Endothelium-Dependent Vasodilation and Oxidative Stress in Chronic Renal Failure

BY

MARGUS ANNUK
Dissertation for the Degree of Doctor of Philosophy (Faculty of Medicine) in Internal Medicine presented at Uppsala University in 2002

ABSTRACT

Cardiovascular disease (CVD) is the major cause of death in patients with chronic renal failure (CRF). Endothelial function and oxidative stress (OS) have previously been shown to be important in the pathogenesis of CVD. In this thesis, the endothelium-dependent vasodilatation (EDV) and OS were investigated in the patients with CRF. Also the influence of L-arginine, erythropoietin and diclofenac on EDV were evaluated in patients with CRF.

Patients with CRF were found to be characterized by a defect EDV even after correction for traditional cardiovascular risk factors. This impairment was related to the degree of renal failure.

Measurement of OS markers in CRF patients demonstrated that these patients were in a state of OS compared to healthy controls. The most informative indices to evaluate the degree of OS in CRF were: oxidized glutathione (GSSG) level, ratio between oxidized and reduced glutathione (GSSG/GSH ratio), lag phase of lipoprotein fraction (LPF) and baseline diene conjugation level of LPF.

Simultaneously investigated OS markers and EDV demonstrated a relationship between OS and EDV in patients with CRF. EDV was positively correlated with total antioxidative activity, reduced glutathione (GSH) and lag phase of LDL.

Local infusion of L-arginine as a substrate for nitric oxide synthesis and diclofenac as an inhibitor of cyclooxygenase-derived vasoconstrictive agents augmented EDV in patients CRF. In contrast, the erythropoietin treatment (both acute and long-term) impaired EDV in CRF patients.

In conclusion, patients with CRF have increased levels of OS markers and impaired endothelial vasodilatory function. These factors may be important with respect to the high morbidity and mortality of CVD found in patients with CRF. One possible mechanism to reduce CVD in patients with CRF is to improve endothelial function and eliminate OS. Locally administrated L-arginine and diclofenac improved EDV but erythropoietin administration impaired EDV in patients with CRF.

Key words: endothelium, vasodilation, oxidative stress, chronic renal failure, L-arginine, diclofenac, erythropoietin.

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To my son Mattias
This thesis is based on the following original studies, which will be referred to in the text by their Roman numerals:


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**ABBREVIATIONS**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>AI</td>
<td>angiotensin I</td>
</tr>
<tr>
<td>AII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethyl-arginine</td>
</tr>
<tr>
<td>AM</td>
<td>adrenomedullin</td>
</tr>
<tr>
<td>BDC-LPF</td>
<td>baseline diene conjugation of lipoproteins fraction</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>CRF</td>
<td>chronic renal failure</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine-monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CCl₃⁻</td>
<td>trichloromethyl radical</td>
</tr>
<tr>
<td>CD</td>
<td>conjugated diene</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>copper</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EFI</td>
<td>endothelium function index</td>
</tr>
<tr>
<td>EDCF</td>
<td>endothelium-derived contracting factor</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDV</td>
<td>endothelium-dependent vasodilation</td>
</tr>
<tr>
<td>EIDV</td>
<td>endothelium-independent vasodilation</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>ETₐ</td>
<td>endothelin receptor type A</td>
</tr>
<tr>
<td>ETₐB</td>
<td>endothelin receptor type B</td>
</tr>
<tr>
<td>FBF</td>
<td>forearm blood flow</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSH</td>
<td>oxidized glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LOOH</td>
<td>lipid hydroperoxide</td>
</tr>
<tr>
<td>LP</td>
<td>lipid peroxidation</td>
</tr>
<tr>
<td>LPF</td>
<td>lipoprotein fraction</td>
</tr>
<tr>
<td>MCh</td>
<td>methacholine</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MmHg</td>
<td>millimeters mercury</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide-adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO⁺</td>
<td>nitric oxide radical</td>
</tr>
<tr>
<td>NO₂⁺</td>
<td>nitrogen dioxide radical</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>cNOS</td>
<td>constitutive nitric oxide synthase</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>O₂⁻⁻</td>
<td>superoxide anion radical</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OH⁺</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>OS</td>
<td>oxidative stress</td>
</tr>
<tr>
<td>oxLDL</td>
<td>oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PGH₂</td>
<td>prostaglandin H₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>prostacyclin</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>R⁺</td>
<td>carbon-centered radical</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>RO⁺</td>
<td>alkoxy radical</td>
</tr>
<tr>
<td>ROO⁺</td>
<td>peroxyl radical</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RS</td>
<td>reactive species</td>
</tr>
<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TAA</td>
<td>total antioxidant activity</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TPA</td>
<td>tissue plasminogen factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
</tbody>
</table>
INTRODUCTION

Cardiovascular disease and chronic renal failure

Cardiovascular causes of death in patients with chronic renal failure (CRF) are many times higher than in general population (Levey SA and Eknoyan G 1999). Atherosclerosis is a major contributor to the accelerated rate of clinically evident cardiovascular disease (Lindner et al 1974). Vascular endothelium plays a pivotal role in the maintenance of vasodilation and the inhibition of platelet aggregation and regulation of smooth muscle cell proliferation through the release of nitric oxide (NO) and other factors. Endothelial dysfunction may precede and promote atherosclerosis by several mechanisms, such as adhesion of monocytes and platelets, increase in vascular permeability, proliferation and migration of smooth muscle cells (Harrison DG 1997). Several studies have demonstrated abnormalities in endothelial function in patients with atherosclerosis and CRF (Morris ST et al. 2000; Celemajer DS et al. 1992; Liao JK et al. 1991; Zeiher AM et al. 1990).

The lipoprotein abnormalities and possible risk factors for atherosclerosis in CRF are elevated very low-density lipoprotein (VLDL), moderately elevated low-density lipoprotein (LDL) and decreased high-density lipoprotein (HDL) levels. An accumulation of small dense LDL also occurs as in hypertriglycerideremic diabetic patients with or without nephropathy and in hemodialysis patients. These lipoprotein particles are potentially “atherogenic” lipoproteins (Joven J et al. 1993; Cressman MD et al. 1993; Senti M et al. 1992). An increased propensity for oxidation of LDL and increased level of oxidized LDL (oxLDL) have also been reported in CRF. Oxidized LDL may also contribute to endothelial dysfunction.
Numerous studies have demonstrated that oxidative stress plays an important role in the development of atherosclerosis. Reactive oxygen species (ROS) oxidize lipid substances, leading to formation of oxLDL, which is a key mediator of atherosclerosis (Holvoet P and Collen D 1998; Steinberg D 1997). Accumulating evidence suggests that CRF is associated with impaired endothelial cell function (Guldener C et al. 1998; Blum M et al. 1998; Kari JA et al. 1997; Takagi M et al. 1994) as well as with enhanced prolonged oxidative stress (Mimic-Oka J et al. 1999; Ceballos-Picot I et al. 1996; Ha TK et al. 1996; Fillit H et al. 1981).

Patients with CRF have a high prevalence of hypertension, and hypertension in CRF is associated with adverse outcomes of both CVD and renal function (Chanard J 2001).

Several epidemiological studies have reported that elevated plasma total homocysteine is a risk factor for cardiovascular disease (Refsum H and Ueland PM 1998; Boushey CJ et al. 1995). Homocysteine contains a reactive sulfhydryl group, and can undergo oxidation to its disulfide at physiological pH in the presence O2. It has been suggested that this pro-oxidant activity of homocysteine is also responsible for the oxidation of LDL cholesterol and the damaging effect of homocysteine on vascular cells and tissues (Parthasarathy S 1987; Harker LA et al. 1976).

Chronic inflammation is a common feature of end-stage renal disease that is gaining increasing attention as a major cause of morbidity and mortality. Accumulating evidence suggests that chronic inflammation is associated with malnutrition and atherosclerosis in patients with CRF (Stenvinkel P et al. 1999).
Endothelial function and endothelium-derived vasoactive substances

The endothelium is a monolayer of cells which constitutes the internal structure of the entire circulatory system. In 1977 Moncada published the first report indicating that the endothelium releases vasoactive substances (Moncada S et al. 1977). Later, in 1980, Furchgott and Zawadzki demonstrated that stimulation of the endothelium with acetylcholine caused the release of a substance they named endothelium-derived relaxing factor (EDRF) (Furchgott RF and Zawadzki JV 1980). In the late 1980s, EDRF was recognized as nitric oxide (NO) (Palmer RMJ et al. 1987).

Several other regulatory functions of the vascular endothelium have also been described. The endothelium can be regarded as a highly metabolic active endocrine gland and is able to generate adhesion molecules (ICAM-1 and VCAM-1), fibrinectin, interleukin-1, heparansulfate, von Willebrand factor (vWF), transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF) tissue plasminogen factor (tPA), components of the renin-angiotensin system, prostaglandins, nitric oxide (NO) and endothelin-1 (Lüscher TF et al. 1997; Vane JR et al. 1990).

Thus the endothelium has the ability to sense changes in the local milieu and respond by releasing a variety of biologically active substances that participate in the regulation of vascular relaxation and contraction, vascular structure, platelet and monocyte function and the coagulation system.

Endothelium-derived relaxing factors

The endothelium has been shown to release a large number of vascular relaxing substances, such as prostacyclin (PG₂), adrenomedullin (AM), C-type natriuretic peptide (CNP), endothelium-derived hyperpolarizing factors (EDHF) and nitric oxide (NO) (Figure 1).
Endothelial-derived vasoactive substances.

Endothelium-derived relaxing factors (EDRFs) are: endothelium derived hyperpolarizing factor (EDHF), prostacyclin (PGI₂), adrenomedullin (AM), C-type natriuretic peptide (CNP) and nitric oxide (NO).

Endothelium-derived contracting factors (EDCFs) are: endothelin (ET), prostaglandin H₂ (PGH₂), thromboxane A₂ (TXA₂), superoxide anion (O₂⁻) and angiotensin II (AII).

PGI₂ is a product of the cyclooxygenase pathway. Prostacyclin increases cyclic adenosine monophosphate (cAMP) in platelets, resulting in a decrease in platelet adhesion and aggregation, and promoting relaxation in vascular smooth muscle cell (VSMC) (Radomski MW et al. 1987; Armstrong JM et al. 1978).

AM is a potent vasodilator peptide, produced by posttranslational splicing of pro-adrenomedullin. It has been proposed that endogenous AM possesses a protective action against cardiovascular damage, possibly through the inhibition of oxidative stress production (Shimosawa T et al. 2002).

CNP is a peptide found within the vascular endothelium (Espiner EA 1994). It is commonly referred to as an endothelium-derived factor (Chen HH and Burnett JC 1998; Brown J et al. 1997) but is also known as an inhibitor of growth and proliferation of vascular smooth muscle cells (Porter JG et al. 1992).
Endothelium-derived hyperpolarization of smooth muscle cells by EDHF is a contributing mechanism to the vasodilatory response to shear stress and other endothelium-dependent vasodilator agents (Chen G et al. 1988). The exact nature of endothelium-dependent hyperpolarization is not fully known, but is thought to be mediated by EDHF and is sensitive to inhibition of calcium-dependent potassium channels (Edwards G et al. 1998).

NO is a soluble gas that diffuses freely in both water and lipid and has a half-life only a few seconds. It is synthesized from the amino acid L-arginine by nitric oxide synthase (NOS) (Palmer RMJ et al. 1988). Two main forms of vascular NOS have been described: the calcium calmodulin-dependent constitutive NOS (eNOS) present in endothelial cells and the calcium-independent inducible NOS (iNOS). The iNOS is primarily expressed in immune cells and VSMC. NO diffuses to the VSMC inducing formation of cyclic guanosine monophosphate. This, in turn, activates protein kinase C resulting in a decline in intracellular ionized calcium in the VSMC leading to relaxation and vasodilation (Moncada S et al. 1993; Moncada S et al. 1991) (Figure 2).

The basal NO production can be upregulated by physical forces, such as shear stress, as well as by several receptor-operating transmitters and hormones such as estrogen, acetylcholine, bradykinin, substance P, serotonin, adrenaline and noradrenaline, adenosine and thromboxane (Lüscher TF and Noll G. 1995; Rubanyi GM et al. 1986).

**Endothelium-derived contracting factors**

In addition to endothelium-derived relaxing factors, endothelial cells also release several vasoconstrictive agents. The vasoconstrictors synthesized by endothelium are endothelins (ET), thromboxane A₂ (TXA₂), prostaglandin H₂ (PGH₂), angiotensin II (AII) and superoxide anion radical (O₂⁻) (Figure 1).
Endothelins exist in three isoforms: endothelin-1 (ET-1), endothelin-2 and endothelin-3 (Inoue A et al. 1989). ET-1 has a wide range of biological actions and is mainly secreted abluminally and binds to endothelin A (ET\textsubscript{A}) and endothelin B (ET\textsubscript{B}) receptor subtypes located on vascular smooth muscle cells. ET-1 is a potent vasoconstrictor and pressor-agent but has also mitogenic effects (Ferro CJ and Webb DJ 1996).

Thromboxane A\textsubscript{2} and prostaglandin H\textsubscript{2} can be produced by the endothelium in response to agonists such as histamine, serotonin and arachidonic acid and may evoke vasocontraction by activating thromboxane receptors on vascular smooth muscle cells (Lüscher TF and Noll G 1995).

Angiotensin II is formed from angiotensin I by the action of angiotensin converting enzyme synthesized and expressed on the endothelial cell.

Recently, a superoxide anion radical has been classified as a vasoconstrictive agent derived by endothelial cells. (Rey FE et al. 2001; Wang HD et al. 1998).

**Endothelial dysfunction and cardiovascular disease**

A primary function of the endothelium is to control the intra- and transcellular traffic of numerous nutrients, hormones and cells. The endothelium can express cell adhesive molecules which have a key role in the adhesion and subsequent trans-endothelial migration of leukocytes into the vascular wall. This process is crucial in the clearance of LDL, and especially oxLDL, in the arterial wall. LDL is cleaned by ligation to LDL receptors and oxLDL may also bind to scavenger receptors on monocytes/macrophages in the subendothelial compartment. The uptake of oxLDL by monocytes/macrophages subsequently leads to formation of foam cells, a primary event in the generation of fatty streak lesions.

The endothelium also prevents exposure of the thrombogenic subendothelium to circulating coagulation factors and thus exerts anti-thrombotic actions (Ross R 1993).
The endothelial function may be impaired by risk factors for CVD such as hypertension, hyperlipidemia (including elevated oxLDL levels), and diabetes. These risk factors, which are also oxidative stress-related, contribute to an increase in endothelial cell permeability leading to intimal edema and the influx of oxLDL into the vessel wall.

Hypertension is an independent indicator of increased risk of coronary events. EDV has been shown to be reduced in patients with essential hypertension (Linder L et al. 1990; Panza JA et al. 1990, von zur Mühlen B et al. 2001a). Reduced levels of endothelium-derived relaxing factor have been demonstrated in hypertensive patients with left ventricular hypertrophy (Treasure CB et al. 1993; Panza JA et al. 1993).

Abnormal endothelium-dependent vasodilation has also been demonstrated to be related to hypercholesterolemia in patients without evidence of atherosclerosis (Creager MA et al. 1990).

Increased endothelial cell permeability has been demonstrated in patients with elevated glucose levels and this leads to interstitial edema and may enhance cell proliferation and matrix production (Nannipieri M et al. 1995). There is substantial evidence that EDV is impaired in insulin-dependent and -independent diabetes mellitus (Johnstone MT et al. 1993; Williams SB et al. 1996).

Endothelial dysfunction is an important initial step in the development of atherosclerosis. Endothelial dysfunction has also been shown to be a predictor of future cardiac events (Zeiher AM et al. 1990; Busse R and Fleming I 1996). Recent studies show that the development of endothelial dysfunction depends substantially on the degree of oxidative stress (Matsuoka H 2001; Heitzer T et al. 2001). A prolonged severe oxidative stress may thus lead to endothelial dysfunction (Figure 2).
**Endothelial dysfunction and chronic renal failure**

Accumulating evidence suggests that chronic renal failure is associated with impaired endothelial function. Previous investigations of endothelial function performed in different CRF patients (in children with CRF and in hemodialysis and peritoneal dialysis patients) were using different methods such as ultrasound, vascular cell markers measurement, vessel wall movement detector and occlusion plethysmography (Bolton CH et al. 2001; van Guldener C et al. 1998; van Guldener C et al. 1997; Kari JA et al. 1997; Takagi M et al. 1994). All these studies demonstrated endothelial dysfunction in chronic renal disease.

The precise mechanisms whereby endothelium function is impaired in CRF patients are still unclear. Several possibilities have been proposed, such as elevated levels of asymmetrical dimethylarginine (ADMA), a competitive inhibitor of endothelial NO production (Zoccali C et al. 2001). Also chronic inflammation, oxidative stress and oxLDL are thought to contribute to endothelial damage in patients with CRF (Stenvinkel P 1999; Steinberg D 1997).

**Effects of drugs on endothelium-dependent vasodilation**

Pharmaceutical agents have different effects on endothelial function. Recently, von zur Mühlen and co-workers demonstrated that angiotensin converting enzyme (ACE) inhibition improved endothelium-dependent vasodilation (EDV) both in patients with untreated hypertension and in normotensive patients (von zur Mühlen B et al. 2001a). They also found that an angiotensin-II subtype-receptor antagonist (irbesartan) and β₁-selective adrenoreceptor antagonist (atenolol) improve EDV (von zur Mühlen B et al. 2001b).

Statins and other lipid-lowering drugs, such as cholestryramine, have been shown to have beneficial effects on EDV in hypercholesterolemic patients (Duffy SJ et al. 2001).
Williams and coworkers demonstrated that vitamin C improved EDV in renal transplant recipients (Williams MJ et al 2001).

In contrast to these findings, accumulating evidence suggests that erythropoietin treatment impairs endothelial function (Aguilera A et al. 1999; Vogel V et al. 1997; Bode-Böger SM et al. 1996).

**Oxidative stress**

Oxidative stress has been defined as a disturbance in the balance between antioxidants and pro-oxidants (free radicals and other reactive oxygen and nitrogen species), with increased levels of pro-oxidants leading to potential damage (Sies H 1991; Halliwell B 1997).

**Reactive oxygen and nitrogen species**

The most known reactive species are free radicals. A free radical is any atom or molecule that contains one or more unpaired electrons (Halliwell B and Gutteridge JMC 1999). An unpaired electron is an electron that occupies an orbital alone, but electrons usually associate in pairs in orbitals of atoms and molecules. The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive. Not only free radicals but also non-free radical compounds can cause a redox imbalance (oxidative damage). Free radicals are generally more reactive than non-radicals due to their unpaired electron, but different types of free radicals vary widely in their reactivity (Halliwell B and Chirico S 1993; Slater TF 1984).

Both free radicals and reactive non-free radical compounds are collectively called reactive species (RS). RS are divided into reactive nitrogen species (RNS) – derivates on the basis of nitrogen and reactive oxygen species (ROS) – derivates on the basis of oxygen.
ROS are superoxide (O$_2^•$), hydroxyl (OH$^•$), peroxyl (LOO$^•$), alkoxyl (LO$^•$) and hydroperoxyl (HOO$^•$) as free radicals, and hydrogen peroxide (H$_2$O$_2$), hydrochlorous acid (HOCI), ozone (O$_3$), singlet oxygen ('O$_2$) and hydroxy alkenals as oxygen-based reactive non-radicals.

RNS are nitric oxide (NO$^•$), nitrogen dioxide (NO$_2^•$) and peroxynitrite (ONOO$^•$) as free radicals and nitrous acid (HNO$_2$), dinitrogen trioxide (N$_2$O$_3$) and alkyl peroxynitrites (LOONO) as nitrogen-based non-radicals.

There are also hydrogen radical (H$^•$), the carbon-centered radical (R$^•$) and trichlormethyl radical (CCI$_3^•$) (Halliwell B and Chirico S 1993).

If two free radicals meet, they can join their unpaired electrons and make a covalent bond. Most molecules in the body are not radicals. Hence, any reactive free radical generated is likely to react with a non-radical. When a free radical reacts with non-radical, a free-radical chain reaction results and new radicals are rapidly formed.

Attack of reactive radicals on membranes or lipoproteins starts lipid peroxidation, which is particularly implicated in the development of atherosclerosis (Halliwell B and Gutteridge JMC 1999). If hydroxyl radicals are generated close to DNA, they can attack the purine and pyrimidine bases and cause mutations (Dizdarglu M 1991).

Free radicals do not only exert disadvantageous effects, but are also formed deliberately in the body for useful purposes and have important physiological functions. One of the well-defined roles of free radicals is when activated phagocytic cells produce superoxide anion radicals and hydrogen peroxide as one mechanism to kill bacteria and fungi and to inactivate viruses (Halliwell B and Gutteridge JMC 1999).

In a biological system free radicals attack takes place in the presence of an unbalanced ratio between free radicals and antioxidants.
**Antioxidants**

Protection against free radicals attack can be achieved by prevention of free radical formation, by blocking of chain reactions or by repairing the oxidatively damaged biomolecules (Halliwell B and Gutteridge JMC 1999). There are a number of antioxidants present in the body and derived from the diet. Based on the location, they can be divided into intracellular and extracellular antioxidants (Gutteridge JMC 1995; Rice-Evans C and Burdon R et al. 1993). Intracellular enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase that convert potential substrates (superoxide anion radicals and hydrogen peroxide) to less reactive forms in the body (Gutteridge JMC 1995; Rice-Evans C and Burdon R et al. 1993). Main non-enzymatic cellular antioxidant is reduced glutathione (GSH).

Several extracellular antioxidants such as proteins (transferrin, lactoferrin, albumin, ceruloplasmin) and urate prevent free radical reaction in the body sequestering transition metal ions by chelation in plasma. Albumin, bilirubin and urate may also scavenge free radicals directly. Furthermore, plasma has a considerable peroxyl radical scavenging ability, which is mainly determined by its content of ascorbic acid (Gutteridge JMC 1995; Rice-Evans C and Burdon R 1993; Frei B et al. 1989).

Some antioxidants are located both intra- and extracellularly, such as $\alpha$-tocopherol, which is the major lipid-soluble antioxidant, present in cellular membranes and in plasma lipoproteins. It is an effective chain-breaking antioxidant that protects polyunsaturated lipids from peroxidation by scavenging peroxyl radicals (Halliwell B and Gutteridge JMC 1999).
Oxidative stress

The processes in which free radicals are involved are not necessarily deleterious. On the contrary, they are of fundamental importance for life in that they take part in key biochemical reactions in all living organisms (Halliwell B and Gutteridge JMC 1999). Thus, free radicals are not only produced as an unwanted product, but their levels are precisely controlled by antioxidants. If antioxidant defense is not completely efficient, elevated permanent free radical formation in the body leads to increased damage. The term “oxidative stress” is used to refer to this effect (Sies H 1991). Under the mild oxidative stress, tissues often respond to express extra antioxidant defenses. Severe and prolonged oxidative stress can cause cell injury and death. The term oxidative stress is commonly used to describe a series of reactions involving the chemistry of a wide spectrum of compounds. These include free radicals and other reactive molecules that are potentially able to affect the integrity of virtually all the biomolecules (Halliwell B and Gutteridge JMC 1999).

However, it is important that the reactions in which free radicals are involved are not necessarily deleterious; on the contrary, they are of fundamental importance for life in that they take part in key biochemical reactions in all living organisms (Halliwell B and Gutteridge JMC 1999).

In an organism, oxidative stress takes place in the presence of an unbalanced ratio between free radical production and antioxidants. Severe prolonged oxidative stress is harmful, and may contribute to the development of atherosclerosis.


**Lipid peroxidation**

Lipid peroxidation (LP) is a process where a free radicals attack on polyunsaturated fatty acids (PUFA) takes place. Initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to remove a hydrogen atom from a PUFA (Halliwell B and Gutteridge JMC 1999). Since a hydrogen atom in principle is a free radical with a single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbon-centered radical is stabilized by a molecular rearrangement to form a diene conjugates (DC), followed by reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide (LOOH), but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from fatty acid, the new carbon-centered radical can react with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation can continue. The length of the propagation chain depends on several factors, e.g. the oxygen concentration and the amount of chain-breaking antioxidants present (Halliwell B and Gutteridge JMC 1999).

Extensive LP is often reflected in increased levels of LP products in blood, whereas lipid peroxides formed at a primary site may accumulate in lipoproteins and be transferred through the circulation with consequent LP propagation (Halliwell B and Gutteridge JMC 1999).

The peroxidation of membrane lipid may lead to enhanced membrane permeability and increased intracellular calcium (Elliot SJ and Koliwad SK 1995). LP products may contribute to endothelial injury (Cohrane CG 1991) and may be involved in intensive oxidative modifications of LDL (Esterbauer H et al. 1992) and in the development of atherosclerosis (Basha BJ and Sovers JR 1996).
Oxidative stress and cardiovascular disease

Experimental evidence suggests that free radical mediated reactions, including LP, can induce endothelial dysfunction and injury (Fraticelli A et al. 1996; Cohrane CG 1991). As a result, the ability of the endothelium to act as a selective permeable barrier to plasma components is reduced, which would facilitate increased entry of cholesterol-rich lipoproteins into the arterial wall (Stender S and Zilversmit DB 1981). Furthermore, endothelial cell dysfunction induces interactions between endothelial cells and smooth muscle cells, platelets and monocytes/macrophages (Suematsu M et al. 1993; Ward PA 1991). This may lead to further alterations in vascular regulatory mechanisms, such as increased synthesis of prothrombotic substances, enhanced release of vasoconstricting and growth factors (Busse R and Fleming I 1996).

Certain forms of ROS may deplete bioavailable NO and exacerbate local oxidant stress by reacting directly with NO to form peroxynitrite and other oxidized species. Peroxynitrite, in turn, causes oxidative injury to the endothelium (Beckman JS et al. 1990).

All these events are crucial in the pathophysiology of atherosclerosis (Pandya DP 2001; Heitzer T et al. 2001; Witztum JL 1994). The early stages of atherosclerosis are characterized by subendothelial accumulation of plasma LDL (including some oxLDL) in lesion-prone arterial sites, where it is thought to undergo more intensive oxidative modification via LP-mediated process (Berliner JA and Neinecke JW 1996; Steinberg D et al. 1989). The atherogenic potential of oxLDL is potent and multiple, including activation of monocytes and T-cells, chemotactic effects on blood monocytes and facilitation of foam cell formation. oxLDL is also cytotoxic to endothelial cells and may cause upregulation of adhesion molecules on endothelial cell (Figure 2). Oxidative stress augments the
Reactive oxygen species (ROS) impair vascular function by:

- Injury of endothelial and vascular smooth muscle cell membranes;
- Reacting with nitric oxide (NO) and inactivating it;
- Oxidizing tetrahydrobiopterin (BH4) as cofactor of nitric oxide synthase (NOS);
- Peroxidizing low-density lipoprotein (LDL) to oxidized LDL (oxLDL);
- Stimulating synthesis of asymmetric dimethylarginine (ADMA) yielding NOS inhibition;
- Inhibiting guanylyl cyclase, which leads to decrease production of cyclic guanosine monophosphate (cGMP), activation of platelet aggregation and adhesion; oxLDL, formed under influence of ROS, which may cause upregulation of receptors of adhesion molecules on endothelial cell, PDGF receptors on smooth muscle cell (SMC), PDGF synthesis by SMC, and HLA-expression on monocytes. ECM – extracellular matrix.
inflammatory response in the blood vessel wall. An increased ROS stimulates the endothelium to express the cell surface adhesion molecules P-selectin (Vora DK et al. 1997) and vascular cell adhesion molecule-1 (Li H et al. 1993).

Effects by oxLDL on smooth muscle cells include upregulation of platelet derived growth factor AA (PDGF AA mRNA and PDGF receptors) and stimulation of proliferation (Raveh O et al. 2001; Jiang F et al. 2001; Cucina A et al 1998). Besides, oxLDL also enhances vasoconstriction (Anderson TJ et al. 1996).

Oxidative reactions in the vasculature activate the expression of the transcription factor nuclear factor-κB (Collins T et al. 1995), which in turn promotes the expression of receptors and chemotactic agents to facilitate the migration of inflammatory cells to the development of atheroma.

**Oxidative stress and chronic renal failure**

It has also been shown that formation of ROS plays an important role in the pathophysiology of a wide variety of clinical and experimental renal diseases (Baud and Ardaillon 1986; Nath and Salahudeen 1990). Several studies have demonstrated OS in renal patients including decreased serum antioxidative activity (Kuroda et al 1985), increased concentration of thiobarbituric acid-reactive substances (TBARS) in uremia (Fillit et al 1981), increased concentration of malondialdehyde (MDA) in plasma and in erythrocytes, platelets and peripheral blood mononuclear cells of hemodialyzed patients (Taccone-Galluci et al. 1989, 1987, 1985; Canestrari et al. 1994; Mimic-Oka et al. 1999).

The development of atherosclerosis in CRF patients seems to be dependent on the oxidative status of lipoproteins and the resistance to oxidation.
AIMS

The aims of the present studies were as follows:

I. To investigate endothelium-dependent and endothelium-independent vasodilation in patients with chronic renal failure in relationship to conventional risk factors and renal function.

II. To investigate oxidative stress markers in patients with chronic renal failure, comparable healthy controls and to characterize relationship to renal function.

III. To investigate the relationship between markers of oxidative stress and endothelium-dependent and endothelium-independent vasodilation in patients with chronic renal failure.

IV. To examine the acute effects of L-arginine and diclofenac, an inhibitor of cyclooxygenase-derived vasocontractive agents, on endothelium-dependent vasodilation in patients with chronic renal failure.

V. To investigate the acute and long-term effects of Epoetin alfa on endothelial cell function in patients with chronic renal failure.
SUBJECTS AND METHODS

Study populations and study protocols

Study I

The study sample consisted of 56 patients (20 women and 36 men) with moderate chronic renal failure (mean creatinine clearance 29.4 ml/min/1.73m²), aged 66.9 ± 14.0 years. All were outpatients recruited from Renal Unit of the Department of Medical Sciences, University Hospital, Uppsala.

A control population consisted of 56 subjects (12 women and 44 men) without renal impairment, aged 62.1 ± 10.0 years. All control subjects were recruited from the general population in Uppsala.

Both patients and controls underwent evaluation of endothelium-dependent and endothelium-independent vasodilation.

Study II

The study population consisted of 38 patients (13 women and 25 men) with chronic renal failure (mean serum creatinine 315 ± 210 µmol/L), aged 64.8 ± 17 years. All were outpatients recruited from the Renal Unit of the Department of Medical Sciences, University Hospital, Uppsala.

The control population (21 women and 40 men, mean age 58.5 ± 7 years) was recruited from the Blood Center at the University Hospital, Uppsala.

Oxidative stress markers were measured both in patients and controls.

Study III

The study population consisted of 37 patients (13 women and 24 men) with mild to moderate chronic renal failure (creatinine clearance 25.1 ± 16.2 ml/min/1.73 m²). All were outpatients recruited from Renal Unit of the Department of Medical Sciences, University Hospital, Uppsala. Both oxidative stress markers and endothelial function were measured in this patient group.
Study IV

Effects of L-arginine and diclofenac on endothelium function were measured in 15 patients (5 women and 10 men, aged 66.0 ± 11.5 years) with chronic renal failure (mean creatinine clearance 28.4 ± 19 ml/min/1.73m²) and in 15 healthy controls (6 women and 9 men, aged 69.7 ± 11.3 years).

All patients and controls underwent evaluation of resting forearm blood flow (FBF), EDV and EIDV before evaluation of FBF during intra-arterial L-arginine infusions (80 mg/min and 40 mg/min). In order to evaluate the effects of L-arginine infusion on EDV and EIDV, re-measurements of EDV and EIDV were performed during local infusions of a low dose of L-arginine (10 mg/min). To investigate the effects of cyclooxygenase (COX) inhibition on EDV and EIDV, the measurements of EDV and EIDV were performed during intra-arterial infusion of diclofenac (30 mg/h) (Figure 3).
Study V

Eighteen patients with renal anemia (mean serum creatinine 238 ± 110 µmol/L, mean hemoglobin 101 ± 8 g/L) underwent evaluation of endothelium function investigations before and 30 min after an intravenous injection of Epoetin alfa (10 000 IU). The measurements were repeated after the hemoglobin had reached a level exceeding 120 g/L by Epoetin alfa treatment.

To control for any effects of intraarterial infusion, ten healthy subjects received local intra-arterial saline infusion and EDV and EIDV were measured before and after infusions.

Venous occlusion plethysmography

There are different methods to investigate the endothelium function in humans (Wilkinson IB et al. 1998; Celemajer DS et al. 1992; Vita JA et al. 1990).

We used venous plethysmography because the studied blood vessels remain in their physiological environment where they still are influenced by vascular nerves, sympathetic activity and vasoactive substances, local and circulating.

We evaluated endothelial function in the vascular bed of the forearm (Benjamin N et al. 1995; Brakkee AJM 1983). Vascular beds are however different, but atherosclerosis in the brachial artery is correlated with both coronary and carotid disease (Sorenson et al. 1997), and there is a close relationship between endothelial function in the coronary and forearm circulation (Anderson TJ et al. 1995). Also the endothelial function of the brachial artery is correlated with the prognosis of coronary artery disease (Neunteufl T et al. 2000).

During the blood flow measurements the subjects were supine in a quiet room maintained at a temperature 21-22ºC. An arterial cannula was inserted into the brachial artery of one arm for regional infusions of methacholine (MCh) and
sodium nitroprusside (SNP). A mercury-in-silastic strain gauge, connected to a calibrated plethysmograph, was placed at the upper third of the forearm, which rested comfortably slightly above the level of the heart.

Venous occlusion was achieved by blood pressure cuff applied proximal to the elbow and inflated to 40 mmHg by a rapid cuff inflator. Approximately four inflations/min for about 7 sec each were performed. After a measurement of resting forearm blood flow (FBF) with venous occlusion plethysmography, local infusion of MCh (2 µg/min and 4 µg/min) was performed. This muscarinergic agonist has been shown to increase the forearm release of nitrite and nitrate, the breakdown products of NO, more than eightfold in healthy subjects (Lind L et al. 2000).

To control the mechanical properties of the vascular bed in the skeletal muscle, the exogenous NO-donor SNP (5 µg/min and 10 µg/min) was infused. The vasoactive drug infusions were given during 5 min for each dose, with a 20-min washout period between the drugs. The order of the vasodilations was randomized.

Endothelium-dependent vasodilation (EDV) was defined as forearm blood flow obtained at the highest dose of metacholine (4 µg/min) minus resting forearm blood flow divided by resting forearm blood flow. Endothelium-independent vasodilation (EIDV) was defined as forearm blood flow obtained at the highest dose of nitroprusside (10 µg/min) minus resting forearm blood flow divided by resting forearm blood flow.

The endothelial function index (EFI) was calculated as the ratio between EDV and EIDV.

We have previously shown the short-term (2 h) and long-term (3 weeks) variability of FBF during vasodilatation with MCh and SNP with this method to be less than 5% (Lind L et al. 1998).
Measurement of oxidative stress markers

Blood samples were obtained from *v.cubitalis* and stored frozen at -195°C (liquid nitrogen) until analyzed. All measurements of LP and antioxidant markers were performed in triplicate. LP products were measured in serum and samples were treated with antioxidant butylated hydroxytoluene (BHT) twice, immediately after obtaining and before adding the test reagents to suppress artefactual changes during handling and assay procedures.

*Lipid peroxidation markers*

LP markers comprised diene conjugates (DC), lipid hydroperoxide (LOOH), thiobarbituric acid (TBA) and TBA reactive substances. The first stage of LP consists of the molecular rearrangement of the double bonds in polyunsaturated fatty acid residues of lipids, which leads to the formation of DC. DC levels were measured according to previously described methods (Ristimäe T et al. 1999). Briefly, samples were incubated at 37°C for 25 min, 0.25% BHT and lipids were extracted by heptane/isopropanol (1:1), and samples were acidified by 5 M hydrochloric acid and extracted by heptane. After centrifugation and absorbance of heptane, the fraction was measured spectrophotometrically at an absorbance maximum between 220 and 250 nm. LOOH level was measured by commercially available kit (K-assay, Kamiya Biomedical Company; Seattle, WA).

TBA reactive substance level of serum was measured as described previously (Ristimäe T et al. 1999). Briefly, samples were incubated with 0.475 M Fe²⁺ at 37°C for 30 min. After incubation, BHT (0.25%) was added to the samples, treated with acetate buffer (pH 3.5), and heated with TBA solution (1%, 80°C, 70 min). Then the samples were cooled, acidified, and extracted with butanol. Next, samples were centrifuged, and absorbance of butanol was measured at 534 nm.
Antioxidant markers

No single component of serum antioxidants complex exists that could fully reflect the protective potency of whole blood. Thus, we assessed serum total activity (TAA), reduced and oxidized glutathione (GSH and GSSG, respectively) content in red blood cells, and oxidation resistance of lipoprotein fraction (LPF). TAA was measured as described earlier (Ristimäe T et al. 1999), assessing the ability of the test sample to inhibit linolenic acid peroxidation. Briefly, standard linolenic acid in isotonic saline (0.4 ml), sodium dodecyl sulfate (0.015 ml), and serum (0.030 ml 1: 3: 3 in isotonic saline) were incubated in the presence of 0.2 mM iron at 37°C for 60 min. BHT was added, and samples were treated with acetate buffer (pH 3.5), heated with TBA solution, and assessed for TBA reactive substances. The results were expressed as percentage of inhibition of linolenic acid peroxidation induced by serum samples.

Glutathione was measured by an enzymatic method (Tietze F 1969), modified by Griffith (1980), described by Bhat et al. (1992), and slightly modified by us. Protein was removed from 0.3 ml of heparinized blood by adding an equal volume of a 10% solution of metaphosphoric acid in water, leaving the mixture at room temperature, and then centrifuging it (4°C, 1200 x g, 10 min). The supernatant was carefully collected and stored at -20°C. The sample was divided into two parts for measurement of total amount of glutathione (TGSH) and GSSG. TGSH = GSH plus GSSG. To assay TGSH or GSSG, the supernatant was mixed with 0.895 ml of 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M ethylenediaminetetra-acetic acid and with 0.5 ml of the same buffer containing 0.5 U GSH-reductase and 0.3 mM NADPH. The reaction was initiated by the addition of 0.1 ml of 1 mM 5,5’dithiobis-(2-nitrobenzonic acid). The change in optical density was measured at 412 nm after 10 min and quantitated by comparison with standard curve.
The content of GSH was calculated as the difference between TGSH and GSSG. The resistance of LPF to copper-catalyzed oxidation (lag phase of LPF) was estimated as described by Ristimäe et al. (1999). Briefly, a non-HDL fraction was isolated by a dextran-magnesium precipitation method (Zhang A et al. 1994). Peroxidation of LPF solutions in phosphate-buffered saline (2 mg protein/ml) was initiated with Cu²⁺ (0.45 mM), and the ability of this fraction to oxidize was evaluated by measuring DC at different time intervals of incubation at 37°C. The change of DC absorption as a function of time reflects the process of LDL oxidation, and the oxidation resistance is defined as the length of lag phase. The lag phase can be estimated as the point of intersection between the tangents to the lag time and the propagation phase.

**Statistical analyses**

Differences between groups were calculated by factorial ANOVA (Study I–IV).

The relationship between studied variables were evaluated by univariate regression analysis (Study I–III).

Interactions between several independent variables were then examined with multiple regression analysis (Study I).

Effects of intervention or treatment were calculated using ANOVA for repeated measurements (Study IV and V). p< 0.05 was regarded significant.
RESULTS AND DISCUSSION

Study I: Impaired endothelium-dependent vasodilation in renal failure in humans

Results

Infusion of MCh increased forearm blood flow (FBF) in both groups: in patients with renal failure from 5.6 ± 1.9 (SD) to 15.3 ± 4.2 ml/min/100 ml tissue at the highest dose and in controls from 4.4 ± 1.4 to 19.6 ± 6.3 ml/min/100 ml tissue. As compared with controls, FBF was significantly lower during MCh infusion in patients with renal failure (p<0.0001).

Infusion of SNP caused an increase in the FBF in renal failure patients from 5.6 ± 1.9 to 16.0 ± 4.6 ml/min/100 ml tissue at the highest dose and from 4.4 ± 1.4 to 16.5 ± 5.3 ml/min/100 ml tissue in controls subjects (no significant difference between the groups).

As significant differences in age, frequency of hypertension treatment, blood pressure and fasting blood glucose levels were seen between CRF patients and controls, multiple regression analyses were performed in order to assess if the differences in EDV still persisted after the differences in these cardiovascular risk factors had been adjusted for.

Without including the risk factors, the independent variable uremia/control (uremia = 1) yielded an $R^2$ of 0.21 and a regression coefficient of -176 (95% CI (-242, -110), t = 5.34, p<0.0001) for EDV and $R^2$of 0.14 and a regression of -0.26 (95% CI (-0.39, -0.13), t = 4.13, p<0.0001) of EFI (ratio between EDV and EIDV) as dependent variables. In these models it could be seen that uremia was the major, significant, independent variable explaining the variation in EDV and EFI. The classical risk factors did not contribute in a significant way in this aspect.

There was a negative correlation between the index of endothelial function (EFI) and serum creatinine ($r = 0.34; p = 0.01$) in patients with renal failure, while
EDV was related to serum creatinine ($r = -0.37; p<0.001$), creatinine clearance ($r = 0.45; p<0.005$) and to serum triglyceride levels ($r = -0.29; p<0.005$).

**Discussion**

The present study demonstrated that patients with CRF showed a significantly less pronounced vasodilation during MCh infusion, when compared to controls. SNP-induced vasodilation indicates that there is no difference in blood vessel smooth muscle function in patients with renal failure. Thus the vasodilatory dysfunction in CRF patients seems to be limited to the bioavailability of NO.

To exclude the possibility that differences in the occurrence of hypertension, hypercholesterolemia, and diabetes could explain the impairment in EDV in CRF patients, we performed multiple regression analysis to evaluate the role of these cardiovascular risk factors on EDV. The multiple regression analysis revealed that EDV was impaired in CRF patients irrespective of these cardiovascular risk factors. Another finding in this study was that EDV was correlated with serum creatinine level and creatinine clearance. These findings allow us to conclude that the renal impairment in itself is related to impaired EDV.

It is well known that uremia is associated with dyslipidemia (Keane WF 1994). Previous studies in animals and humans have demonstrated that hypercholesterolemia may lead to an impaired delivery of nitrovasodilators to the vascular media and that elevated circulating free fatty acid levels could induce an impaired EDV (Kari JA et al. 1997; Creager MA et al. 1990). In the present study, total serum cholesterol and serum triglyceride levels were mainly in the normal range in CRF patients. Although a significant correlation between serum triglycerides and EDV, but not serum cholesterol and EDV, was found in
patients with renal failure, indicating that altered triglyceride metabolism and associated changed plasma lipoprotein metabolism may have an important influence on EDV in this patient group.

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthase, is elevated in patients with CRF and may also contribute to endothelial dysfunction in this patient group (Zoccoli C et al. 2001; Vallance P et al 1992).

**Study II: Oxidative stress markers in pre-uremic patients**

**Results**

A significant difference between patients with renal failure and healthy controls was found in the majority of the oxidative stress (OS) indices. The renal patients had significantly higher levels of DC (p<0.02), BDC-LPF (p<0.05), LOOH (p<0.05) and GSSG (p<0.0001) compared with controls. The lag time of LPF, as a marker of the resistance of the lipoprotein fraction to oxidation was significantly longer in healthy controls (p<0.01).

A significant correlation was found between serum creatinine and GSSG concentrations (r = 0.34; p<0.05) and between serum creatinine and glutathione redox ration or GSSG/GSH ratio (r = 0.37; p>0.05). In addition, positive correlations were found between serum urea and GSSG concentrations (r = 0.45; p<0.01) and between serum urea and GSSG/GSH (r = 0.56; p<0.01), whereas an inverse relation was found between serum urea and GSH concentration (r = -0.43; p<0.01). Inverse relationships were also demonstrated between GSSG/GSH and lag phase of LPF (r = -0.62; p<0.01) and between GSSG level and BDC-LPF (r = -0.55; p<0.03).
Discussion

Patients with CRF have increased risk of development of CVD, in the sense that severe atherosclerotic vascular disease develops much more quickly compared with healthy subjects (Foley RN et al. 1998). The development of atherosclerosis is related to severe prolonged oxidative stress, expressed via elevated lipid peroxidation (de Cavanagh EM et al. 1999; Jackson P et al. 1995; Maggi E et al. 1994; Berger HM et al. 1990).

To get more adequate information about OS in patients with CRF, we measured a complex of OS markers (GSSG, GSH, GSSG/GSH, lag phase of LPF, BDC-LPF, TAA, DC, LOOH and TBARS) in patients with impaired renal function and in healthy controls.

Patients had significantly higher levels of DC, BDC-LPF, LOOH and GSSG compared to healthy controls. The lag time was significantly longer in healthy controls.

An increased level of GSSG as well as increased glutathione redox status (GSSG/GSH) have been described as markers of severe cellular oxidative stress (Halliwell B and Gutteridge JMC 1999; Canestrari F et al. 1994). A decreased resistance to oxidation of LDL (shortened lag phase of LPF) and higher baseline of oxLDL indicate a clearly atherogenic nature of plasma lipoproteins (Holvoet P et al. 2000; Ahotupa M and Vasankari TJ 1999).

High values of diene conjugates and lipid hydroperoxides are also considered to be markers of systemic oxidation stress (Halliwell B and Gutteridge JMC 1999).

In the present study, it was demonstrated that GSSG and GSSG/GSH correlated significantly with serum creatinine and urea, which indicates a possibility that glutathione redox status and GSSG level are related to the degree of renal failure. Thus, these elevated cellular OS may have implications for the
atherogenic propensity that is also supported by inverse relation between GSSG/GSH, and by lag phase of LPF, and by the inverse relation between serum urea level and GSH level.

In conclusion, it is suggested that GSSG level, GSSG/GSH status, lag phase of LPF and BDC-LPF are markers of oxidative stress, and implicate a degree of OS in CRF.

A high GSSG/GSH ratio, GSSG level, short lag phase of LPF or high value of BDC-LPF in patients with CRF all indicated increased risk for atherosclerosis.

Study III: Oxidative stress and endothelial function in chronic renal failure

Results

Endothelial vasodilatory function and oxidative stress markers were measured simultaneously in patients with CRF.

Compared with controls, the patients with renal insufficiency had an impaired endothelium-dependent vasodilation, a shorter lag phase of lipoprotein fraction, and higher levels of diene conjugates, lipid hydroperoxide, and GSSG. The GSSG/GSH ratio was lower in patients with CRF.

Endothelium-dependent vasodilation was positively correlated with total antioxidative activity ($r = 0.41$, $p = 0.016$), GSH ($r = 0.44$, $p<0.0098$; Figure 4A), and lag phase of LDL ($r = 0.35$, $p = 0.036$) and negatively correlated with GSSG ($r = -0.40$, $p<0.018$; Figure 4B), GSSG/GSH ($r = -0.47$, $p = 0.0057$; Figure 4C), and diene conjugates ($r = -0.53$, $p<0.0015$) in patients with CRF.
Figure 4

Relationship between endothelium-dependent vasodilation (EDV) and reduced glutathione GSH (A) \( r = 0.44, p<0.0098 \), oxidized glutathione GSSG (B) \( r = -0.40, p<0.018 \) and GSSG/GSH ratio (C) \( r = 0.47, p<0.0057 \)
Discussion

Experimental evidence suggests that free radical mediated reactions, including LP, can induce endothelial dysfunction and injury (Fraticelli A et al. 1996; Cohrane CG 1991).

The results of this study show a close positive relationship between endothelium-dependent vasodilation and GSH (a main cellular antioxidant) and a negative relationship between endothelium-dependent vasodilation and GSSG (or glutathione redox ratio), indicating the important role of GSH in the regulation of endothelial dysfunction in patients with CRF.

The free radical attack on cell membrane polyunsaturated fatty acids results in formation of LP products such as DC, LOOH, and malondialdehyde. High levels of DC, LOOH, and malondialdehyde (the latter is expressed as TBA activity) are considered to be markers of systemic oxidative stress (Halliwell B and Gutteridge JMC 1999). Studies of predialysis patients have demonstrated a relationship between lipid peroxidation activity and the degree of renal failure (Fillit H et al. 1981). In this study, a significant inverse correlation was found not only between DC levels and endothelium-dependent vasodilation but also between DC and endothelium-independent vasodilation. These findings indicate that endothelial cell function and vascular smooth muscle cell function may be modulated by LP.

The substantial role of lipoprotein oxidation in atherosclerosis is well established. A decreased resistance to oxidation of LDL (shortened lag phase) indicates an atherogenic nature of LDL (Holvoet P et al. 2000; Ahotupa M and Vasankari TJ 1999) and is associated with an increased risk of atheromatosis (Rengström J et al. 1992). The present study demonstrated a significant correlation between the lag phase of LPF and endothelium-dependent vasodilation in patients with CRF. Thus, the shorter the lag phase, the higher propensity to produce
oxLDL. This is associated with reduced endothelium-dependent vasodilation in a patient group known to have an increased risk for atherosclerosis, including cardiac and peripheral vascular disease.

**Study IV: Cyclooxygenase inhibition improves endothelium-dependent vasodilation in patients with chronic renal failure**

*Results*

Infusion of L-arginine (40 and 80 mg/min) increased FBF in renal patients from $4.5 \pm 0.8$ to $7.4 \pm 1.6$ ml/min/100 ml tissue ($p<0.001$) at the highest dose and in controls from $4.3 \pm 1.0$ to $5.8 \pm 1.4$ ml/min/100 ml tissue, $p = 0.003$). The increase in FBF was significantly higher in renal patients ($p = 0.005$).

Infusion of L-arginine (10 mg/min) increased resting FBF both in patients with renal failure (from $4.5 \pm 0.8$ to $5.3 \pm 1.4$ ml/min/100 ml tissue, $p = 0.004$) and in healthy controls (from $4.3 \pm 1.0$ to $4.9 \pm 0.9$ ml/min/100 ml tissue, $p = 0.03$). Similarly, infusion of diclofenac increased resting FBF in patients with renal failure from $4.5 \pm 0.8$ to $5.9 \pm 1.2$ ml/min/100 ml tissue ($p = 0.004$) and in controls from $4.3 \pm 1.0$ to $4.9 \pm 0.9$ ml/min/100 ml tissue ($p = 0.01$).

L-arginine infusion (10 mg/min) resulted in a significant increase in FBF during MCh infusion at the highest dose in renal patients from $13.8 \pm 2.1$ to $15.0 \pm 3.1$ ml/min/100 ml tissue ($p = 0.007$) and in controls from $16.6 \pm 3.0$ to $18.9 \pm 3.2$ ml/min/100 ml tissue ($p = 0.01$). Infusions of L-arginine (10 mg/min) did not result in a significant change in FBF during SNP infusion in renal patients and in controls.

Diclofenac infusion increased significantly FBF during MCh infusion at the highest dose from $13.8 \pm 2.1$ to $15.5 \pm 2.4$ ml/min/100 ml tissue in patients with renal failure ($p<0.05$). There was no significant change in the FBF during MCh infusion in healthy controls. Infusion of diclofenac did not increase FBF during SNP infusions in any of the groups.
Discussion

In end-stage renal failure there is accumulation of methylated analogs of L-arginine, such as asymmetrical dimethyl arginine (ADMA). These substrates are known as competitive inhibitors of NO-synthase (Vallance P et al. 1992).

Therefore we administrated L-arginine, the precursor of NO, to patients with CRF and to the healthy controls to examine if the responses of MCh and SNP improved. The low dose of L-arginine (10 mg/min) resulted in an increase in resting FBF, as well as in EDV, both in renal patients and in controls with no significant difference between the groups, suggesting that lack of L-arginine, or competition by ADMA, do not play a role for CRF patients, but might be a general factor in this age group. Chauhan and co-workers (1996) have also reported a selective impairment of endothelium-dependent vasodilatation with aging and that administration of L-arginine can restore endothelial function.

L-arginine infusion at higher doses resulted in an increase in FBF both in patients with CRF and in healthy controls, being more pronounced in patients with CRF. This finding supports the idea that patients with CRF have low L-arginine levels resulting in an impaired EDV or the presence of competitive inhibitors of NOS, i.e. ADMA, possibly counteracted by infusion of L-arginine leading to an improvement in NO production. It is quite possible that increased levels of L-arginine may overcome effects of ADMA.

A prostanoid-derived contracting factor has been found to be co-released by the endothelium during muscarinic receptor stimulation in hypertensive patients (Taddei S et al. 1997) and in spontaneously hypertensive rats (Lüscher TF et al. 1990) This can be disclosed by inhibition of the cyclooxygenase (COX) pathway, as used in the present study.

Lipid peroxidation products might inactivate NO by the formation of ONOO-. However, as this mechanism also would affect exogenously given NO, and no effect on FBF during SNP infusion was seen, this mechanism seems less likely. It
could be seen, that although COX inhibition improved EDV, EDV was not normalized. This implies that other mechanisms are involved in the impairment of EDV seen in CRF. COX inhibition increased resting FBF in both CRF and controls suggesting that during resting conditions a tonic effect of endothelium-derived contracting factors is present in this age group.

**Study V: Both acute and long-term erythropoietin treatment impairs endothelial vasodilatory function in patients with renal anemia**

**Results**

EDV was significantly attenuated after acute Epoetin alfa injection (from 19.2 ± 4.2 to 15.4 ± 3.9 ml/min/100 ml tissue for MCh 4µg/min, p<0.0001) but there was no significant difference in SNP responses.

Long-term anemia treatment (15 ± 4 weeks) also attenuated EDV (to 17.2 ± 4.5 ml/min/100 ml tissue for MCh 4 µg/min, p<0.05). There was no significant effect on the SNP response.

Also, there was no significant difference in blood pressure after acute injection of Epoetin alfa or after long-term treatment of anemia.

**Discussion**

Erythropoietin (EPO) treatment is nowadays a standard treatment in renal anemia and usually improves the quality of life. However, hypertension often develops during EPO treatment by an unknown mechanism (Singbarth G 1994).

An impairment of endothelium-dependent vasorelaxation as a result of EPO administration has been described in healthy male volunteers (Wada Y et al. 1999) and Aguilera and co-workers (1999) showed in peritoneal dialysis patients that EPO treatment caused endothelial cell damage.
Experiments in anaesthetized rabbits demonstrated that treatment with EPO selectively attenuated endothelium-dependent vasodilation via inhibition of endothelial nitric oxide synthase activity (Noguchi K et al. 2001), while Wada and co-workers demonstrated an impairment through a cyclooxygenase-dependent mechanism (Wada Y et al. 1999).

The study in peritoneal dialysis patients showed that long-term treatment with EPO caused an increase in endothelial cell damage markers (thrombomodulin and tissue-type plasminogen activator), suggesting that EPO could also alter other functions of the endothelium than the vasoregulatory action (Aguilera A et al. 1998).

We did not find any significant change in arterial blood pressure after long-term EPO administration. Therefore, it is not conclusive from the present study if an impaired EDV causes the increased prevalence of hypertension described by other investigators (Taddei S et al. 1993; Linder L et al. 1990; Panza JA et al. 1990).

The present findings allow us to conclude that EPO treatment (both acute and long-term) impairs EDV, which may be of importance with respect to the high incidence of cardiovascular complications in this patient group.
GENERAL DISCUSSION

Despite different kinds of renal replacement therapy, the mortality from CVD in patients with CRF is many times higher than in the general population. The traditional risk factors are frequently present in CRF patients. However, based upon conventional risk factor analysis, these factors would not fully explain the extraordinary increase in morbidity and mortality in CVD among patients with CRF. This prompted us to investigate other factors which may contribute to and explain the high morbidity and mortality in uremic patients.

In recent years, the role of endothelial cell dysfunction and oxidative stress for development of cardiovascular disease has been highlighted (Heinecke JW 1998; Diaz MN et al. 1997). These findings prompted us to investigate endothelial function and oxidative stress in patients with chronic renal failure.

We could demonstrate (Study I) that renal impairment in itself is related to impaired endothelial vasodilatory function. Several factors have been hypothesized as potential causes of endothelial dysfunction in patients with CRF.

Such factors include accumulation of uremic toxins poisoning the endothelium, hypertension and shear stress as well as dyslipidemia with cytotoxic lipoprotein species such as small dense LDL particles. Another attractive concept is competitive inhibition of endothelial NO production by asymmetrical dimethylarginine (ADMA), which has been reported to be increased in patients with CRF (Vallance P et al. 1992).

Oxidative stress may damage endothelial function through several mechanisms. Reactive oxygen species (ROS), especially hydroxyl radicals may injure the endothelial cell membrane. An interaction between ROS and endogenous vasoactive mediators formed in endothelial cells, has been demonstrated, i.e. superoxide anions reacting with endothelium-derived NO leading to inactivation of NO (Wever RM et al. 1998). In addition, ROS may cause oxidation of an essential...
cofactor of nitric oxide synthase (NOS), tetrahydrobiopterin (BH4), yielding synthesis of superoxide anion, instead of NO (Ueda S et al. 2000; Forstermann U et al. 1998).

ROS may also oxidize lipid components, leading to formation of oxLDL, which is one of the key mediators of atherosclerosis (Holvoet P and Collen D 1998; Steinberg D 1997). Oxidized LDL is not only incorporated into macrophages leading to the formation of foam cells, but is also directly cytotoxic to the vascular wall, including endothelial cells (Matsuoka H 2001). Furthermore, oxLDL has been shown to potentially participate in the atherosclerotic process by upregulation of receptors of adhesion molecules on endothelial cells, stimulating synthesis of IL-1 and growth factors by monocytes and upregulation PDGF-AA synthesis and receptor expression in smooth muscle cells.

There are, however, also studies demonstrating that oxLDL concentration and endothelial function are unrelated in patients with CRF (Bolton CH et al. 2001). Thus, endothelial dysfunction is multifactorial.

Accumulating evidence points to the role of chronic inflammation as a contributor to cardiovascular morbidity and mortality. Chronic inflammation includes increased formation of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin -1 (IL-1) and interleukin-6 (IL-6) and cell adhesion molecules (CAMs) as well as expression of CAM receptors. Uremic patients have been shown to have increased levels of chronic inflammatory markers such as C-reactive protein (CRP) (Bolton HC et al. 2001; Stenvinkel P 2001). Oxidative stress may be a link between endothelial dysfunction and chronic inflammation in CRF patients. The endothelium produces a number of substances that not only regulate vasomotor tone but also the recruitment and activity of inflammatory cells by upregulation of receptors of adhesion molecules and the propensity for thrombosis formation. Oxidative stress also activates pro-inflammatory signalling pathways such as nuclear factor κB, which is probably a key event in the inflammatory response by oxidative stress (Das UN 2000). Our findings (Study II
and III) support role of oxidative stress in endothelial dysfunction. We found that impaired EDV was related to increased levels of oxidative stress markers and reduced levels of antioxidants.

Potential options for reduction of CVD in patients with CRF may be to improve endothelial function or reduce the degree of oxidative stress by pharmacological treatment. We examined the effect of L-arginine as a substrate for NO synthetase, and diclofenac as an inhibitor of COX-derived vasoconstrictor agents on EDV in patients with CRF (Study IV). L-arginine infusions increased EDV both in renal patients and in healthy controls, suggesting that lack of L-arginine, or competition by ADMA, may play a role and that substitution of L-arginine may be an option. There are conflicting findings regarding effects of L-arginine administration on endothelial dysfunction in chronic renal failure.

Studies have demonstrated beneficial effects of L-arginine on vascular responses in experimental animals and in humans under different pathological conditions, including renal failure-associated endothelial dysfunction (Hand MF et al. 1998). Oral intake of L-arginine was also found to prevent glomerular hyperfiltration and to decrease proteinuria in rats (Reyes AA et al. 1993). On the other hand, Cross and co-workers did not find any beneficial effects of acute L-arginine administration on endothelium function in patients with chronic renal failure (Cross JM et al. 2001). It remains to be disclosed whether long-term oral substitution with L-arginine leads to improved endothelial function.

Another option to improve endothelial function is treatment with antioxidants. Recent studies showed that vitamin C administration improved endothelial dysfunction in renal allograft recipients (Williams MJ et al. 2001) and vitamine E-modified dialysis membrane prevented an increase in lipid peroxidation during dialysis (Eiselt J et al. 2001). Vaziri and co-workers (2002) demonstrated that antioxidant therapy with vitamin E ameliorated CRF-induced hypertension,
improved vascular tissue NO production, lowered tissue nitrotyrosine burden, and reversed downregulations of NOS isoforms in rats with CRF. However, they did not find any similar effects in healthy controls (Vaziri ND et al. 2002). These findings implicate a contribution of oxidative stress to chronic renal failure-induced hypertension and dysregulation of NO metabolism.

As demonstrated in Study V, erythropoietin (EPO) treatment may impair endothelial function in patients with CRF. Our findings indicated that the hormone *per se* may act on the endothelial cells and reduce their capacity to induce vasodilation. High concentrations of EPO have been shown to exert a direct action of smooth muscle cells *in vitro*. However, in the present investigation (Study V) the endothelial independent vasodilation remained unaltered after the Epoetin alfa injection. This indicates that Epoetin alfa, when given at doses commonly used in clinical praxis, exert vascular effect by an alteration in the endothelial function.

The recent detection of receptors of erythropoietin on endothelial cells may support this theory (Brines ML et al. 2000). It is of interest, however, that the increase in blood pressure seen during EPO treatment only occurs in patients with anemia due to renal failure (Demetri GD et al. 1998). We have shown (Study I) that the endothelial function is impaired already before start of EPO treatment in these patients. The pre-existing endothelial dysfunction may contribute to the increased vascular sensitivity to EPO treatment in patients with renal failure as compared with those with anemia of other origin.

The decreased endothelial dependent vasodilatation persisted when the anemia had been corrected and when an EPO concentration comparable with that in healthy subjects was present. Persisting effects of the previously given EPO may have contributed to this. A lack of the principle substrate for NO-formation, L-arginine, may be one factor contributing to the impaired endothelial function in renal patients. During renal anemia treatment with EPO and increased blood
viscosity due to an increased hematocrit, a decreased erythrocyte deformability has been found (Linde T et al. 1992). These changes may increase the shear stress resulting in an increased NO formation and an aggravation of this substrate deficiency.

The net effect of the reduction in endothelial function found in Study V is hard to predict. Left ventricular hypertrophy is frequently present in uremic patients and has been shown to be a risk factor for death in hemodialysis patients (Foley RN et al. 1998). Since anemia is a risk factor for the development of left ventricular hypertrophy and anemia treatment with EPO causes a decrease in the left ventricular mass, early renal anemia treatment has been recommended (Singh NP et al. 2000). Our findings of an EPO-induced reduction of the endothelial dependent vasodilation may counteract the beneficial cardiovascular effects of anemia treatment, emphasizing the need for controlled studies with solid endpoints regarding cardiovascular effects of renal anemia treatment.

In conclusion, the present studies have demonstrated endothelial dysfunction in patients with chronic renal failure, which may contribute the cardiovascular morbidity and mortality in these patients. A state of oxidative stress is present in chronic renal failure and related to endothelial dysfunction. Local infusion of L-arginine improves endothelial function, and may be an optional treatment in the future. Erythropoietin treatment seems to have an adverse effect on endothelial function.

Future investigations should focus on the importance of chronic inflammation for endothelial dysfunction in CRF and thus on the process of atherosclerosis and cardiovascular disease in patients with CRF.
GENERAL SUMMARY

- Patients with moderate chronic renal failure have an impaired endothelium-dependent vasodilation even after correction for traditional cardiovascular risk factors and this impairment is related to the degree of renal failure.

- Patients with chronic renal failure are in a state of severe cellular and systemic oxidative stress.

- Impairment of endothelium-dependent vasodilation is related to increased levels of oxidative stress markers and reduced levels of antioxidants in patients with chronic renal failure.

- L-arginine infusion improves endothelium-dependent vasodilation in patients with chronic renal failure and in healthy controls, and cyclooxygenase inhibition improves endothelial-dependent vasodilation only in patients with chronic renal failure.

- Both acute and long-term treatment with Epoetin alfa impairs endothelium-dependent vasodilation, but not endothelium-independent vasodilation in patients with chronic renal failure.
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