo H, Nurminen M-L (2000). Comparison of enalapril and valsartan in cyclosporine A-induced hypertension and nephrotoxicity in spontaneously hypertensive rats on high-sodium diet. Br J Pharmacol 130: 1339–1347.

III Lassila M, Finckenberg P, Pere A-K, Vapaatalo H, Nurminen M-L (2000). Enalapril and valsartan improve cyclosporine A-induced vascular dysfunction in spontaneously hypertensive rats. Eur J Phamacol 398: 99–106.

IV Lassila M, Santisteban J, Finckenberg P, Salmenperä P, Riutta A, Moilanen E, Virtanen I, Vapaatalo H, Nurminen M-L. Vascular changes in cyclosporine A-induced hypertension and nephrotoxicity in spontaneously hypertensive rats on high-sodium diet. Submitted.

ABBREVIATIONS

ACE	angiotensin converting enzyme
AT_1	angiotensin II type 1 receptor
AT_{1A}	angiotensin II type 1 subtype A receptor
AT_{1B}	angiotensin II type 1 subtype B receptor
AT_2	angiotensin II type 2 receptor
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CsA	cyclosporine A
CYP450	cytochrome P450
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
mRNA	messenger ribonucleic acid
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PRA	plasma renin activity
RAS	the renin-angiotensin system
SHR	spontaneously hypertensive rat(s)
TGF-β_1	transforming growth factor-β_1
TRAP	total radical trapping parameter

ABSTRACT

Cyclosporine A is an immunosuppressive drug widely used to prevent rejection of transplanted organs and to treat autoimmune diseases. Cyclosporine A therapy is often limited by adverse effects such as hypertension and nephrotoxicity. Cyclosporine A nephrotoxicity is characterised by renal dysfunction and by renal morphological damage with interstitial fibrosis and arteriolopathy. The exact mechanisms of cyclosporine A-induced hypertension and nephrotoxicity are unknown.

Renal and cardiovascular effects of long-term administration of cyclosporine A were studied in spontaneously hypertensive rats on high-sodium diet. The animal model was selected to overcome the resistance of normotensive rats to adverse renal and cardiovascular effects of cyclosporine A. The role of the renin-angiotensin system, the renal kallirein-kinin system, the L-arginine–nitric oxide pathway and oxidative stress in cyclosporine A-induced renal and cardiovascular toxicity were evaluated. Furthermore, the effects of cyclosporine A on arterial function and structure were examined.

Six-weeks administration of cyclosporine A caused hypertension and nephrotoxicity in spontaneously hypertensive rats in the developmental phase of hypertension. Cyclosporine A-induced hypertension and nephrotoxicity were related to vascular dysfunction, which was more pronounced in renal than in mesenteric arteries. The vascular dysfunction included both impaired endothelium-dependent (acetylcholine) and endothelium-independent (sodium-nitroprusside) relaxations as well as decreased contractile responses to noradrenaline, potassium chloride and angiotensin II. Cyclosporine A also caused morphological damage to intrarenal arterioles and small arteries and, to a lesser extent, mesenteric resistance vessels. Long-term cyclosporine A administration decreased aortic smooth muscle cell viability in cell cultures.

Cyclosporine A caused a rise in plasma renin activity, but did not affect urinary cyclic guanosine 3',5'-monophosphate, kallikrein or 8-isoprostaglandin $F_{2\alpha}$ excretion. Neither did it affect endothelial or inducible nitric oxide synthase expression in the kidney, heart or aorta nor plasma total radical trapping parameter or urate concentration. Concomitant inhibition of the renin-angiotensin system with angiotensin converting enzyme inhibitor enalapril or with angiotensin AT_1 receptor antagonist valsartan protected against cyclosporine A toxicity. Bradykinin B_2 receptor antagonist icatibant

(HOE 140) did not reduce the beneficial effects of enalapril. The precursor of nitric oxide, L-arginine, did not markedly affect cyclosporine A-induced hypertension, renal dysfunction or morphological damage to kidneys or vessels. L-arginine did, however, tend to improve arterial dysfunction in spontaneously hypertensive rats treated with cyclosporine A.

These results suggest that the activation of the renin-angiotensin system plays an important part in cyclosporine A toxicity in this hypertensive animal model. Inhibition of the renin-angiotensin system by angiotensin converting enzyme inhibitor or by AT_1 receptor antagonist is effective in protecting against renal and cardiovascular toxicity of cyclosporine A. In contrast, these studies indicate no role for the renal kallikrein-kinin system, the L-arginine–nitric oxide pathway or oxidative stress as primary mediators of cyclosporine A toxicity. However, the participation of these mechanisms cannot be totally excluded.

1. INTRODUCTION

Cyclosporine A (CsA) is an immunosuppressive drug widely used to prevent rejection of transplanted organs and to treat autoimmune diseases. CsA treatment is often limited by adverse effects such as hypertension and nephrotoxicity (for review, see Andoh and Bennett 1998; Taler *et al.* 1999). CsA nephrotoxicity is characterised by renal dysfunction as evidenced by a rise in serum creatinine and a decrease in creatinine clearance and by morphological damage with interstitial fibrosis and arteriolopathy (Andoh and Bennett 1998).

The exact mechanisms of CsA-induced hypertension and nephrotoxicity remain obscure. Clinical and experimental studies have revealed that several mechanisms may be involved (Table 1). These include activation of the renin-angiotensin system (RAS) (Murray *et al.* 1985; Edwards *et al.* 1994; Burdmann *et al.* 1995; Shihab *et al.* 1996), enhanced sympathetic tone (Murray *et al.* 1985) or increased synthesis of endothelins (Fogo *et al.* 1992; Lanese and Conger 1993). Use of CsA is also reported to be related to increased vasoconstriction (Murray *et al*. 1985) and vascular endothelial dysfunction (De Nicola *et al.* 1993; Amore *et al.* 1995; Stephan *et al.* 1995). The renal and cardiovascular effects may be related to reduced prostacyclin (PGI_2) production (Neild *et al.* 1983), increased thromboxane synthesis (Perico *et al*. 1986), transforming growth factor-β expression (TGF-β_1) (Wolf *et al*. 1995) or leukotriene production (Butterly *et al.* 2000). Some studies suggest a defect in intracellular calcium handling (Lo Russo *et al.* 1996), magnesium deficiency (Mervaala *et al.* 1997), decreased renal dopamine production (Pestana *et al.* 1995), induction of cytochrome P450 isoenzymes in renal microsomes (Basu *et al.* 1994; Nakamura *et al.* 1994), oxidative stress (De Nicola *et al*. 1993) or rise in serum cholesterol level (Satterthwaite *et al.* 1998) as possible mechanisms of renal and cardiovascular effects of CsA. Finally, a renal defect in sodium handling may participate in the CsA toxicity (Curtis *et al.* 1988; Curtis 1994).

Many of the clinically seen adverse effects of CsA have been difficult to produce in animals. Sodium-depleted normotensive Sprague Dawley rats have been used to study the nephrotoxicity of CsA (Burdmann *et al.* 1995; Pichler *et al.* 1995; Porter *et al.* 1999). In this rat model, a fairly high dose of CsA (15 mg/kg/day s.c.) causes morphologically similar renal toxicity, mainly characterised by tubulointerstitial fibrosis of striped pattern and arteriolopathy of the afferent arterioles, to that

seen in CsA-treated patients. However, sodium-depleted Sprague Dawley rats develop no hypertension, which is a frequent adverse effect in patients.

To study the hypertensive mechanisms of CsA, a new rat model has been developed by Mervaala *et al.* (1997). In spontaneously hypertensive rats (SHR) on high-sodium diet, long-term treatment with CsA (5 mg/kg/day s.c.) produces clinically relevant plasma concentrations and markedly elevates blood pressure concomitantly with kidney dysfunction and histological renal damage. The present study was aimed at investigating the mechanisms of CsA toxicity using the SHR model, which is a well-known genetic hypertension model with many similarities to human essential hypertension (Okamoto *et al.* 1964; Phillips 1999). The study focused on the role of RAS, the kallikrein-kinin system, the L-arginine–nitric oxide (NO) pathway, oxidative stress and vascular changes in renal and cardiovascular effects of CsA.

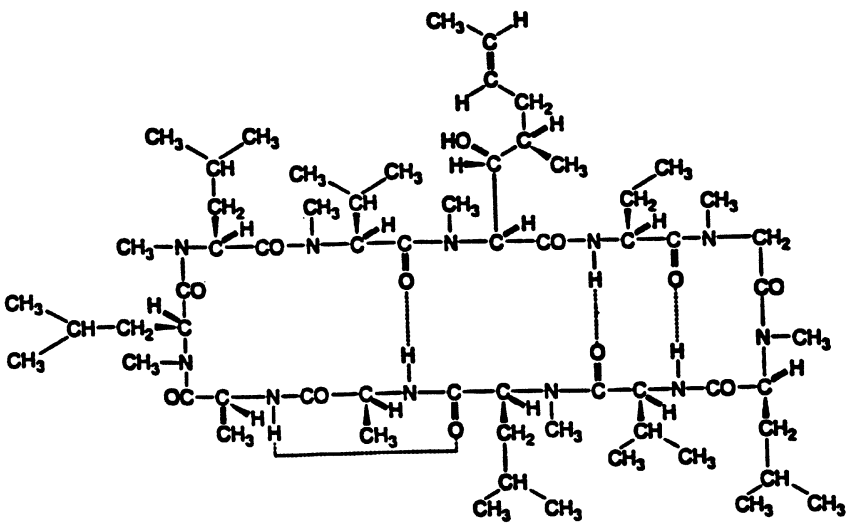

Figure 1. Chemical structure of cyclosporine A.

Table 1. Proposed mechanisms of cyclosporine A-induced renal and cardiovascular toxicity.

Mechanism	**Reference**
activation of the renin-angiotensin system	Murray *et al.* 1985; Edwards *et al.* 1994; Burdmann *et al.* 1995; Shihab *et al.* 1996
vasoconstriction	Murray *et al*. 1985; Epstein *et al.* 1998
defect in intracellular calcium handling	Lo Russo *et al.* 1996; Avdonin *et al.* 1999
endothelial dysfunction	De Nicola *et al.* 1993; Amore *et al.* 1995; Stephan *et al.* 1995
increased endothelin synthesis	Fogo *et al.* 1992; Lanese and Conger 1993
reduced prostacyclin production	Neild *et al.* 1983
increased tromboxane production	Perico *et al*. 1986
increased leukotriene production	Butterly *et al.* 2000
decreased dopamine synthesis	Pestana *et al.* 1995
oxidative stress	De Nicola *et al*. 1993
induction of cytochrome P450 isoenzymes	Nakamura *et al.* 1994
enhanced sympathetic tone	Murray *et al.* 1985; Scherrer *et al*. 1990
renal defect in sodium handling	Curtis *et al.* 1988; Curtis 1994
induction of transforming growth factor-β_1	Wolf *et al*. 1995
magnesium deficiency	Mervaala *et al.* 1997
changes in serum cholesterol level	Satterthwaite *et al.* 1998

2. REVIEW OF THE LITERATURE

2.1. General aspects of cyclosporine A

CsA is a cyclic endecapeptide which is derived from extracts of *Tolypocladium inflatium Gams*, a member of the *Fungi imperfekti* family (Figure 1) (Kahan 1999).

> "The high treeless plain where this fungus was discovered, Hardarger Vidda, is a land populated by malevolent spirits. One man, Askelad, a Nordic folk hero, used his ingenuity and perseverance to subvert the spirits' schemes and preserve the peace and harmony of the community" (Kahan 1999).

Since the early 1980's, CsA has been widely used to prevent rejection of organ transplants. It has increased graft survival and decreased patient mortality (Kahan *et al.* 1987; 1999). The use of CsA to treat autoimmune diseases has increased during recent years (Bach 1999). In Finland, indications of CsA include rheumatoid arthritis, psoriasis, atopic dermatitis, endogenous uveitis and nephrotic syndrome. The dose of CsA in autoimmune diseases is usually lower than that used in organ transplantations (Bach 1999). Unlike in organ transplantations, in autoimmune diseases the use of CsA is often temporary.

CsA was the first T-lymphocyte selective drug to inhibit lymphocyte function without concomitant myelosuppression. CsA binds to cyclophilin to inhibit phosphatase calcineurin, and thereby prevents the dephosphorylation of nuclear factor of activated T-cells that is essential for upregulation of messenger ribonucleic acid (mRNA) (Figure 2) (Liu *et al.* 1992). Thus, CsA interferes with the synthesis of many lymphokine mediators, particularly interleukin-2, which is critical for T-lymphocyte proliferation and maturition, and interferon-γ, which is critical for macrophage activation. It has recently been proposed that increased production of TGF-β_1 is at least partly responsible for the immunosuppressive effects of CsA (Hojo *et al.* 1999; Nabel 1999). The mechanism for the induction of TGF-β_1 by CsA remains obscure (Nabel 1999).

The extent to which the adverse effects of CsA are related to the immunosuppressive mechanisms of the drug is controversial. Inhibition of calcineurin by CsA may alter the production of many biologically active agents, such as endothelin, NO and TGF-β_1, which have been implicated in adverse renal and cardiovascular effects (Curtis 1998; Hemenway and Heitman 1999). On the other hand, calcineurin inhibition may protect against the development of left ventricular hypertrophy (Sussman *et al.* 1998).

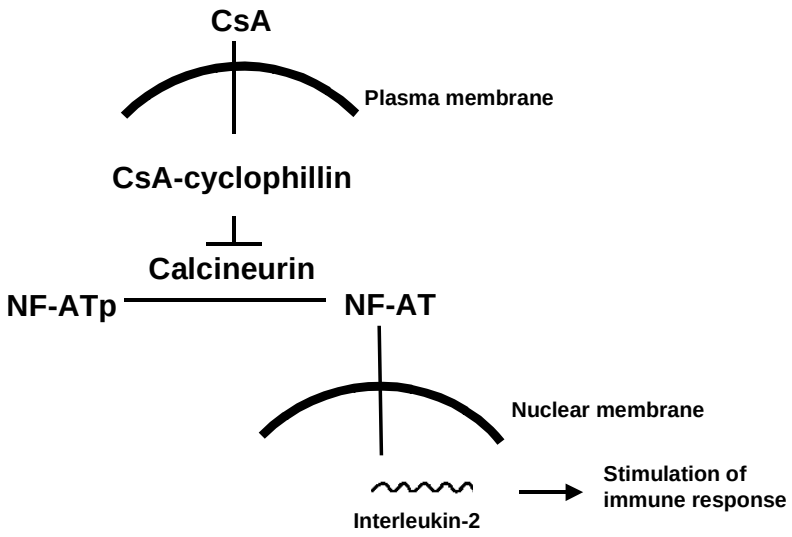

Figure 2. Main mechanism for the immunosuppressive action of cyclosporine. CsA, cyclosporine; NF-AT, nuclear factor of activated T-cells; p, phosphate group. Adapted from Nabel (1999).

2.2. Adverse renal and cardiovascular effects of cyclosporine A

2.2.1. Human studies

The most important adverse effects that restrict the use of CsA are nephrotoxicity and hypertension (Mason 1989; Taler *et al.* 1999). These effects have been detected in transplant recipients and in autoimmune patients. Other factors besides CsA may, however, also contribute to these abnormalities, and thus, determining the effects of CsA alone is difficult. In transplant patients, these confounding factors include rejection of the transplanted organ and concomitant

glucocorticoid and other immunosuppressive treatments. In some autoimmune patients, for example those suffering from rheumatoid arthritis, the disease itself may cause renal and cardiovascular sequalae (Bach 1999).

A single dose of CsA causes a transient rise in blood pressure in healthy volunteers (Hansen *et al.* 1997). Long-term CsA treatment in organ transplant recipients increases the risk for hypertension compared with the therapy with azathioprine and glucocorticoids (Kahan *et al.* 1987; Schorn *et al.* 1988; Taler *et al.* 1999). CsA also induces hypertension in autoimmune patients (Bach 1999). While the incidence of hypertension is 29–54% in CsA-treated autoimmune patients, in transplant patients the corresponding figure is 71–100% (Taler *et al.* 1999). Hypertension is an independent risk factor for atherosclerosis in CsA-treated transplant patients (Kasiske 1988). Furthermore, hypertension after transplantation is related with lower rates of graft survival (van Ypersele de Strihou *et al.* 1983). Hypertension is usually reversible after discontinuation of short-term CsA therapy (Taler *et al.* 1999), whereas continued treatment even at reduced doses frequently results in sustained hypertension (Schwartz *et al.* 1996).

Adverse renal effects of CsA can be divided into two categories: functional and histological abnormalities. CsA-induced renal dysfunction is related to reduced glomerular filtration rate and renal blood flow. This is generally thought to be secondary to vasoconstriction of the glomerular afferent arterioles, which causes a decrease in glomerular pressure (Murray *et al.* 1985; Myers *et al.* 1988; Hansen *et al.* 1997; Andoh and Bennett 1998). Renal dysfunction, in turn, causes an increase in serum creatinine concentration and a decrease in creatinine clearance. These functional changes are dose-dependent and are usually reversible after short-term CsA treatment (Andoh and Bennett 1998).

CsA-induced histological renal damage is mainly characterised by tubulointerstitial fibrosis of striped pattern and arteriolopathy of the afferent arterioles (Antonovych *et al.* 1988; Mihatsch *et al.* 1988; Andoh and Bennett 1998). Interstitial fibrosis consists of streaks of fibrosis. Arteriolopathy is characterised by luminal narrowing and arteriolar wall thickening. These changes are composed of subendothelial edema, swelling of hypertrophied endothelial cells protruding into the lumen and eosinophilic granular transformation of vascular smooth muscle cells. However, other types of renal

lesions, including glomerular and tubular damage, have also been linked with CsA treatment (Antonovych *et al.* 1988; Andoh and Bennett 1998). The histological changes develop mainly during long-term use and are usually irreversible once established even if CsA treatment is ceased, but the risk of histological changes is diminished with reducing the dose of CsA (Andoh and Bennett 1998).

2.2.2. Animal studies

As a consequence of the resistance of rats to adverse renal and cardiovascular effects of CsA, there was very little if any preclinical information on them in early clinical use (for review, see Curtis 1994). Therefore, the adverse renal and cardiovascular effects of CsA were somewhat unexpected. A great deal of effort was put into developing suitable animal models to study the mechanisms of these CsA-induced abnormalities.

The adverse renal effects of CsA were slight or absent in normotensive rats on normal-sodium diet (Elzinga *et al.* 1993; Porter *et al.* 1999). An exception to this was that CsA in a year-long study induced tubulointerstitial fibrosis (Lafayette *et al.* 1993). To study the nephrotoxicity of CsA, a rat model using sodium-depleted normotensive Sprague Dawley rats was developed (Burdmann *et al.* 1995; Pichler *et al.* 1995; Young *et al.* 1995b; Porter *et al.* 1999). In this rat model, a fairly high dose of CsA (15 mg/kg/day s.c.) for four weeks caused morphological and functional renal toxicity similar to that seen in CsA-treated patients.

According to several studies, CsA does not induce hypertension in normotensive rats even if high doses of up to 50 mg/kg/day are used (Nahman *et al.* 1988; Diederich *et al.* 1992; Lafayette *et al.* 1993; Basu *et al.* 1994; Stephan *et al*. 1995). A moderate rise in blood pressure by CsA at 25 mg/kg/day has, however, been reported in some studies of normotensive rats (Takeda *et al.* 1995; Oriji and Keiser 1998). The SHR model is a well-known genetic hypertension model that has many similarities to human essential hypertension (Okamoto *et al.* 1964; Phillips 1999). A few previous studies of CsA with SHR have been reported. Interestingly, CsA at a dose of 10 mg/kg/day delayed the onset of hypertension in two-week-old SHR (Sitsen and de Jong 1987). In SHR in the developmental phase of hypertension, CsA at doses of 20–100 mg/kg/day induced elevation of

blood pressure and renal vascular lesions (Ryffel *et al.* 1986; Nahman *et al.* 1988). At 5 mg/kg/day, CsA caused a rise in blood pressure on normal-sodium diet (Basu *et al.* 1994; Mervaala *et al.* 1997). On high-sodium diet in SHR, CsA-induced hypertension manifests with concomitant renal dysfunction and glomerular damage (Mervaala *et al.* 1997; Pere *et al.* 1998).

2.3. Mechanisms of cyclosporine A toxicity

2.3.1. Renin-angiotensin system and kallikrein-kinin system

2.3.1.1. Renin-angiotensin system in control of cardiovascular functions

The renin-angiotensin system (RAS) is an important regulator of blood pressure and renal function. The most important effector of RAS is octapeptide angiotensin II (Figure 3). It is formed in an enzyme cascade initiated by renin, which is secreted from juxtaglomerular cells of the kidney. While RAS is a circulatory hormone system, all of its components also exist locally in tissues, e.g. in the kidney, blood vessel wall, heart, brain and adrenal cortex (for review, see Ganten *et al.* 1983; Kim and Iwao 2000). Therefore, RAS seems to be a regional regulator as well.

The main stimulators of renin release are decreased sodium concentration in the distal tubule, decreased renal perfusion pressure and adrenergic β_1-receptor stimulation. Angiotensin II, potassium, antidiuretic hormone and atrial natriuretic peptide reduce renin release. Renin cleaves angiotensinogen, which is derived from the liver, into inactive decapeptide angiotensin I (Kim and Iwao 2000).

There are different enzymatic routes of angiotensin II formation from angiotensin I (Figure 3). Angiotensin I is activated by angiotensin converting enzyme (ACE), which is mainly located on the surface of the vascular endothelium and the lung epithelium. Other enzymes, including chymase, which is present in the heart, can also convert angiotensin I to angiotensin II. The importance of these non-ACE-pathways is still controversial. In humans, the non-ACE pathways are reported to represent at least 40% of total angiotensin II formation (Hollenberg *et al.* 1998). Some evidence exists that ACE-independent pathways become quantitatively more important under pathological

conditions such as diabetes (Hollenberg *et al.* 1998). In rats, however, ACE seems to be the most important enzyme for angiotensin II formation (Okunishi *et al.* 1993).

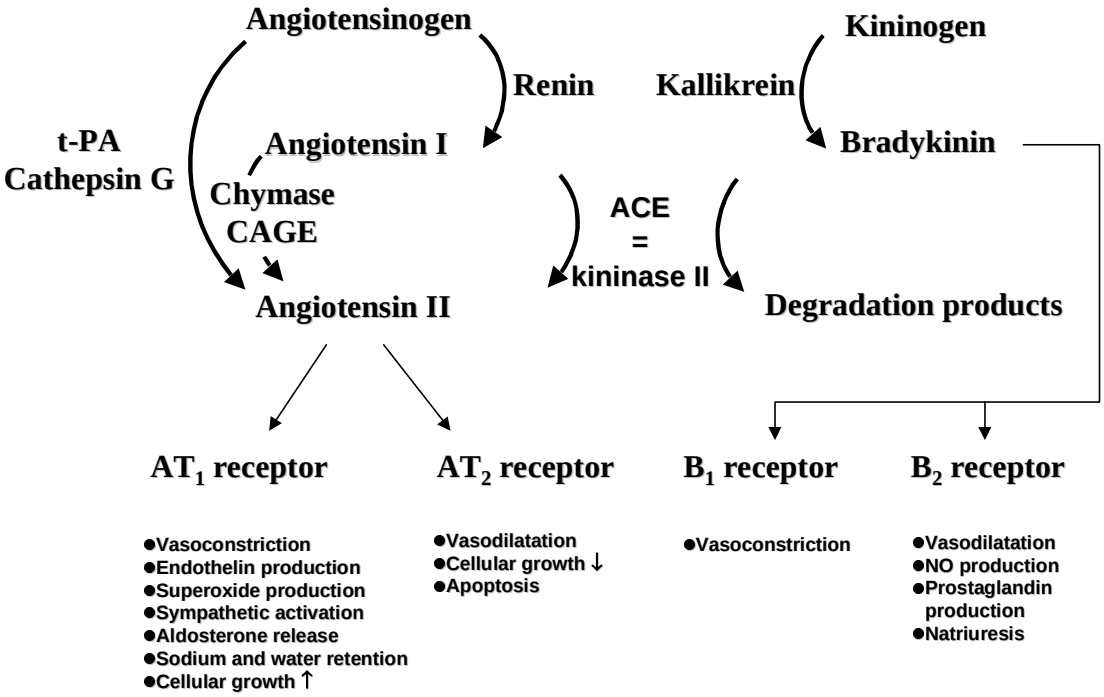

Figure 3. Renin-angiotensin system and kallikrein-kinin system. ACE, angiotensin converting enzyme; CAGE, chymostatin-sensitive angiotensin II-generating enzyme; NO, nitric oxide; t-PA, tissue plasminogen activator.

At least two types of angiotensin receptors are present on the surface of the target cells: Angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors (for review, see Stroth and Unger 1999). AT_1 receptors can be further sudivided into subtype A (AT_{1A}) and subtype B (AT_{1B}) receptors. AT_{1A} receptor is the major subtype mediating cardiovascular and renal effects of angiotensin II (Llorens-Cortes *et al.* 1994). AT_1 receptors are G-protein-coupled receptors with several second-messenger systems, including activation of phospholipase C. This results in activation of the phosphoinositol pathway, which leads to increased intracellular calcium levels and protein kinase C activation. These, in turn, activate growth-related immediate transcription factors, such as c-fos, c-myc and c-jun. Furthermore, inhibition of adenylate cyclase by angiotensin II reduces cyclic adenosine 3', 5'-

monophosphate (cAMP) levels. AT_2 receptors are also coupled to G-proteins, but the signal transduction pathways are not fully known (Stroth and Unger 1999).

Most of the known cardiovascular effects of angiotensin II are mediated via AT_1 receptors. These include contraction of vascular smooth muscle, enhancement of calcium sensitivity of the contractile apparatus of the smooth muscle, increased endothelin production, facilitation of noradrenaline biosynthesis and release and inhibition of its reuptake in sympathetic nerve terminals, stimulation of catecholamine release from the adrenal medulla, positive inotropic and chronotropic action on the heart and facilitation of aldosterone biosynthesis and secretion in the adrenal cortex. Furthermore, AT_1 receptor stimulation augments tubular sodium reabsorption in the kidney, inhibits renin release, induces polydipsia and releases vasopressin from the pituitary gland (for review, see Stroth and Unger 1999; Kim and Iwao 2000).

Growing evidence shows that AT_2 receptors are also important in controlling the cardiovascular system (for review, see Stroth and Unger 1999). It seems that most of the effects mediated by AT_2 receptors are the opposite of those mediated by AT_1. AT_2 receptor knock-out mice develop an increase in blood pressure and an increased sensitivity to the pressor actions of angiotensin II (Ichiki *et al.* 1995). The stimulation of AT_2 receptors is proposed to antagonise the AT_1-mediated blood pressure increase (Scheuer and Perrone 1993), to cause vasodilation and an inhibition of angiogenesis (Munzenmaier and Greene 1996) and to increase endothelial NO production (Seyedi *et al.* 1995). *In vitro* evidence shows that stimulation of AT_2 receptors may cause apoptosis of cells (Yamada *et al.* 1996). However, it seems that both receptor subtypes can mediate vascular apoptosis in rats *in vivo* (Diep *et al.* 1999).

Even though RAS has an important role in regulating blood pressure, its role in hypertension is unclear. In some renovascular forms of hypertension, activation of RAS is the major reason for the development of hypertension. Otherwise, increased activity of RAS, measured as plasma renin activity (PRA) elevation or as increased plasma angiotensin II concentrations, is an irregular finding in SHR or in human essential hypertension (Paran *et al.* 1995). However, antagonism of RAS by ACE inhibitors and AT_1 receptor antagonists is effective in reducing blood pressure in SHR and in hypertensive patients (Timmermans and Smith 1996; Gavras 1997).

Moreover, angiotensin II may play a role in the development of renal and vascular damage independently of blood pressure. Via AT_1 receptors, angiotensin II can cause cell growth, differentiation and proliferation directly by affecting several kinase pathways and indirectly by inducing several growth factors, including TGF-β_1 and platelet-derived growth factor (for review, see Stroth and Unger 1999).

2.3.1.2. Kallikrein-kinin system in control of cardiovascular functions

The kallikrein-kinin system plays a role in controlling blood pressure, renal haemodynamics and excretory functions (for review, see Majima and Katori 1995). Tissue and plasma kallikreins generate kinins from kininogens by hydrolysis (Figure 3). Kinins are oligopeptides that contain the bradykinin sequence in their structure. There are two types of bradykinin receptors, B_1 and B_2 receptors. G-protein-coupled B_2 receptors mediate most of the known effects of bradykinin. The activation of B_2 receptors causes phospholipase C activation and thus an increase in intracellular calcium concentration which stimulates the production of NO and vasoactive prostanoids such as prostaglandin E_2 (PGE_2) and PGI_2. (Linz *et al.* 1995). The effects mediated via B_1 receptors are not well known, but recent evidence suggests that activation of these receptors results in vasoconstriction and decreased glomerular filtration rate (Schanstra *et al*. 2000). The expression of B_1 receptors is reported to be induced during inflammation in rats (Schanstra *et al*. 2000) and in renal disease in patients (Naicker *et al.* 1999). Kinins are destroyed by kininases. The two main kininases are kininase II, which has been found to be identical with ACE, and neutral endopeptidase.

A decreased activity of the renal kallikrein-kinin system has been found in essential hypertension and in rat models of genetic hypertension (for review, see Majima and Katori 1995). Thus, the renal kallikrein-kinin system may have a crucial role in the development of hypertension.

The cardiovascular effects of the kallikrein-kinin system have been studied, e.g., by using B_2 receptor antagonist icatibant (HOE 140). Inhibition of bradykinin breakdown by ACE inhibitors leads to increased B_2 receptor activation. Icatibant has been reported to partly antagonise the antihypertensive effects of ACE inhibitors in experimental animals (Bao *et al.* 1992; O'Sullivan and

Harrap 1995). Therefore, increased availability of bradykinin seems to be responsible for some of the beneficial effects of ACE inhibitors.

2.3.1.3. Cyclosporine A and renin-angiotensin system

Several lines of evidence suggest an involvement of RAS in CsA toxicity. In rats, activation of RAS by CsA is a consistent finding. An increase in PRA has been demonstrated in CsA-treated rats on sodium-depletion (Burdmann *et al.* 1995), normal-sodium (Abassi *et al.* 1996) or high-sodium diets (Mervaala *et al.* 1997). Increased PRA by CsA has been related to increased renin expression in the juxtaglomerular apparatus (Young *et al.* 1995a). *In vitro*, CsA stimulated renin production and release from juxtaglomerular cells (Kurtz *et al.* 1988). The mechanism for this direct effect is obscure, but it is proposed to be linked to cyclophilin binding (Kurtz *et al.* 1988; Lee 1997). Furthermore, CsA increased local expression of angiotensin II in the outer medulla and medullary rays (Ramirez *et al.* 2000).

CsA has been reported to elevate PRA in cardiac and liver transplant patients (Julien *et al*. 1993) and plasma angiotensin II levels in patients with psoriasis (Edwards *et al.* 1994). Data of the effects of CsA on RAS in man are, however, to some extent contradictory; normal or even low PRA have been reported (Bantle *et al.* 1987; Myers *et al.* 1988; Mason *et al.* 1991). There is evidence of CsA-induced juxtaglomerular hyperplasia related to increased total renin content and impaired intrarenal conversion of inactive prorenin to active renin (Bantle *et al.* 1987; Myers *et al.* 1988; Mason *et al.* 1991). CsA may raise angiotensin II levels by increasing the activity of ACE which has been detected in serum of patients (Letizia *et al*. 1995) as well as in serum and in lung tissue of rats (Erman *et al*. 1990). Therefore, the lack of increase in PRA in some clinical studies does not exclude activation of RAS. It has been suggested that CsA causes activation of tissue RAS, which is not always seen as a change in PRA (Mason *et al.* 1991; Young *et al.* 1995a; Ramirez *et al.* 2000).

Chronic administration of angiotensin II in rats produces renal injury resembling that observed in CsA nephropathy in man (Giachelli *et al.* 1994; Noble and Border 1997). Both angiotensin II and CsA cause an overexpression of TGF-β_1, a growth factor which has been implicated in the pathophysiology of many fibrotic diseases of the kidney and other organs (Pichler *et al.* 1995; Noble

and Border 1997). In addition, both angiotensin II (Giachelli *et al.* 1994) and CsA (Pichler *et al.* 1995) increase tubulointerstitial expression of osteopontin, an adhesion molecule associated with renal fibrosis. Thus, activation of RAS by CsA seems to be at least partly responsible for interstitial fibrosis through stimulation of TGF-β_1 and osteopontin expression.

Antagonism of RAS by ACE inhibitors and AT_1 receptor antagonists lowers blood pressure in CsA patients (Mourad *et al*. 1993; Sennesael *et al*. 1995; Hannedouche *et al*. 1996; Del Castillo *et al*. 1998; Taler *et al*. 1999). Clinical studies suggest that the CsA-induced hypertension can be treated with several classes of antihypertensive drugs including ACE inhibitors, AT_1 receptor antagonists, calcium antagonists and adrenergic β-receptor antagonists. However, some studies suggest that ACE inhibitors alone have limited antihypertensive effects directly after transplantation (Textor *et al.* 1988; Taler *et al*. 1999).

In normotensive rats during sodium deprivation, antagonism of RAS with enalapril and losartan prevented CsA-induced morphological nephrotoxicity without affecting blood pressure (Burdman *et al.* 1995; Pichler *et al.* 1995). In the same animal model, arteriolopathy and interstitial fibrosis were diminished by RAS inhibition with enalapril and losartan, but not by some other classes of antihypertensive drugs such as calcium antagonist nilvadipine, vasodilatator dihydralazine or diuretic furosemide (Shihab *et al*. 1997), while blood pressure remained unaffected by any of the treatments. AT_1 receptor antagonist losartan reduced CsA-induced interstitial fibrosis in sodium-deprived rats concomitantly with decreasing renal TGF-β_1 and osteopontin expression (Pichler *et al.* 1995). Additionally, in a one-year study of normotensive rats on normal-sodium diet, CsA caused tubulointerstitial fibrosis without affecting blood pressure (Lafayette *et al.* 1993). Both concomitant enalapril and a combination of potassium channel opener minoxidil, monoamine transporter inhibitor reserpine and diuretic hydrochlorothiazide lowered blood pressure, but only enalapril prevented morphological damage of the kidneys. These studies suggest that angiotensin II has a specific role in the development of CsA-induced morphological renal damage. The effect of angiotensin II seems to be, at least partly, independent of blood pressure changes.

The effects of drugs suppressing RAS on CsA-induced renal dysfunction are unclear. Theoretically, it is possible that suppression of RAS might be harmful. CsA reduces the glomerular filtration rate

and renal blood flow by causing vasoconstriction of the glomerular afferent arterioles (Murray *et al.* 1985; Myers *et al.* 1988). When renal blood flow is substantially reduced, angiotensin II contributes to the maintenance of the glomerular filtration rate by constricting the efferent glomerular arterioles (Hall *et al.* 1999). Under such conditions, dilation of the efferent arterioles by drugs suppressing RAS may potentiate the reduction in the glomerular filtration rate.

In sodium-depleted normotensive rats, enalapril and losartan prevented CsA-induced renal morphological changes but not the decrease in glomerular filtration rate (Burdmann *et al.* 1995; Kon *et al.* 1995; Pichler *et al.* 1995). In this rat model, it seems that renal dysfunction is related to activation of the endothelin system, whereas morphological damage is related to activation of RAS (Burdmann *et al.* 1995; Kon *et al.* 1995; Pichler *et al.* 1995). There are case reports in which ACE inhibitors have worsened the renal function of CsA-treated kidney transplant patients (Garcia *et al.* 1994). In most human studies, however, ACE inhibitors have lowered blood pressure, but have not affected renal function in CsA-treated renal-allograft patients (Sennesael *et al.* 1995; Hausberg *et al.* 1999). Furthermore, enalapril prevented the CsA-induced decline in glomerular filtration rate in diabetic patients (Hannedouche *et al.* 1996). Likewise, in CsA-treated normotensive rats on normal-sodium diet, ACE inhibitor captopril and AT_1 receptor antagonist saralasin increased glomerular filtration rate and renal blood flow (Kaskel *et al.* 1987). Taken together, it seems that suppression of RAS is not harmful for renal function of all CsA-treated patients and may in fact improve it in some cases. However, a subgroup of patients may exist, for example, kidney transplant patients, who are more susceptible than others to renal dysfunction due to cotreatment of CsA and a drug suppressing the activity of RAS.

CsA may cause cardiovascular effects via RAS by mechanisms which are independent of the rate of angiotensin II formation. Long-term treatment with CsA upregulated AT_1 receptors in rat vascular and renal tissues (Iwai *et al.* 1993; Regitz-Zagrosek *et al.* 1995). Similarly, CsA-incubation upregulated AT_1 receptors in human vascular cells and potentiated the rise in intracellular calcium concentration in response to angiotensin II (Avdonin *et al.* 1999). According to culture studies of rat vascular smooth muscle cells, it seems that CsA increases the intracellular calcium concentration by several mechanisms: CsA stimulated transmembrane calcium influx (Lo Russo *et al.* 1996; Meyer-Lehnert *et al.* 1997), and augmented angiotensin II-stimulated calcium influx and efflux

(Pfeilschifter and Ruegg 1987). There is evidence that CsA may also mobilise calcium from the intracellular stores by activating the phosphoinositol pathway (Lo Russo *et al.* 1997). In addition, calcium may be released from intracellular stores by CsA by a mechanism which does not involve activation of the phosphoinositol pathway, but is due to a direct effect on intracellular calcium stores (Gordjani *et al*. 2000). In accordance with the above-mentioned mechanisms, repeated administration of CsA has been reported to increase the vasoconstrictive effect of angiotensin II (Auch-Schwelk *et al.* 1993; Takeda *et al.* 1995).

Very recently, a different view about the interaction of CsA and RAS has been presented. CsA antagonised the harmful effects of RAS overexpression on end-organ damage in double transgenic rats harbouring human renin and angiotensinogen genes (Mervaala *et al.* 2000). The protection was due to anti-inflammatory properties of CsA by inhibition of interleukin-6 and inducible nitric oxide synthase (iNOS) expression. It seems that CsA may not only cause renal and cardiovascular toxicity via induction of RAS, but can also antagonise inflammatory responses to various stimuli, including angiotensin II.

2.3.1.4. Cyclosporine A and renal kallikrein-kinin system

Only a few reports exist about the role of the renal kallikrein-kinin system in CsA toxicity. In normotensive rats, CsA at 20 mg/kg/day for three days decreased renal kallikrein and bradykinin B_2 receptor levels (Bompart *et al*. 1996). In another study, CsA-administration at 25 mg/kg/day for 28 days increased renal kallikrein and bradykinin B_2 receptor levels (Wang *et al.* 1997). This was proposed to occur as compensation for drug-induced vasoconstriction and hypertension. In patients with psoriasis, a reduced urinary kallikrein excretion was detected after six-month treatment with CsA (Raman *et al.* 1998). No studies of CsA with icatibant have been reported. Thus, the renal kallikrein-kinin system may have a role in CsA toxicity, but requires further studies before any conclusions can be drawn.

2.3.2. Endothelium, nitric oxide and oxidative stress

2.3.2.1. Role of endothelium in control of cardiovascular functions

The endothelial cell layer participates in the control of vascular tone and blood pressure by producing relaxing and contracting factors, thus modulating the tone of the underlying smooth muscle. Dysfunction of vascular endothelium, characterised by a decrease in endothelium-dependent relaxation, is shown in human essential hypertension and in various models of experimental hypertension (for review, see McIntyre *et al.* 1999). In SHR, the dysfunction occurs already before the onset of hypertension (Jameson *et al.* 1993). Impaired endothelial dysfunction is reported in the normotensive offspring of hypertensive patients (Taddei *et al.* 1996). Endothelial dysfunction can be improved by antihypertensive therapy in rats and in humans (for review, see Vapaatalo *et al.* 2000).

Nitric oxide (NO) is a lipophilic gas which is formed enzymatically from its precursor L-arginine by three isoforms of NO synthase (NOS), endothelial eNOS, neuronal nNOS and inducible iNOS (for review, see Stuehr 1999). NO diffuses to the vascular smooth muscle cells and activates guanylate cyclase, which leads to the increased production of cyclic guanosine 3',5'-monophosphate (cGMP) (Figure 4). cGMP-dependent kinase regulates several pathways involved in calcium homeostasis. NO also hyperpolarises vascular smooth muscle cells via cGMP-dependent pathway as well as direct effects on calcium-dependent potassium channels and Na^+-K^+ ATPase (for review, see Busse and Fleming 1995). *In vivo*, NO is destroyed mainly by superoxide anion (for review, see Stuehr 1999). Because of its short half-life (0.1 seconds *in vivo*), NO acts primarily as a paracrine hormone (Loscalzo and Welch 1995). Activity of NOS isoforms is regulated in different ways. Constitutive NO production by eNOS is controlled by intracellular calcium concentration following physical (shear stress) or chemical stimuli of various receptors for endogenous substances. iNOS is controlled mainly by inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (Rao 2000). High amounts of NO are released by iNOS in response to inflammatory stimuli from a variety of cell types (Rao 2000).

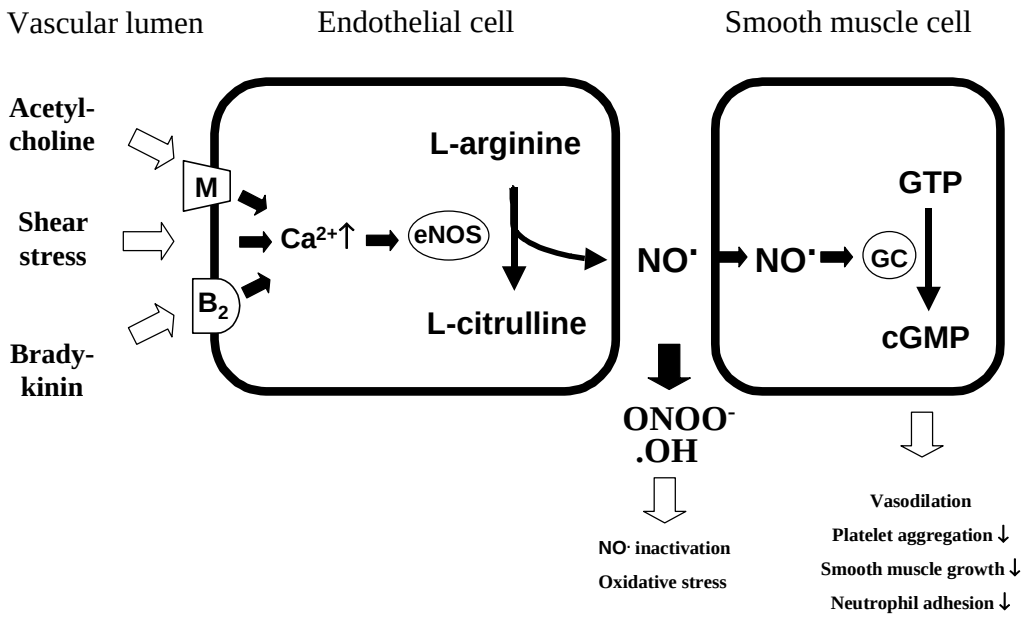

Figure 4. L-arginine–nitric oxide system. B_2, bradykinin B_2 receptor; cGMP, cyclic guanosine 3',5'-monophosphate; eNOS, endothelial nitric oxide synthase; GC, guanylate cyclase; GTP, guanosine 5'-triphosphate; M, muscarinic receptor; NO, nitric oxide; .OH, hydroxyl radical; $ONOO^-$, peroxynitrite.

In addition to NO, prostanoids have a major role in the control of vascular function. Prostanoids, including PGI_2 and PGE_2, are produced from arachidonic acid, which is liberated from the cell membrane phospholipids by phospholipase A_2. The effects of PGI_2, the most important vasodilating prostaglandin, are mediated by membrane receptors on the smooth muscle cells. Stimulation of these receptors activates adenylate cyclase, which leads to increased intracellular concentrations of cAMP, and further to hyperpolarisation of the cell membrane, reduction of intracellular calcium concentration and decreased sensitivity of contractile proteins to calcium (for review, see Cohen and Vanhoutte 1995).

Recent evidence suggests that a factor called endothelium-derived hyperpolarising factor contributes to endothelium-mediated vasodilation by increasing the conductance of smooth muscle cells to potassium ions (for review, see Cohen and Vanhoutte 1995). The release of endothelium-derived

hyperpolarising factor is believed to be initiated by an increase in free calcium concentration in the endothelial cell. The chemical structure of the substance or possibly of several different substances is not known. Endothelium-derived hyperpolarisation may, at least partly, account for nonprostanoid products of arachidonic acid metabolism (Cohen and Vanhoutte 1995; Fisslthaler *et al.* 2000).

The endothelium is also a source of contracting factors. A gene codes for three isoforms of endothelins (1, 2 and 3), which are generated in two steps from preproendothelins. First, neutral endopeptidase forms the intermediate product proendothelins, which are further activated by endothelin converting enzyme (Benigni and Remuzzi 1999). They act via two receptor subtypes (A and B). Other important endothelium-derived contractile factors include cyclooxygenase product thromboxane A_2 and prostaglandin endoperoxide intermediates prostaglandin G_2 and H_2 (Cohen 1995). All of these substances contract smooth muscle cells via thromboxane A_2 receptors (Cohen 1995).

2.3.2.2. Oxidative stress

Endothelial cells are both significant sources and targets of reactive oxygen products, including superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite (for review, see McIntyre *et al.* 1999). These products can inhibit vascular relaxation by neutralising the effects of NO, but they may also have direct vasoconstrictive properties (Cosentino *et al.* 1994). The main sources of reactive oxygen products are respiratory chain enzymes in the mitochondria, such as aldehyde oxidase, dihydroorotic dehydrogenases, flavin dehydrogenases, peroxidases, cyclooxygenase, NOS, and auto-oxidation of a large group of compounds including catecholamines, flavins and ferredoxin. Many enzymes, including superoxide dismutases, can inactivate reactive oxygen products (McIntyre *et al.* 1999).

Increased superoxide production has been shown in endothelial cells from stroke-prone SHR (Grunfeld *et al.* 1995). In addition, aortic rings from SHR are more sensitive to contractions induced by oxygen-derived free radicals than those from Wistar-Kyoto rats (Auch-Schwelk *et al.* 1989). Increased vascular oxidative stress has also been demonstrated in SHR *in vivo* (Suzuki *et al.* 1995; 1998). These studies suggest that genetic hypertension is associated with increased oxidative stress.

Endothelial dysfunction was earlier thought to be attributed mostly to decreased production of NO either due to decreased activity of eNOS or to a deficiency in the availability of L-arginine (Palmer *et al.* 1988). L-arginine causes vasodilation and lowering of blood pressure in experimental animals and in man (for review, see Li and Förstermann 2000). However, recent evidence suggests that impaired endothelium-dependent relaxation is associated with increased rather than decreased expression of eNOS in vasculature (Boulomie *et al.* 1997). eNOS can also catalyse superoxide formation in a reaction which is primarily regulated by cofactor tetrahydrobiopterin rather than L-arginine (Vasquez-Vivar *et al.* 1998). Thus, it seems that for normal endothelial function the balance between NO and superoxide anion is more important than the absolute levels of either alone (McIntyre *et al.* 1999).

2.3.2.3. Effects of cyclosporine A on vascular function

Repeated *in vivo* administration of fairly high doses of CsA has been found to impair endothelium-dependent relaxation to acetylcholine in the rat aorta (Mikkelsen *et al.* 1992; Auch-Schwelk *et al.* 1994; Verbeke *et al.* 1995; Kim *et al.* 1996), in femoral arteries (Gallego *et al.* 1994) and in mesenteric arteries (Rego *et al.* 1990). In CsA-treated transplant recipients, renal vasodilatory response to L-arginine infusion was reduced with a concomitant lowering of urinary nitrate excretion (Gaston *et al.* 1995). Likewise, CsA treatment in rheumatoid arthritis patients attenuated the vasodilatory responses to isoprenalin and PGE_2 (Stein *et al.* 1995). CsA inhibited endothelium-dependent relaxation *in vivo* in liver transplant recipients (Albillos *et al.* 1998) and *in vitro* in subcutaneous resistance vessels obtained from normal subjects undergoing routine surgery (Richards *et al.* 1990).

Some evidence suggests that endothelial dysfunction is due to direct toxicity of CsA on endothelial cells. CsA-induced dysfunction was associated with concomitant endothelial damage in rats (Kim *et al*. 1996). In addition, incubation of bovine aortic endothelial cells with CsA at a therapeutically relevant concentration of 1 μM resulted in cell lysis (Zoja *et al.* 1986).

CsA has been reported to impair endothelium-independent relaxations in rats (Gallego *et al*. 1993; Kim *et al*. 1996), but endothelium-independent relaxations have also been reported to remain

unaltered (Oriji and Keiser 1998). Response to nitroglycerin was unchanged by CsA in patients (Stein *et al.* 1995).

CsA contracted renal arteries and arterioles of rats *in vitro* (Lanese *et al.* 1994; Epstein *et al.* 1998). Repeated administration of CsA has been reported to increase (Rego *et al.* 1990), decrease (Roullet *et al.* 1995) or have no effect (Ressureicao *et al.* 1995) on contractile responses in mesenteric arteries of rats. Likewise, repeated administration of CsA has been reported to sensitise renal arteries of rats to noradrenaline (Mikkelsen *et al.* 1992), but this has not been confirmed by all studies (Diederich *et al.* 1992). In patients, contractile responses to noradrenaline remained unaltered by CsA (Stein *et al.* 1995).

Taken together, CsA causes vascular dysfunction *in vivo* which is characterised by decreased endothelial relaxations in man and in rats. In addition, CsA increases vascular contractility *in vitro*.

2.3.2.4. Effects of cyclosporine A on nitric oxide synthase

According to previous studies, CsA seems to have different effects on iNOS and eNOS expression (Table 2 and 3). Increased NO synthesis and eNOS mRNA and protein levels have been reported in endothelial cells exposed to CsA *in vitro* (Lopez-Ongil *et al*. 1996). The induction of eNOS seems to be due to transcriptional induction of the eNOS gene (Navarro-Antolin *et al.* 2000). CsA also caused monocyte eNOS upregulation in renal transplant patients (Calo *et al.* 1998) and eNOS overexpression in the rat kidneys (Amore *et al*. 1995) and in the renal cortex of uninephroctomised rats (Bobadilla *et al.* 1998). In addition, acute infusion of CsA into human forearm vessels increased NO activity *in vivo* (Stroes *et al*. 1997). However, long-term treatment with CsA did not affect renal or vascular eNOS expression in rats (Vaziri *et al.* 1998), suggesting that induction of eNOS by CsA is unclear *in vivo*.

Table 2. Effects of cyclosporine A on iNOS activity and/or expression. iNOS, inducible nitric oxide synthase; IL-1α, interleukin-1α; IL-1β, interleukin-1β; LPS, lipopolysaccaride; NOS, nitric oxide synthase; TNF-α, tumor necrosis factor-α.

iNOS

in vitro	**Cell type**	**Stimulus**	**Effect of CsA**	**Reference**
	murine peritoneal macrophages	LPS, IFN-γ	iNOS activity ↓	Conde *et al.* 1995
	murine J774 macrophages	LPS	iNOS activity ↓	Hattori and Nakanishi 1995
	rat renal mesangial cells	IL-1β	iNOS expression ↓	Kunz *et al.* 1995
	rat aorta,	IL-1α, TNF-α	iNOS expression and activity ↓	Marumo *et al.* 1995
	murine J774 macrophage and			
	vascular smooth muscle cells	LPS	iNOS expression and activity ↓	Dusting *et al.* 1999
	murine L929 fibroblasts	IFN-γ	iNOS expression ↓	Trajkovic *et al.* 1999

in vivo	**Animal model**	**Effect of CsA**	**Reference**
	uninephrectomised Wistar rats, 30 mg/kg, 7 days	iNOS mRNA ↓ in renal medulla	Bobadilla *et al.* 1998
	Sprague Dawley rats, 18 mg/kg, 3 weeks	renal and vascular iNOS expression ↓	Vaziri *et al.* 1998
	double transgenic rats harbouring human renin		
	and angiotensinogen genes, 5 mg/kg, 3 weeks	renal iNOS expression ↓	Mervaala *et al.* 2000

Table 3. Effects of cyclosporine A (CsA) on eNOS activity and/or expression. cGMP, cyclic guanosine 3',5'-monophosphate; eNOS, endothelial nitric oxide synthase; NOS, nitric oxide synthase.

in vitro	**Cell type**	**Effect of CsA**	**Reference**
	bovine aortic endothelial cells	eNOS expression and activity ↑	Lopez-Ongil *et al.* 1996
	bovine aortic endothelial cells	eNOS expression ↑	Navarro-Antolin *et al.* 1998
in vivo	**Animal model**	**Effect of CsA**	**Reference**
	healthy volunteers, acute infusion 75 µg/min	NOS activity ↑	Stroes *et al.* 1997
	uninephrectomised Wistar rats, 30 mg/kg, 7 days	eNOS expression ↑ in renal cortex	Bobadilla *et al.* 1998
	Sprague Dawley rats 25 mg/kg, 7 days	urinary nitrate + nitrite ↓, urinary cGMP↓	Oriji and Keiser 1998
	renal transplant patients	monocyte eNOS expression ↑, Plasma nitrate + nitrite ↑	Calo *et al.* 1998
	Sprague-Dawley rats; CsA 50 mg/kg, 1 day	eNOS activity ↓ in heart ventricles	Rao *et al.* 1998

CsA inhibited transcription of iNOS in murine macrophage and vascular smooth muscle cells (Dusting *et al.* 1999). In addition, depressed renal iNOS expression has been reported *in vivo* in rats after CsA treatment (Bobadilla *et al.* 1998; Vaziri *et al.* 1998; Mervaala *et al*. 2000). CsA also impaired iNOS in rat aortic smooth muscle cells (Marumo *et al.* 1995) and inhibited NO production in cultured macrophages (Conde *et al.* 1995).

Little is known about the effects of CsA on nNOS. Long-term CsA administration has been reported to decrease nNOS expression in uninephroctomised rats (Bobadilla *et al.* 1998).

L-arginine has been shown to have a beneficial effect on CsA-induced endothelial dysfunction (Gallego *et al*. 1993; Prieto *et al*. 1997; Oriji and Keiser 1998) and on renal haemodynamics in CsA-treated rats (De Nicola *et al*. 1993). Short-term treatment with L-arginine also reversed CsA-induced hypertension (Oriji and Keiser 1998).

2.3.2.5. Effects of CsA on oxidative stress

Several lines of evidence suggest that CsA-induced renal and cardiovascular toxicity may be due to oxidative stress. CsA increases hypoxia and free radical production in the rat kidney (Zhong *et al*. 1998). Antioxidants, such as α-tocopherol, ascorbate, lazaroids and superoxide dismutase/catalase, have been shown to diminish CsA-induced renal toxicity (Wang and Salahudeen 1994; Wolf *et al*. 1994).

The mechanisms and the role of oxidative stress in CsA toxicity are unknown. Free radicals may be derived directly from CsA or its metabolites (for review, see Buetler *et al.* 2000). Reperfusion subsequent to CsA-induced renal vasoconstriction and hypoxia may also increase formation of free radicals. One possible mechanism of CsA-evoked oxidative stress is eNOS hyperactivity. Chronically administered CsA increased eNOS activity in the rat kidney parallel with decreased renal function (Amore *et al*. 1995). The functional nephrotoxicity by CsA was prevented by L-arginine (Amore *et al*. 1995). The protective effect of L-arginine was reversed by NOS inhibitor, suggesting that NO was crucial in the effect of L-arginine (Amore *et al*. 1995). While the intracellular L-arginine is sufficient for basal NO production in endothelial cells (Arnal *et al*. 1995),

the substrate supply may be inadequate for the escalated demands of activated NOS. During insufficient L-arginine supply, the increase in NOS activity would not correspond to increased production of NO. Instead, NOS may generate reactive superoxide anion and other oxygen radicals (Stamler *et al*. 1992). L-arginine treatment might prevent this by shifting NOS activity toward NO generation and reduced oxidant injury.

Some mechanisms of CsA-induced radical oxygen species generation besides eNOS hyperactivity have been suggested. These include alterations in calcium homeostasis, leading to vasoconstriction and CsA metabolism by cytochrome P450 system (CYP450), more specifically, the CYP450 3A isoenzyme (Ahmed *et al.* 1995; Buetler *et al.* 2000).

2.3.3. Renal sodium handling

2.3.3.1. Role of renal sodium handling in blood pressure control

Kidneys play a pivotal role in the regulation of sodium balance. Several modulatory factors can control sodium excretion to a certain degree. These include RAS, aldosterone, the renal renal kallikrein-kinin system, dietary intake of potassium, calcium and magnesium, natriuretic peptides and endothelium-derived factors. According to the pressure-natriuresis theory presented by Guyton *et al.* (1972), an increase in dietary sodium beyond the control of these regulatory factors causes a rise in blood pressure to get rid of excess sodium. High sodium intake is one of the risk factors for development of hypertension in rats and in humans (Mervaala *et al.* 1992; Dyer *et al.* 1994).

2.3.3.2. Effects of cyclosporine A on renal sodium handling

In rats, short-term CsA administration reduced urinary excretion of sodium (Ryffel *et al*. 1986; Kaskel *et al*. 1987). Vasoconstriction of renal arterioles causes a reduction in perfusion pressure that may cause sodium retention and, therefore, a rise in blood pressure. In SHR, CsA caused a pronounced rise in blood pressure and renal dysfunction during high intake of sodium (Mervaala *et al.* 1997). In contrast, during normal-sodium diet, the blood pressure increasing effect of CsA was only moderate and no effect on renal function was observed. Evidence also exists for disturbances

in renal sodium handling playing a role in CsA-induced hypertension in patients. Sodium restriction has been shown to lower blood pressure in hypertensive transplant recipients treated with CsA, but not in hypertensive transplant recipients treated with azathioprine and glucocorticoids (Curtis *et al.* 1988; Curtis 1994). These findings together suggest that high intake of sodium is a risk factor for CsA-induced hypertension and renal toxicity.

3. AIMS OF THE STUDY

The exact mechanisms of cyclosporine A-induced hypertension and nephrotoxicity remain unknown. The renin-angiotensin system is proposed to play an important role in cyclosporine A toxicity, although some studies contradict this. The present study aimed at relating possible alterations in this system to functional and morphological changes in the vasculature and the kidney, also taking into consideration the kallikrein-kinin system, the L-arginine–nitric oxide pathway and oxidative stress. Spontaneously hypertensive rats on high-sodium diet were used as a model of cyclosporine A-induced hypertension and nephrotoxicity in patients.

The specific aims were to:

1. examine the long-term effects of cyclosporine A on blood pressure and on end-organ functions and morphology.

2. relate cyclosporine A-induced changes in vascular function with morphology.

3. investigate the role of the renin-angiotensin system and the renal kallikrein-kinin system in cyclosporine A toxicity.

4. evaluate the importance of the L-arginine–nitric oxide pathway and oxidative stress in cyclosporine A toxicity.

4. MATERIALS AND METHODS

4.1. Experimental animals

Male SHR (Harlan Sprague Dawley, Indianapolis, IN, USA) aged 7–9 weeks at beginning of the experiment were used. The rats were housed in a standard animal laboratory room (temperature 22–24°C, a 12-hour light-dark cycle). The rats had free access to tap water and feed, and were weighed daily during the experiment. All rats were placed on high-sodium diets during the six-week treatment (sodium 2.6%, magnesium 0.2%, potassium 0.8%, calcium 1.0%, phosphorous 0.75% of the dry weight of the feed; R36, Finnewos Aqua, Helsinki, Finland). The food was prepared by adding sodium chloride to the commercially available rat feed.

The protocols of the studies were approved by the Animal Experimentation Committee of the Institute of Biomedicine, University of Helsinki, Finland.

4.2. Drug treatments

CsA was administered subcutaneously at a dose of 5 mg/kg/day. This dose has been shown to result in CsA plasma concentrations similar to those measured in CsA-treated patients (Mervaala *et al.* 1997). Control rats received the same volume (1 ml/kg) of the vehicle. Enalapril and valsartan were mixed in the food to produce the planned daily doses. The dose of enalapril (30 mg/kg/day) was chosen to be comparable with previous studies to reduce blood pressure in SHR (Nunez *et al.* 1997). The higher dose of valsartan (30 mg/kg/day) has been reported to produce a marked antihypertensive effect, whereas the lower dose (3 mg/kg/day) caused a slight antihypertensive effect during long-term administration (Yamamoto *et al*. 1997). Icatibant was administered by a subcutaneous osmotic minipump for two weeks. The dose of icatibant (500 µg/kg/day) has been shown to inhibit the cardiovascular effects of bradykinin in rats (Bao *et al.* 1992; O'Sullivan and Harrap 1995). L-arginine (1.7 g/kg/day) in drinking water has been shown to antagonise the effects of CsA in Sprague Dawley rats during sodium deprivation (Andoh *et al.* 1997). The design of the individual studies is presented in Table 4.

Table 4. Design of the individual studies. CsA, cyclosporine A.

Study	**Drugs studied**		**Major measurements**
	DRUG 1	DRUG 2	
I	CsA	Enalapril	Blood pressure, renal function and morphology, heart morphology, plasma renin activity, urinary electrolytes
II	CsA	Enalapril, valsartan, Enalapril+icatibant	Blood pressure, renal function and morphology, plasma renin activity, urinary kallikrein and electrolyte excretion
III	CsA	Enalapril, valsartan, Enalapril+icatibant	Renal and mesenteric arterial functions
IV	CsA	L-arginine	Blood pressure, renal function and morphology, total radical trapping parameter, plasma urate, urinary 8-isoprostaglandin $F_{2\alpha}$, cyclic guanosine 3',5'-monophosphate and electrolyte excretion, nitric oxide synthase expression, arterial function and morphology, aortic smooth muscle cell cultures

4.3. Measurement of systolic blood pressure and heart rate

Systolic blood pressure and heart rate were measured every second week (I, II) or every week (IV) using a tail cuff blood pressure analyser (Apollo-2AB Blood Pressure Analyser, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). This method has been reported to show good correlations with parallel recordings from intra-arterial catheters in rats (Bunag and Butterfield 1982).

4.4. Collection of samples

During the last week of the experiment, the rats were housed individually in metabolic cages for 24–48 hours. The consumption of food and tap water was measured by weighing the feed and the water bottles, respectively. Urine was collected for biochemical determinations.

At the end of the experiments, the animals were made unconscious with CO_2/O_2 70/30% (AGA, Riihimäki, Finland) and decapitated 24 hours after the last CsA administration. Blood samples were taken into chilled tubes with or without EDTA (4.5 mM). The heart was excised, the great vessels, atria and free wall of the right ventricle were dissected and the left ventricular mass was weighed. The kidneys were washed with ice-cold saline and weighed.

4.5. Arterial responses

A 2 to 3-mm-long section of the right renal artery and a 3-mm-long section of the mesenteric artery 5 mm distally from the mesenteric artery-aorta junction were cut (III, IV). The rings were placed between stainless steel hooks, mounted in an organ bath chamber in Krebs-Ringer buffer and equilibrated for 30 min at 37°C, with a resting tension of 0.2 g for renal and 1.0 g for mesenteric arteries. The isometric force of contraction was registered with a polygraph (FTO3 transducer, Model 7P122E Polygraph; Grass Instrument, Quincy, MA, USA).

Cumulative concentration-response curves for noradrenaline (III, IV) and potassium chloride (IV) and the response to a single administration of angiotensin II (III) were determined. Angiotensin II

was administered only once to avoid tachyphylaxis (Khairallah *et al.* 1966). Cumulative relaxation responses to acetylcholine and sodium nitroprusside were examined after precontraction with noradrenaline (III, IV).

4.6. Histology

4.6.1. Sample preparation

Cross-sections of the kidney, a section of the mesenteric arterial trunk with the first- and second-order branches and the arterial bed, a section of the renal artery and coronal sections of the left ventricle were cut. The samples were fixed for 24–48 hours with 10% formaline and then dehydrated and embedded in paraffin. Thereafter, the samples were deparaffinised, hydrated and stained with Masson's trichrome. All slides were evaluated by light microscopy without knowledge of sample identity.

4.6.2. Kidneys

Interstitial, tubular and glomerular changes were looked for (I, II, IV). In study II, the renal slides were scored according to arterioglomerular changes using the method described by Pere *et al.* (1998): 100 consecutive glomeruli and their afferent arterioli from each kidney slide were assigned for severity of changes using scores from 0 to 3 (0=normal, 1=slight, 2=severe, 3=necrosis). Thereafter, a glomerular damage index was calculated by assessing 100 consecutive arterioglomerular units in each kidney and counting the number of affected glomeruli in each score group.

4.6.3. Arteries

Periadventitial, adventitial, medial and intimal changes were registered in renal and mesenteric vessels. Vessels from mesenteric beds were recorded by morphometric analysis with an image operating system (AnalySIS, 3.0 Soft Imaging System GmbH, Munich, Germany). The corrected area of the media was determined using the cross-sectional area of the media and a correction for

eccentricity of the sections (Lee *et al.* 1983). The corrected medial area was expressed as a ratio to the mean diameter of the whole vessel to exclude the effect of vessel size on the measured area. The mean axis was determined as a mean of the longest and shortest axes seen in the picture. On the basis of vascular diameter, the arteries were divided into two categories: less than 100 μm (resistance arteries and arterioles) and 100–400 μm (the first- and second-order branches from the superior mesenteric artery).

4.6.4. Heart

The effect of CsA and enalapril on vascular and myocardial changes of the heart were investigated (I).

4.7. Biochemical determinations

4.7.1. Urinary electrolytes

In study I, urinary sodium, potassium, magnesium, calcium and phosphorous concentrations were determined using a Baird PS-4 inductively coupled plasma emission spectrometer (Baird Co, Bedford, MA, USA). Otherwise, urinary magnesium concentration was measured using an atom absorption spectrophotometer (II) (BM/Hitachi 917 analyser, Boehringer Mannheim, Germany/Hitachi Ltd, Tokyo, Japan). Urinary sodium and potassium were determined by flame photometer using an ion selective electrode-compensator (II, IV) (human serum pool, IL Model 943, Instrumentarium Laboratory, Milan, Italy).

4.7.2. Plasma renin activity, urinary kallikrein and protein, serum and urinary creatinine

Plasma renin activity (PRA) was determined by a radioimmunoassay of angiotensin I (Medix Angiotensin I test®, Medix Biochemica, Kauniainen, Finland). Total protein concentration of urine was determined by the method of Lowry *et al*. (1951) after precipitation of proteins with 10% trichloroacetic acid. Urinary and serum creatinine were analysed by the Jaffe method (Bartels *et al.* 1972) (BM/Hitachi 917 analyser) without deproteinisation. Creatinine clearance as an index of glomerular filtration rate was calculated by dividing the average 24-hour urinary creatinine

excretion rate by serum concentration of creatinine. Urinary kallikrein activity was measured by amidolytic assay using a chromogenic tripeptide substrate H-D-valyl-L-leucyl-L-arginine-p-nitroaniline dihydrochloride (Amundsen *et al.* 1979).

4.7.3. Urinary cyclic guanosine 3',5'-monophosphate, tissue nitric oxide synthase expression, urinary 8-isoprostaglandin $F_{2\alpha}$, plasma total radical trapping parameter and urate

Urinary cGMP was measured by radioimmunoassay (Axelsson *et al.* 1988). eNOS and iNOS expression in heart, aorta, kidney medulla and kidney cortex were analysed by Western blotting using primary antibody mouse monoclonal anti-eNOS IgG_1 (1:2500, Transduction Laboratories, Lexington, KY, USA) or rabbit polyclonal anti-iNOS IgG (1:6000, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Urinary 8-isoprostaglandin $F_{2\alpha}$ was analysed by radioimmunoassay. Nonlabeled 8-isoprostaglandin $F_{2\alpha}$ was obtained from Cayman Chemical Co (Ann Arbor, MI, USA). 8-[^{125}I] isoprostaglandin $F_{2\alpha}$-tyrosine methyl ester and 8-isoprostaglandin $F_{2\alpha}$ antibody were supplied by Dr. István Mucha, Institute of Isotopes Co, Budapest, Hungary. Total radical trapping parameter (TRAP) was measured by a chemiluminescence method (Metsä-Ketelä 1991) using 2,2-azo-bis(2-aminopropane) hydrochloride (ABAP; Polysciences, Warrington, PA, USA) as peroxyl radical producer. A water-soluble tocopherol, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Hoffmann-La Roche Ltd, Basel, Switzerland) was used as a standard to calculate TRAP values (Wayner *et al.* 1987). Urate concentrations in plasma were measured colorimetrically (Sigma Chemical Co, St. Louis, MO, USA).

The urinary excretions and creatinine clearance were expressed per 100 g of body weight in studies II and IV.

4.8. Cell cultures

Smooth muscle cells were taken for cell culture from the aorta of the SHR on high-sodium diet which had received CsA or vehicle for six weeks (n=4 per group). The procedure for obtaining the

vascular smooth muscle cells for culture was slightly modified from the explant technique (Pang and Venance 1992). The viability of the cells was tested in a culture for 7 days. Cells from the third passage were used for the experiment. The cells from the control and CsA groups were plated in a concentration of 10^6 per petri dish. After seven days, the cells were detached from the dishes and counted.

4.9. Compounds

The following compounds were used: Cyclosporine A (Sandimmun® 50 mg/ml infusion concentrate, Novartis Ltd, Basel, Switzerland), valsartan (Novartis Ltd, Basel, Switzerland), enalapril maleate (Leiras Ltd, Turku, Finland), icatibant (d-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin, Hoechst AG, Frankfurt am Main, Germany), a lipid solution (Intralipid®, Kabi Pharmacia, Stockholm, Sweden), L-arginine, acetylcholine chloride, angiotensin II, noradrenaline bitartrate (Sigma Chemical Co), sodium nitroprusside dihydrate (F. Hoffmann-La Roche AG, Basel, Switzerland) and sodium pentobarbital (The University Pharmacy, Helsinki, Finland). The stock solutions of the compounds used in *in vitro* studies were dissolved in distilled water. All solutions were freshly prepared before use and protected from light.

4.10. Statistical analysis

The results are expressed as means ± S.E.M. The results for cumulative arterial responses and systolic blood pressure were analysed using two-way analysis of variance (ANOVA) with repeated measures for overall treatment effect. Other data were analysed by one-way ANOVA. Tukey's test (II, III, IV) or Duncan's test (I) were used for pairwise comparisons between treatment groups. The results for PRA were not normally distributed and were, therefore, analysed by the Kruskal-Wallis ANOVA and the Mann-Whitney *U*-test (II). Bonferroni correction ($p'=p*k$) was applied to the resultant *P* values to allow pairwise comparisons of multiple groups (Ludbrook 1994). Data for the experiment with icatibant were analysed by the Student's *t*-test. $P<0.05$ was considered significant.

5. RESULTS

5.1. Blood pressure, heart rate and left ventricle weight

The effect of six-week CsA treatment on blood pressure, heart rate and the left ventricle weight-to-body weight ratio was evaluated in SHR during the developmental phase of hypertension. Systolic blood pressure was 37–55 mmHg higher in CsA-treated SHR than in control SHR on high-sodium diet (I, II, IV) (Table 5). Concomitant enalapril at 30 mg/kg/day (I and II) and valsartan at 3 and 30 mg/kg/day dose-dependently (II) antagonised the hypertensive effect of CsA. Icatibant at 500 μg/kg/day had no effect on blood pressure during CsA and enalapril administration (II). L-arginine at 1.7 g/kg/day did not significantly influence CsA-induced hypertension (IV).

CsA increased heart rate during the six-week treatment (I, II, IV) (Table 5). Concomitant enalapril, valsartan, icatibant or arginine treatment had no effect on heart rate as compared with CsA alone.

The cardiac effects of CsA varied: CsA increased the left ventricle weight-to-body weight ratio in study IV and tended to do so in study II (P=0.098), whereas in study I the left ventricle weight-to-body weight ratio was unaffected by CsA (Table 5). Concomitant enalapril and both doses of valsartan lowered this ratio as compared with CsA alone or with the control group (I, II). Icatibant had no effect on the left ventricle weight-to-body weight ratio during CsA and enalapril administration (II). The left ventricle weight-to-body weight ratio remained unaltered with concomitant L-arginine (IV).

5.2. Body weight gain, food and water consumption, urinary volume and electrolyte excretion

To find out whether CsA or other drugs affected normal well-being of the animals, body weight gain and food and water consumption were studied. CsA reduced body weight gain 7–15% as compared with control rats in each study. Concomitant enalapril, valsartan, icatibant or L-arginine had no effect on body weight gain when compared with CsA alone.

No significant differences in food intake between the experimental groups were detected at the end of the experimental period (I, II, IV). Increased water intake and urinary volume by CsA was observed in studies I and IV, whereas enalapril normalised these parameters. In other studies, no differences were present between groups in the intake of water (II, IV). Enalapril and the higher dose of valsartan, but no other treatment, lowered urinary volume below that of control rats (II).

A lowered (I, II) or similar (IV) sodium excretion was detected of the CsA group compared with the control group at the end of the study. Potassium excretion was detected to be either lowered (I, II) or unaffected (IV) by CsA. CsA increased (I) or had no effect (II) on magnesium excretion. Any concomitant treatment did not influence sodium, potassium or magnesium excretions during CsA treatment (I, II, IV). CsA increased calcium and phosphorous excretion, whereas enalapril attenuated this effect (I).

5.3. Renal function

CsA caused renal dysfunction with marked proteinuria, an increase in serum creatinine and a decrease in creatinine clearance (I, II, IV) (Table 5). Enalapril and both doses of valsartan protected against CsA-induced renal dysfunction (I, II). Icatibant had no effect on renal function during CsA and enalapril administration (II). L-arginine had no significant effect on CsA-induced renal dysfunction (IV).

5.4. Arterial function

The effects of CsA on arterial function were assessed to find out whether CsA-induced hypertension and nephrotoxicity were related to arterial dysfunction. CsA severely impaired renal arterial contractions induced by noradrenaline (III, IV), angiotensin II (III) and potassium chloride (IV) (Table 6). Similarly, CsA impaired endothelium-dependent relaxation responses to acetylcholine (III, IV) and endothelium-independent responses to sodium nitroprusside (III, IV) in renal arteries.

The effects of CsA on mesenteric arteries were less clear than in renal arteries: potassium chloride contractions were significantly impaired by CsA (IV), but contractions induced by noradrenaline

(III, IV) or angiotensin II (III) were not. Relaxation responses to acetylcholine were not significantly affected by CsA in mesenteric arterial rings (III, IV). Sodium nitroprusside relaxations in mesenteric arteries were impaired by CsA in study IV, but not significantly affected in study III.

Enalapril and both doses of valsartan improved the CsA-induced impairment of renal arterial contractions to noradrenaline and angiotensin II as well as the sodium nitroprusside relaxations (III). The higher dose of valsartan improved the acetylcholine response markedly, but the effects of enalapril (P=0.06) or the lower dose of valsartan (P=0.24) did not reach significance. Icatibant had no effect on arterial responses during CsA and enalapril administration (III). L-arginine protected from the CsA-induced impairment of renal artery responses to potassium chloride, but did not affect other contractile or relaxation responses significantly (IV).

5.5. Histology

5.5.1. Kidneys

Long-term CsA administration damaged one-third of glomeruli; the glomerular damage index was about 8 times higher than in the control group (II) (Table 5). Pathological changes varied from a slight mesangial matrix expansion to severe necrosis and capillary collapse (II, IV). Interstitial fibrosis of striped pattern was apparent in the renal cortex of CsA-treated rats (II, IV). Atrophied tubuli were surrounded by interstitial fibrotic tissue.

Enalapril and both doses of valsartan prevented all CsA-induced pathological renal changes (II). Icatibant did not affect renal histology during CsA and enalapril treatment. L-arginine had no effect on renal histology (IV).

5.5.2. Arteries

In small renal arterioles, CsA caused medial changes that ranged from minor smooth muscle cell vacuolisation to massive smooth muscle and endothelial cell proliferation, leading to complete occlusion of the vessel (II, IV) (Table 5). An increased amount of connective tissue surrounding the

arterioles was also observed in CsA-treated rats. Large intrarenal arteries in the CsA group were mostly normal; only a slight thickening of the adventitia was seen (II, IV). In study IV, interstitial and perivascular abundance of leukocytes was detected.

The structure of the mesenteric arterial trunk, the first- and second-order branches and the smaller arteries and arterioles of both the mesenteric arterial bed and the renal artery was mostly preserved in the control group (IV).

CsA caused adventitial and perivascular fibrosis associated with the presence of leukocytes in small arteries as well as in arterioles of the mesenteric bed, whereas medium-sized arteries were only slightly affected (IV). The bigger arteries (mesenteric and renal artery) remained unaffected by CsA. The medial and intimal layers were mostly normal in CsA-treated rats. However, focal medial hypertrophy was present in some small arteries and arterioles of the mesenteric bed, sometimes leading to occlusion of the vessel. L-arginine did not protect from CsA-induced morphological damage to small arteries (IV).

CsA reduced the corrected area of the medial layer and its ratio to the mean diameter of the vessel in the under 100 μm category by about 30% (IV). CsA affected neither the medial layer nor the medial layer ratio to the diameter of the bigger vessels. Concomitant L-arginine tended to cut about half of the CsA-induced decrease in medial hypotrophy in small vessels, but the effect was not significant (P=0.2 vs. both the control and CsA group) (IV).

5.5.3. Heart

CsA administration caused histological damage to the hearts of SHR on high-sodium diet (I) (Table 5). The damage consisted of thickening of the vessel walls and fibrotic scars in the parenchyma. Enalapril prevented damage to the arteries of the heart (I).

5.6. Plasma renin activity and urinary kallikrein excretion

Plasma renin activity was measured to reflect the activity of RAS. The average PRA levels in control rats were 0.5–0.8 ng angiotensin I/ml/h (I, II). CsA caused a 20 to 50-fold increase in PRA (I, II). Concomitant enalapril or valsartan tended to further increase PRA during CsA treatment, but the effect was not significant when compared with the group receiving CsA alone (I, II). Icatibant had no effect on PRA during CsA and enalapril treatment (II).

Urinary kallikrein activity was assessed to reflect the renal kallikrein-kinin system activity. CsA alone or combined with enalapril or valsartan had no effect on urinary kallikrein excretion (II). Icatibant did not influence kallikrein excretion during CsA and enalapril treatment (II).

5.7. Tissue nitric oxide synthase expression and urinary cyclic guanosine 3',5'-monophosphate

Tissue NO synthase expression and urinary cGMP excretion were analysed to find out whether CsA toxicity was related to dysfunction of the L-arginine–NO pathway. CsA alone or combined with L-arginine had no effect on eNOS expression in the renal cortex, medulla, aorta or heart (IV). iNOS expression was not detected in any of the study groups (IV). CsA alone or combined with L-arginine had no effect on urinary cGMP excretion (IV).

5.8. Plasma total radical trapping parameter and urate, and urinary 8-isoprostaglandin $F_{2\alpha}$

To explore the hypothesis that CsA may induce oxidative stress, plasma urate concentration, TRAP and urinary 8-isoprostaglandin $F_{2\alpha}$ excretion were assessed. CsA alone or combined with L-arginine had no effect on these variables (IV).

5.9. Cell cultures

The effect of long-term CsA treatment on cell viability was tested in cell culture. The viability of the cultured aortic smooth muscle cells was preserved in the control group (IV): after the seven-day culture, the number of aortic cells from the control group increased approximately 4-fold as

Table 5. Summary of the effects of cyclosporine A (CsA), enalapril, enalapril + icatibant, valsartan and L-arginine on systolic blood pressure (SBP), the left ventricle weight-to-body weight ratio (LV), renal function, glomerular damage, interstitial fibrosis and arterial damage in SHR on high-sodium diet. U-Prot, urinary protein excretion; CrCl, creatinine clearance.

	SBP	LV	U-Prot	CrCl	RENAL DAMAGE			Resistance vessel damage	Coronary artery damage
					Glomerular damage	Interstitial fibrosis	Arteriolopathy		
CsA	↑	↑	↑	↓	↑	↑	↑	↑	↑
+enalapril	↓	↓	↓	↑	↓	↓	↓		↓
+icatibant	↔	↔	↔	↔	↔	↔	↔		
+valsartan 3 mg/kg	↓	↓	↓	↑	↓	↓	↓		
+valsartan 30 mg/kg	↓	↓	↓	↑	↓	↓	↓		
+L-arginine	↔	↔	↔	↔	↔	↔	↔	↔	

↑ increased
↓ decreased
↔ no effect

compared with the beginning of the culture. In contrast, the six-week CsA administration *in vivo* caused a clear decrease in the viability of the smooth muscle cells. After the seven-day culture, only one plate out of four had a noticeable number of living cells.

Table 6. Summary of effects of CsA, enalapril, enalapril + icatibant, valsartan and L-arginine on arterial function of SHR on high-sodium diet. NA, noradrenaline; Ang II, angiotensin II; KCl, potassium chloride; Ach, acetylcholine; SNP, sodium nitropusside.

Renal arteries	**NA**	**Ang II**	**KCl**	**Ach**	**SNP**
CsA	↓	↓	↓	↓	↓
+enalapril	↑	↑		(↓)	↑
+icatibant	↔	↔		↔	↔
+valsartan 3 mg/kg	↑	↑		↔	↑
+valsartan 30 mg/kg	↑	↑		↑	↑
+L-arginine	↔		↑	↔	↔

Mesenteric arteries	**NA**	**Ang II**	**KCl**	**Ach**	**SNP**
CsA	↔	↔	↓	↔	↔
+enalapril	↔	↔		↔	↔
+icatibant	↔	↔		↔	↔
+valsartan 3 mg/kg	↔	↔		↔	↔
+valsartan 30 mg/kg	↑	↔		↔	↔
+L-arginine	↔		↔	↔	↔

↑ increased
↓ decreased

6. DISCUSSION

Renal and cardiovascular effects of long-term administration of CsA were studied in SHR on high-sodium diet during the developmental phase of hypertension. The animal model of SHR on high-sodium diet was selected to overcome the resistance of normotensive rats to adverse renal and cardiovascular effects of CsA. The present study evaluated the role of RAS, the renal kallikrein-kinin system, the L-arginine–NO pathway and oxidative stress in CsA-induced renal and cardiovascular effects. Furthermore, the effects of CsA on arterial function and morphological changes were examined.

6.1. Relevance of the animal model used to toxicity of cyclosporine A in man

In CsA-treated patients, factors other than the drug may also have renal and cardiovascular effects. These include rejection of the transplanted organ, the autoimmune disease against which CsA is used and concomitant medications. Therefore, experimental models are needed to study the adverse effects of CsA. However, species' differences in response to toxic effects of CsA have hampered research (Porter *et al*. 1999; Taler *et al*. 1999).

In humans, the dietary intake of sodium in industrialised countries is several times greater than the physiological need (Dyer *et al.* 1994). The excess sodium is a major risk factor for the development of hypertension and end-organ damage (Mervaala *et al.* 1992; Dyer *et al.* 1994). Laboratory rats, in contrast, are normally fed a diet containing sodium in an amount corresponding to their physiological need. According to several studies, CsA-induced hypertension is sodium-sensitive (Ryffel *et al*. 1986; Kaskel *et al*. 1987; Curtis *et al.* 1988; Curtis 1994; Mervaala *et al.* 1997). Therefore, the differences in renal and cardiovascular effects of CsA observed between humans and rats may, at least in part, be due to differences in sodium intake.

A decreased amount of excreted sodium was detected in CsA-treated rats at the end of the six-week experiment. Even though sodium excretion remained unaffected by CsA in one study, this may be seen as evidence of the sodium-retaining effect of chronic CsA treatment in SHR. However, according to the pressure-natriuresis theory of Guyton *et al*. (1972), this reasoning is not logical. An

increase in sodium intake may have an acute sodium-retaining effect, but to avoid chronic sodium and water retention, the excess sodium excretion by kidneys is facilitated by an increase in blood pressure (Guyton *et al*. 1972). Alternatively, the observed decrease in the amount of excreted sodium with CsA may have been due to increased sodium stool loss, which has been observed in rats (Devarajan *et al*. 1989). Thus, the present study does not greatly clarify the effects of CsA on sodium handling in SHR.

CsA causes both hypertension and renal toxicity in patients. However, this is not the case in the most frequently used animal model of CsA nephrotoxicity. Sodium-depleted Sprague Dawley rats do not develop hypertension despite renal damage (Burdmann *et al*. 1995; Porter *et al*. 1999). In contrast, in the presently used model of SHR on high-sodium diet, CsA caused nephrotoxicity concomitantly with an increase in blood pressure. Some confounding factors may exist, such as the effect of high-sodium intake and the pathophysiology underlying the development of hypertension in SHR, which is still mostly unclear (Phillips 1999). However, the CsA-induced nephrotoxicity with renal dysfunction, proteinuria, arteriolopathy, interstitial fibrosis and glomerular damage was similar in character to that observed in patients (Mihatsch *et al*. 1988; Griffits *et al*. 1996; Andoh and Bennett 1998). Thus, it is possible that clinical cardiovascular and renal toxicity of CsA in humans is a combination of drug-induced hypertension and direct renal and vascular effects. The present studies with this hypertensive model are, therefore, expected to shed some light on the mechanisms of CsA toxicity in man.

6.2. Cyclosporine A-induced vascular changes

Long-term treatment of SHR with CsA caused an impairment of arterial function, including both impaired relaxations and decreased contractile responses. This dysfunction was clear in renal but less clear in mesenteric arteries. Both endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) relaxations were impaired. Furthermore, CsA impaired contractile responses of renal arteries to all test substances (noradrenaline, potassium chloride, angiotensin II). These results show that CsA toxicity in SHR is related to arterial dysfunction. The relationship between increased blood pressure and vascular dysfunction in different forms of hypertension has been widely debated. The majority of evidence suggests that vascular dysfunction

is secondary to hypertension (McIntyre *et al.* 1999; Vapaatalo *et al.* 2000), although some studies stress the importance of vascular dysfunction as a preceeding phenomenon (Jameson *et al.* 1993; Taddei *et al.* 1996). CsA at therapeutically relevant concentrations is toxic to endothelial cells *in vitro* (Zoja *et al.* 1986) and induces functional and morphological vascular damage in normotensive rats without affecting blood pressure (Kim *et al*. 1996). CsA also interferes with intracellular calcium homeostasis in vascular smooth muscle cells (Pfeilschifter and Ruegg 1987; Lo Russo *et al.* 1997; Meyer-Lehnert *et al.* 1997; Avdonin *et al.* 1999; Gordjani *et al*. 2000) and causes vasoconstriction *in vitro* (Lanese *et al.* 1994; Epstein *et al.* 1998). Therefore, it is tempting to speculate that the CsA-induced vascular dysfunction occurs first, and hypertension and renal toxicity are, at least partly, secondary to the dysfunction.

CsA-induced hypertension was associated with endothelial, medial and adventitial damage to intrarenal small arteries and arterioles and, to a lesser extent, to mesenteric resistance vessels. The damage of resistance vessels was related with medial hypothrophy. CsA is known to cause structural damage to intrarenal arteries and arterioles, while integrity of extrarenal vessels is mostly preserved (Antonovych *et al.* 1988; Mihatsch *et al*. 1988; Andoh and Bennett 1998). Moreover, experimental hypertension is usually associated with hypertrophy of the resistance vessels (Lee *et al.* 1983). In this light, the CsA-induced medial hypotrophy was unexpected, and it seems likely that the vascular damage was not solely due to hypertension but to some other effects of CsA in vascular remodelling of the medial layer. One potential reason for this is apoptosis, which seems to be involved in CsA-induced nephrotoxicity in normotensive rats during sodium deprivation (Thomas *et al*. 1998). Accordingly, the decreased viability of the cultured aortic smooth muscle cells by CsA, which was observed in the present study, might also have been due to apoptosis.

6.3. Renin-angiotensin system and renal kallikrein-kinin system in cyclosporine A toxicity

PRA is a widely used parameter to estimate the activity of the systemic RAS. Normally, high-sodium intake physiologically depresses PRA. CsA, however, caused a substantial increase in PRA in SHR during high-sodium diet. This finding is in agreement with several rat studies which conclude that CsA increases PRA regardless of the sodium content of the diet (Burdmann *et al.* 1995; Abassi *et al.* 1996; Mervaala *et al.* 1997). However, variable effects of CsA on PRA in

humans have been presented (Bantle *et al.* 1987; Myers *et al.* 1988; Mason *et al.* 1991; Julien *et al.* 1993).

The mechanism of the activation of RAS by CsA in SHR, in other animal models or in some clinical settings is somewhat obscure. It may be due to a direct induction of renin excretion in juxtaglomerular cells observed *in vitro* (Kurtz *et al.* 1988). On the other hand, CsA has vasoconstrictive properties (Murray *et al.* 1985; Myers *et al.* 1988; Hansen *et al.* 1997; Andoh and Bennett 1998) which may lead to reduction in blood supply to kidneys. Evidence exists that renin secretion is tonically elevated in ischemic nephrons (Sealey *et al.* 1988). Therefore, it is possible that increase in PRA is, at least partly, secondary to haemodynamic changes in kidneys. Some observations with transplant patient have shown that PRA rises gradually after transplantation, suggesting that activation of RAS is partly due to ischemic damage to kidneys (Taler *et al*. 1999). An increase in blood pressure with CsA may also contribute to renal damage and a subsequent increase in PRA in SHR. Taken together, CsA may, in fact, cause a vicious circle: RAS activation by CsA reduces renal blood flow and induces local ischemia in kidneys that further increases renin release and potentiates CsA toxicity. Thus, the rise in PRA may be both a cause and a consequence of CsA-induced renal damage in SHR on high-sodium diet.

The effects of ACE inhibitors are usually somewhat weaker during high intake of sodium, when RAS already is physiologically suppressed (Waeber *et al.* 1990). In the present study, however, RAS antagonism with either enalapril or valsartan protected against the CsA-induced hypertension effectively. This was probably due to activation of RAS by CsA. The effectiveness of enalapril and valsartan in reducing blood pressure is consistent with several long-term clinical studies (Mourad *et al*. 1993; Sennesael *et al*. 1995; Hannedouche *et al*. 1996; Del Castillo *et al*. 1998).

Angiotensin II has been proposed to have a major role in the development of CsA-induced renal injury via induction growth factors and adhesion molecules (Pichler *et al.* 1995). ACE inhibitors and AT_1 receptor antagonists protect against renal injury without affecting blood pressure in the sodium-depletion rat model of CsA toxicity (Burdmann *et al.* 1995; Andoh and Bennett 1998). An ACE inhibitor and an AT_1 receptor antagonist protected against renal injury in our hypertensive model as well. The protection may have been due to directly reducing the local effects of

angiotensin II, but prevention of CsA-induced hypertension may have also participated. The results showed that RAS antagonism prevents CsA-induced renal injury, not only in blood pressure-independent models of CsA toxicity, but also during concomitant rise in blood pressure. From the data of the present study, however, it is not possible to differentiate the extent to which lowering of blood pressure contributed to the renal protection**.**

Enalapril and valsartan protected against CsA-induced renal dysfunction, which was measured as decreased creatinine clearance and increased serum creatinine concentration. The results are similar to those of CsA-treated normotensive rats (Kaskel *et al.* 1987) and in diabetic patients (Hannedouche *et al.* 1996). The findings are, however, in conflict with the effects of CsA in the model of sodium deprivation, in which ACE inhibitors and AT_1 receptor antagonists do not normalise renal function, even though structural changes to the kidneys are protected against (Burdmann *et al.* 1995; Andoh and Bennett 1998). The difference may be due to higher blood pressure and glomerular perfusion pressure in SHR with high-sodium intake than in rats on sodium-depleted diet, which had very low systemic blood pressure that was below the renal autoregulatory range (Burdmann *et al.* 1995). It is also possible that preservation of kidney function by RAS antagonists is at least partly due to prevention of glomerular damage rather than direct effects on renal haemodynamics.

The present study also evaluated the role of the renal kallikrein-kinin system in long-term CsA administration. Urinary kallikrein excretion is proposed to reflect renal glandular kallikrein activity (Bönner and Marin-Grez 1981; Majima and Katori 1995). CsA had no effect on urinary kallikrein excretion. Likewise, B_2 receptor antagonist icatibant did not antagonise the beneficial effects of ACE inhibition. Therefore, it seems that the renal kallikrein-kinin system does not have a crucial role in CsA toxicity in this animal model. A study with normotensive rats suggested that in short-term administration CsA may decrease the activity of the renal kallikrein system (Bompart *et al.* 1996). A similar finding in six-month CsA treatment in psoriasis patients suggested that suppression of the renal kallikrein-kinin system may also have a role in long-term effects of CsA (Raman *et al.* 1998). However, increased renal kallikrein and bradykinin B_2 receptor levels have been reported in rats after long-term CsA administration (Wang *et al.* 1997). Physiological differences between man and rat as well as duration of the experiment might explain the observed differences.

In summary, activation of RAS, but not the dysfunction of the renal kallikrein-kinin system, seems

to be a major mechanism of CsA toxicity in this hypertensive animal model. Equally, inhibition of RAS is effective in protecting against CsA toxicity.

6.4. L-arginine–nitric oxide pathway and oxidative stress in cyclosporine A toxicity

The endothelial dysfunction detected *in vitro* could have been due to a defect in the L-arginine–NO pathway induced by CsA. The long-term effects of CsA on the L-arginine–NO pathway were evaluated by the expression of eNOS and iNOS as well as by urinary excretion of cGMP. CsA had, however, no effect on urinary cGMP excretion or on eNOS and iNOS expression in the kidney, heart and aorta. L-arginine, at a dose that has antagonised the toxic effects of CsA in normotensive rats (Andoh *et al.* 1997), tended to have some beneficial effects during CsA administration in the present study, but failed to reverse the CsA toxicity. According to literature, CsA induces eNOS (Lopez-Ongil *et al*. 1996; Navarro-Antolin *et al.* 2000) and depresses iNOS *in vitro* (Dusting *et al.* 1999), but only some of the *in vivo* studies show similar results (Amore *et al*. 1995; Bobadilla *et al.* 1998). The present results are in agreement with those *in vivo* studies in which CsA did not affect the L-arginine–NO pathway, even though L-arginine slightly attenuated CsA toxicity (Bobadilla *et al.* 1994; Vaziri *et al.* 1998; Hansen *et al.* 1999). Thus, long-term toxic effects of CsA on endothelial cells result in dysfunction, which seem to be mainly mediated by mechanisms other than a defect in the L-arginine–NO pathway.

TRAP has been proposed to reflect the antioxidative status of plasma (Wayner *et al*. 1987). Urate concentration of plasma is a major contributor to TRAP. Urinary excretion of 8-isoprostaglandin $F_{2\alpha}$, which is a non-enzymatic product of free-radical catalysed peroxidation of arachidonic acid, was used as a marker of oxidative products (Patrono and FitzGerald 1997; Kanji *et al*. 1999). CsA did not affect TRAP, plasma urate concentration or urinary excretion of 8-isoprostaglandin $F_{2\alpha}$. While some studies suggest that CsA may induce oxidative stress (Wang and Salahudeen 1994; Wolf *et al*. 1994), its actual role in the development of the renal and cardiovascular toxicity is far from clear (Buetler *et al.* 2000). The present results of unaltered indicators of oxidative stress suggest that oxidative stress does not play a major part in long-term effects of CsA in this hypertensive animal model. However, because the effects of antioxidant administration on CsA

toxicity were not studied, and, analysis of plasma TRAP was performed at the end of the experiment only, the participation of oxidative stress cannot be totally excluded.

7. SUMMARY AND CONCLUSIONS

Clinically, CsA causes hypertension and nephrotoxicity, the latter of which are characterised by renal dysfunction with a rise in serum creatinine and a decrease in creatinine clearance, and by morphological damage with interstitial fibrosis and arteriolopathy. The exact mechanisms of CsA-induced hypertension and nephrotoxicity are unknown. Renal and cardiovascular effects of long-term administration of CsA were studied in SHR on high-sodium diet. The model was selected to overcome the resistance of normotensive rats to adverse renal and cardiovascular effects of CsA.

The major findings and conclusions of this study are as follows:

1. CsA caused hypertension and nephrotoxicity that resemble those seen in CsA-treated patients.
2. Long-term CsA administration caused vascular dysfunction, which was more pronounced in renal than in mesenteric arteries. The dysfunction included both impaired endothelium-dependent and endothelium-independent relaxations and reduced contractile responses to noradrenaline, potassium chloride and angiotensin II.
3. CsA toxicity was related to morphological damage in intrarenal arterioles and small arteries and, to a lesser extent, in extrarenal resistance vessels.
4. Activation of RAS is an important mechanism of CsA toxicity in this hypertensive animal model. Inhibition of RAS by ACE inhibitor or AT_1 receptor antagonist protected against renal and cardiovascular CsA-induced toxicity.
5. No major role was found for the renal kallikrein-kinin system, the L-arginine–NO pathway or oxidative stress as primary causes of CsA toxicity.

8. ACKNOWLEDGEMENTS

The present study was carried out at the Institute of Biomedicine, Department of Pharmacology and Toxicology, University of Helsinki during the years 1997–2000.

I was fortunate to have had two experts of cardiovascular research as my supervisors. My sincere gratitude to Professor Heikki Vapaatalo, the head of the Department of Pharmacology and Toxicology, for providing me with the opportunity to work in the field of pharmacology and for supporting me in all possible ways throughout the study. I also thank Marja-Leena Nurminen, MD, PhD, for conscientious supervising. I appreciate the informative lessons she gave on writing scientific manuscripts.

I thank Docent Eero Mervaala for suggesting the present work to me and for sharing his vast knowledge on the study.

I respectfully thank Professor Olli Vuolteenaho and Docent Mika Kähönen, the referees appointed by the Medical Faculty of the University of Helsinki, for careful revision and constructive criticism.

I am also grateful to Carol Ann Pelli, HonBSc, for linguistic revision of this thesis.

I was fortunate to work with experts of several areas of science including medicine, dentistry, pharmacy, nutrition, biochemistry, animal biology, cell biology and biotechnology. I thank all personnel in our department for creating an inspiring working climate and for sharing the joys and sorrows of the everyday work.

My deepest gratitude to my closest workmate Piet Finckenberg, MSc, a man with a thousand skills, for fruitful cooperation. I warmly thank my highly professional co-authors Professor Juhani Ahonen, Professor Heikki Karppanen, Professor Ismo Virtanen, Professor Eeva Moilanen, Leena Krogerus, MD, Tuula Lähteenmäki, PhD, Anna-Kaisa Pere, MD, Asko Riutta, MSc**,** Pertteli Salmenperä, BSc, Jesus Santisteban, MD, and Timo Vaskonen, MD, for their collaboration. Marika Sipola, MSc, also deserves to be acknowledged for her unselfish contribution.

I am deeply grateful to Anneli von Behr, Pirjo Saarelainen, Toini Siiskonen, Aira Säisä, Aili Takkinen, Maarit Mäntynen, Esa Oja, Eino Ruusula, Erkka Solatunturi, LicPhil, and the office personnel for skilful technical assistance. I am also much obliged to Marja-Liisa Räsänen and other staff of the Experimental Animal House for their kind help.

I wish to thank my parents for unconditional love and support during these trying years.

This study was supported by Novartis Finland, Director of Research Kari Harjula, Einar and Karin Stroem's Foundation, Emil Aaltonen Foundation, Kidney Foundation and Pharmacal Research Foundation, Finland.

Helsinki, December 2000.

Markus Lassila

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