Studies on Antioxidant and Lipid Lowering Effects on Human Microcirculation

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Abstract

In previous work from this laboratory, vital microscopy was successfully used to study microcirculatory effects of hypercholesterolemia in a rabbit model. Hypercholesterolemia caused a dramatic depression of the blood flow velocity in the conjunctiva microvessels of the rabbits. In accordance with the contention that hypercholesterolemia is associated with oxidative stress that is of importance for the microcirculation, antioxidants were shown to have a clear preventive effect on the cholesterol-induced changes in the microcirculation.

It was considered to be of interest to study if similar effects by hypercholesterolemia and antioxidants might be demonstrated also in human microcirculation. A technique for computerized video capillary microscopy imaging was set up for evaluation of the microcirculation in the nailfold. In two of the studies, the flying-spot technique was primarily used for measurement of the blood cell flow velocity (CBV). In three of the studies the Capiflow system and the time to peak (TtP) after a brief arterial occlusion (PRH) were used. According to current concepts, the latter technique is a more reliable assessment. In an evaluation of the TtP method, the between-day variation was found to have a coefficient of variation of less than 13%, provided that the mean of at least two assessments were used.

Smoking is known to induce a considerable oxidative stress that would be expected to affect the microcirculation. In accordance with this, smoking a single cigarette caused a 40-50% decrease in microcirculatory blood flow velocity in 23 of 24 subjects studied. This change was reduced by more than 50% in the same subjects after intake of 2 g of vitamin C 2 h before smoking. Interestingly, intake of 1 g of vitamin C was without effect. Pretreatment for 2 weeks with N-acetylcysteine (200 mg×3 per day) also significantly reduced the smoking-induced effect on the microcirculation in a group of healthy volunteers with mixed smoking habits. The preventive effect of N-acetylcysteine was however considerably lower than the effect of the high dose of vitamin C.

Plasma apheresis offers a unique possibility to study effects in the microcirculation of abrupt changes in plasma lipid levels. We studied four patients regularly treated with LDL-apheresis every third week. In spite of a reduction of the cholesterol levels by almost 50%, there was no obvious change in time to peak at PRH registered prior to apheresis and two days later. The smoking-induced effect on TtP did not seem to be affected by the LDL-apheresis either. Essentially the same results were obtained when measuring the change in the diameter of the brachial artery (FMD) as a response to arterial occlusion and nitroglycerin. Thus the method used for evaluation of the microcirculation and that used for evaluation of conduit vessels showed concordant results.

Diabetes is known to be associated with a disturbed endothelial function and microcirculation. In a double blind cross over study on 17 patients with type 2 diabetes, microvascular reactivity was found to be markedly reduced. Treatment for 2 weeks with a daily dose of 1g × 3 of vitamin C did not significantly improve the vascular reactivity. A slight effect could however be demonstrated in capillary blood flow velocity.

It is concluded that it is possible to use capillary microscopy for evaluation of effects on the microcirculation in the nailfold, particularly in connection with acute studies. Patients with hypercholesterolemia and diabetes as well as volunteers inhaling cigarette smoke had a disturbed microcirculation, probably related to an oxidative stress. The most significant effects of antioxidants observed in the present work appeared in connection with acute oxidative stress (smoking) and a very high dose of the antioxidant (vitamin C).

Key Words: microcirculation, oxidative stress, antioxidant, smoking, hypercholesterolemia, diabetes, vitamin C, N-acetylcysteine, lipid lowering, capillary blood flow velocity, post reactive hyperemia, endothelial function.

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Introduction

Endothelial function and microcirculation

Endothelial function

The endothelium is a cell monolayer that constitutes the internal structure of the entire circulatory system. The endothelial cell (EC) surface in an adult human is composed of approximately $6 \times 10^{13}$ cells, weigh about 1 kg [1]. The total area of all the capillaries in the body, which are composed of single layer of endothelial cell, is estimated to be over 6000 m$^2$ in adults [2].

The endothelium is an anatomical barrier between blood and interstitium at the capillary level, and in the entire vascular tree. Endothelial layers generates NO which together with other mediators play a pivotal role in the regulation of blood flow [3-5], in different vascular beds under different physiological, pathological or pharmacological stimuli. Adhesive molecules are expressed by endothelial cell involved in the process of leukocyte rolling, adhesion on the endothelium, and migration through it, especially under inflammatory conditions. The endothelium is also of importance as an antithrombotic factor. Thus it prevents exposure of the thrombogenic sub-endothelium to the circulating factors of coagulation, and inhibits aggregation of platelets [6]. More generally, the endothelium controls the inter- and transcellular traffic of numerous nutrients and hormones in the metabolic process. Endothelial cells are also involved in the initiation of vessel formation and subsequent branching, establishment of capillary beds, and vessel wall formation [7, 8].

NO is synthesized in endothelial cells from L-arginine by the endothelial isoform of NO synthetase (eNOS) [9]. This enzyme is constitutively expressed and located to caveolae in the plasma membrane. It is catalytically inactive when bound to caveolin. In the presence of calcium, calmodulin displaces caveolin and binds to eNOS, thereby activating the enzyme [10]. Thus, NO production by endothelial cells is stimulated by factors that increase intracellular agonists like acetylcholine, bradykinin, substance P, and thrombin [11]; and by physical stimuli including shear stress [12]. NO produces vasodilation primarily by activating guanyl cyclase in vascular smooth muscle cells, which increases intracellular concentrations of cGMP. The latter compound acts in turn as a second messenger, activating cGMP-dependent protein kinase, which decreases cytosolic calcium concentration and modulates ion channel function leading to relaxation of vascular smooth muscle cells.

The hemodynamic shear stress, as the friction of blood flow on the endothelium, is regarded to be the main stimulus of eNOS activity. As a consequence of increased metabolic activity, the local metabolic changes ($PO_2 \downarrow$, $PCO_2 \uparrow$, pH$\downarrow$) promote the relaxation of precapillary
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sphincters, causing an increase in capillary blood flow. Blood flow also increases in the upstream arterioles and arteries. The increased blood flow is accompanied by an increase in endothelial shear stress, which in turn enhances endothelial NO synthesis and release, and also prostacyclin synthesis and release. These vasodilator factors diffuse locally and relax the underlying smooth muscle cells. This flow-mediated vasodilation occurs both in arteries and veins and allows the adaptation of the diameter of the vessel to the flow. Flow-dependent vasodilation (proximal), combined with metabolic vasodilation (distal), allows a perfect adaptation between tissue O₂ consumption and tissue O₂ supply.

**Microcirculation**
The microcirculation is widely defined as the blood flow through vessels that have a diameter less than 150µm. It includes arterioles (with the diameter 10-100µm), capillaries (4-10µm), and venules (10-100µm). However, there is no universally accepted definition of the microcirculation. Whereas the arterioles have one or two layers of smooth muscles in the tunica media, a small artery (0.1-2mm) may have up to about eight layers. Capillaries are responsible for transport of nutrients to the tissues and removal of cellular excreta. The capillaries consist of a single layer of endothelial cells. Their distribution, arrangement and structure vary from organ to organ. Blood flow through capillaries is controlled by the contraction of vascular smooth muscle at the level of arteriole. The arterioles are the major site of the resistance to blood flow [13, 14], due to the sudden decrease of the diameter and the presence of smooth muscle cells in the vessels that are able to dynamically adjust the diameter.

A definition based on arterial vessel physiology rather than diameter or structure has been proposed, based on the response of the isolated vessel to increased internal pressure [15]. According to this definition, all the arterial vessels that respond to increasing pressure by a myogenic reduction in lumen diameter should be included in the microcirculation, in addition to the capillaries and venules. Such a definition would include the smallest arteries and arterioles in the microcirculation and would be in line with the recent suggestion that the small arterial and arteriolar components should be considered a continuum rather than distinct sites of resistance control [16]. Several aspects of the physiology of arterial vessels, including flow-dependent responses, are not restricted to particular vessel categories. Magnitude of flow dependency is obviously greater in smaller vessels [17].

The regulation of the vascular bed blood flow is mainly controlled by local factors such as blood pressure and metabolites, as well as by nerves and circulating hormones. The endothelial-mediated dilation is one of the local control factors of peripheral blood flow. If the arteriole is denuded of endothelium, the dilatation of the vessel in response to
increase flow is abolished [18]. The vasodilation is presumably caused by endothelial-derived relaxing factor (EDRF), which is released from the endothelium in response to the shear stress occurring as a consequence of the increase in flow velocity. Now it is clear that this is mainly caused by endothelial NO release [19]. Reactive hyperemia is another mechanism for intrinsic or local control of blood flow regulation. When the circulation to a tissue is released after a short period of vascular occlusion, there is usually a sharp rise in blood flow to the tissue followed by a gradual and roughly exponential return to the resting level. It can be easily observed in cutaneous areas such as the hand. It is not dependent on vasomotor nerves [20]. The exact mechanism that accounts for the coordination of arterial and arteriole dilation is not well understood [21]. One factor believed to be of important is accumulation of vasodilator metabolites. The increase of shear stress caused by the release of the occlusion may induce NO release and thus increase the flow.

Oxidative stress and antioxidant defense

Oxidative stress is of importance in many human disease processes, especially in cardiovascular disease, aging and cancer. Human has evolved a particularly efficient antioxidant defense system. The balance between oxidant stress and antioxidant defense is very important in relation to the endothelium derived NO function.

Oxidative stress

Oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species and antioxidant defenses, which may lead to tissue injury [22]. Free oxygen radicals are generated endogenously as an unavoidable by-product of many biochemical processes. It has been calculated that 1-3% of the oxygen consumed is used to make superoxide and that human beings may produce over 2 kg of superoxide in the body every year [23, 24]. In the vascular system, there is a membrane bound oxidase, NADH/NADPH oxidase [25, 26] , both in endothelium and smooth muscle. This enzyme is an important source of vascular superoxide production, which can reduce endothelial NO activity.

In addition, free radicals may be generated exogenously in response to electromagnetic radiation from the environment, metabolism of drugs; or acquired directly in the form of oxidizing pollutants such as ozone, nitrogen dioxide, and tobacco smoke [27].

Molecules such as superoxide anion (O$_2^-$), hydroxy radical (HO), nitric oxide (NO) and lipid radicals are free radicals which possess an unpaired electron. Others such as hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO$^-$), and hypochlorous acid (HOCl), are not free radicals but have oxidizing effects that contribute to the oxidant
stress. The latter chemical species are often referred to as ROS (Reactive Oxygen Species) due to their higher reactivity relative to molecular O$_2$. The reaction between superoxide and NO is extremely rapid with a rate constant which is very near the limit of diffusion [28].

Superoxide production is increased in a number of disease states including hypercholesterolemia [29, 30], hypertension [31], diabetes mellitus [32] and inflammation [33-35]. The precise mechanisms leading to this increase remain uncertain.

Another consequence of the increased production of reactive oxygen species in the vasculature is lipid peroxidation, and there is strong experimental evidence that this process adversely influences endothelial function and the bioactivity of NO. For example, ox-LDL is cytotoxic to endothelial cells [36]. Ox-LDL promotes the recruitment of inflammatory cells to the vessel wall, which may increase local production of reactive oxygen species leading to ‘inactivation’ of NO as described above [37]. There is evidence that ox-LDL and products of lipid peroxidation can react with NO directly and thus eliminate its biological activity [38, 39]. Ox-LDL also seems to decrease eNOS protein levels in endothelial cells [40], thus potentially decreasing production of NO. Finally, products of lipid peroxidation including lysophosphatidylcholine, may interfere with signal transduction and receptor-dependent stimulation of NOS activity [41, 42] resulting in reduced activation of guanylyl cyclase [43].

**Antioxidant defense**

Human body has evolved strong antioxidant defense mechanisms to protect against free radical attacks [44, 45]. The possibility has been discussed that the high efficiency of this defense is one of the reasons for the relative long life of the human species [46-48]. Numerous natural defenses exist either to prevent ROS formation or to neutralize them after they have been generated. The defenses include enzymes as well as low-molecular-weight compounds. SOD catalyzes the dismutation of superoxide anion (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) [49-51]. In the presence of transition metals, H$_2$O$_2$ is rapidly converted to the potent hydroxyl radical HO• through the Fenton reaction. The two most important enzymes that are able to neutralize H$_2$O$_2$ are catalase and glutathione peroxidase. The latter enzyme is also able to reduce peroxides, including lipid peroxides. There are several metal binding proteins, such as ferritine, transferrin, ceruloplasmin, and albumin that are able to keep transition metal ions bound. All these defenses are largely extracellular.

Aside from these enzymes and proteins, there is an array of antioxidant molecules capable to scavenge free radicals in extracellular compartments [52]. The most notable are vitamin C and E, various carotenoids, glutathione, ubiquinone, uric acid and bilirubin. Several dietary micronutrients may also contribute to
protective mechanisms (vitamins, oligominerals and polyphenols) [53], [54].

Ascorbic acid is a very potent water-soluble antioxidant in human plasma[55]. Ascorbic acid is present in plasma at levels of about 30-60µM in unsupplemented healthy individuals [56]. After oral supplements these concentration can be about doubled [57, 58]. The two-electron oxidized form of ascorbate, dehydroascorbic acid (DHA), is taken up by facilitated diffusion into the cell and, is rapidly reduced or recycled to ascorbate by either GSH or NADPH-dependent mechanisms [59]. Ascorbic acid might prevent endothelial function through multiple mechanisms: (a) decreased oxidation of LDL [60, 61], (b) release of NO from S-nitrosothiols [62], (C) direct reduction of nitrite (NO\textsubscript{2} -) to NO [63, 64], (d) scavenging of superoxide [63, 65], (e) stimulation of conversion of protein disulfides to sulfhydryls [66], and (f) regeneration of vitamin E [67, 68].

Vitamin E is the major natural lipid-soluble antioxidant in human plasma[69]. The effects of lipid-soluble antioxidant α-tocopherol supplementation on vascular function has been examined in a great number of clinical trials [70-74]. Supplement of individuals with vitamin E results in increased level of LDL-associated vitamin E and enhanced resistance of LDL to in vitro oxidation [75]. Vitamin E can directly scavenge superoxide [65] but it is unlikely to compete with NO for superoxide in vivo because of the low reaction rate constant. Thus it may be more efficient in improving endothelial function by its inhibitory effects on lipid peroxidation [76]. However the results of clinical trials with vitamin E in humans have been mixed [77, 78].

NAC is an antioxidant drug commonly used in clinical practice. Two possible antioxidant mechanisms have been proposed for this thiol containing antioxidant. Firstly, NAC may have direct free radical scavenging properties. ROS may react with NAC resulting in the formation of NAC disulphide [79, 80]. The significance of this mechanism of action in vivo is however questionable in view of the fact that the bioavailability of total NAC is only about 9% [81, 82]. The ability of the reduced form is even lower (about4%)[82]. A direct scavenging action of NAC in vivo is therefore only likely to be significant when administered intravenously or by inhalation. Secondly, and of probably more importance, NAC may exert its antioxidant effects indirectly by facilitating GSH biosynthesis [83]. Due to this mechanism NAC may contribute to the regeneration of endothelium-derived relaxing factor [84, 85], Increasing evidence indicates that the action of NAC may be pertinent to microcirculatory blood flow and tissue oxygenation [86, 87].
Oxidative stress and NO availability in connection with smoking, hypercholesterolemia, and diabetes

**Cigarette smoking**
The exposure to cigarette smoke is associated with progression of atherosclerosis is well documented [88]. According to a recent study, current cigarette smoking is associated with 50% increase in the progression of atherosclerosis, and past smoking is associated with 25% increase. Exposure to environmental tobacco smoke (ETS) is associated with 20% increase. The impact of smoking on atherosclerosis progression has been found to be increased by diabetes and hypertension [89].

Cigarette smoke can be divided into two phases: particle-phase (tar) and gas-phase smoke. The concentrations of radicals in these fractions differ. Tar contains more than $10^{17}$ stable, long-lived radicals per gram. The most important free radical in the tar phase is regarded to be quinone/hydro-quinone (Q/QH$_2$) complex, which is an active redox system that can reduce molecular oxygen to produce superoxide. Gas-phase smoke contains more than $10^{15}$ free radicals per puff. Most of the free radicals in the gas phase of cigarette smoke, have been identified as alkoxy and alkyl free radicals with the latter making up approximately two third of the total components [90-92]. It is evident that the exposure and burden of free radicals to the body is much higher in cigarette smokers than in nonsmokers.

Cigarette smoking is associated with impairment of endothelium-dependent arterial dilation [93, 94]. The latter is dose-related and potentially reversible in asymptomatic young adults. Cigarette smoking also leads to acute coronary vasoconstriction effects [95-97]. Recently it was shown that the impairment of endothelium-dependent vasodilation in healthy smokers is associated with reduced endothelial NO generation and eNOS activity [98-100].

Smokers’ exhaled NO concentrations are significantly lower than that of nonsmokers, and smoking a single cigarette also significantly but transiently reduced exhaled NO. Passive smoke inhalation also decreases exhaled NO in healthy subjects. It has been shown that in pulmonary artery endothelial cells, NOS is inhibited by cigarette smoke extract [101], and that the expression of eNOS is reduced in smoker pulmonary artery [102]. Cigarette smokers have been found to have about 5 folds higher mean expired breath H$_2$O$_2$ concentration than nonsmokers. This oxidative stress might be generated both directly by the free radicals in aqueous solution, and indirectly by activated phagocytes [103]. It has also been shown that cigarette smoking increases the numbers of circulating leukocytes and neutrophils [104-106]. In animal models, cigarette smoke increase neutrophil adhesion to the endothelium of both arteries.
and venules [107, 108]. Some studies also suggest that the production of lipid peroxidants is increased in plasma of healthy smokers [109, 110].

Cigarette smoking also affects the antioxidant defense. Smoker have an inverse association between serum levels of vitamin C and smoking [111]. Kallner et al demonstrated a 40% increase of metabolic turnover of ascorbic acid in smokers compared with nonsmokers [112]. In children exposed to ETS, the serum level of ascorbic acids is significantly reduced [113]. In adults, beside from lower serum ascorbic level, the intake of the antioxidant is also affected by smoking [114]. Plasma anti-oxidant capacity was found decreased in healthy smokers. Interestingly, this reduction was also associated with 30% decrease in plasma protein thiol levels [110, 115]

**Hypercholesterolemia**

Considerable epidemiological, experimental, and clinical investigations have given strong support for the conclusion that elevated plasma cholesterol levels plays a dominant role in development of atherosclerosis. Multiple clinical trials have established the efficacy of lipid-lowering for both primary and secondary prevention of cardiovascular disease (CVD) [116]. The "oxidation hypothesis", first established by Steinberg and colleagues [117], states that oxidative modification of LDL is central to the atherogenic process. It is now clear that oxLDL, with its many oxidatively modified lipids and degradation products, contributes to both the initiation and progress of atherosclerosis.

Considerable experimental and clinical data suggest that elevated plasma levels of total and LDL cholesterol are associated with impaired endothelial function [118-120]. Experimentally induced hyperlipidemia induced by either a fatty meal or an infusion with lipids impairs FMD [121]. In patients with hypercholesterolemia, endothelium-dependent vasodilation in both coronary and peripheral vessels is impaired before the development of clinical atherosclerosis [122]. The endothelial dysfunction in coronary microcirculation of hypercholesterolemic patients can be corrected by treatment with L-arginine [123, 124]. These data clearly suggest a decrease in availability of NO in hypercholesterolemic patients.

In cholesterol-fed rabbits, hypercholesterolemic vessels produce about three fold more $O_2^-$ than normal vessels [29]. A reduction of serum cholesterol is associated with normalization of endothelial superoxide anion production [125]. In human studies, endothelial superoxide generation has been suggested to be elevated in hypercholesterolemia [126]. The above findings suggest that hypercholesterolemia may be associated with oxidative stress.
eNOS has been reported to be present in caveolae but not in other part of the plasma-membrane [127-129]. Cholesterol is one of the main constituents of caveolae consistent with a direct interaction between cholesterol and the enzyme [118]. There are several studies on the effect of endothelial cholesterol loading on eNOS activity. Endothelial cells incubated in the presence of cholesterol have been reported to display a 50% increase in NO release in response to calcium ionophore. Expression of eNOS and the number of caveolae was increased to about the same extent [130]. In contrast, incubations with higher amounts of cholesterol inhibited eNOS activity [131]. Incubation of endothelial cells with oxLDL resulted in trans-location of both caveolin-1 and eNOS from the caveolae, and inhibition of acetylcholine induced eNOS activation [132].

Hypercholesterolemia is known to increase endothelin-1 production through increased gene expression and via protein kinase C [133-136]. Hypercholesterolemia also seems to enhance coronary artery vasoconstrictor response to endothelin [136].

**Diabetes**

Oxidative stress has been considered to be of importance for the increased vascular disease in diabetes. Increased lipid peroxidation has been reported in leucocytes of patients with type 2 diabetes, using the MDA (malondiadehyde) test [137]. Another study reported plasma MDA to be particularly increased in type 2 diabetes compared with both type 1 and health control [138]. A specific non-enzymatic peroxidation product of arachidonic acid, esterified 8-epi-prostaglandin F$_{2\alpha}$ [139], was found to be significantly higher in type 2 diabetes patients than in healthy subjects [140-143]. This increase was significantly correlated with increased platelet activation. Improvement of metabolic control or vitamin E supplementation reduced urinary 8-epi-PGF$_{2\alpha}$ level and reduced the platelet activation [143]. In contrast Vessby et al. reported no significant difference in urinary 8-epi-PGF$_{2\alpha}$ excretion between type 1 diabetes and control subjects [144]. Electron spin resonance methods have also been used for evaluation the oxidative stress in type 2 diabetes, suggesting ascorbate depletion [145].

A number of studies have demonstrated a reduced antioxidant defenses in diabetes [144, 146-148]. During an oral glucose tolerance test, plasma concentrations of protein-bound sulphydryl (SH) groups, vitamin C, vitamin E and uric acid are significantly decreased in normal subjects as well as in patients with type II diabetes mellitus [146]. Akkus et al. demonstrated reduced vitamin C levels in leucocytes from type 2 diabetes patients, but not lowered SOD [137]. In contrast erythrocyte SOD levels were reported to be lower in both type 1 and 2 diabetes [138, 149]. In the lens, glutathione peroxidase activity and ascorbic acid levels were significantly decreased in diabetic patients, especially those suffering from retinal damage [150]. Nourooz-Zadeh et al reported
significantly increased levels of hydroperoxides and decreased levels of alpha-tocopherol in plasma of diabetes type 2 patients [151]. Vessby et al. reported vitamin E level to be relatively high in subjects with type 1 diabetes mellitus, suggesting that vitamin E supplementation may not be required in Swedish type 1 diabetes patients [144].

The cause of diabetic oxidative stress is still unclear. Different mechanisms may contribute to the increased production of reactive free radicals, occurring as a consequence of the elevated glucose levels. Under conditions of hyperglycemia, nonenzymatic glycoxidation may occur as a result from the interaction of aldoses, such as glucose, with free amino groups on polypeptides or lipids. Formation of glycation products such as hemoglobin A\textsubscript{1c}, are probably not of direct pathophysiological significance for the complications of diabetes mellitus. Further molecular rearrangements, often involving oxidation, may result in the formation of advanced glycation end products (AGEs), such as N\textsuperscript{ε}-(carboxymethyl)lysine, pentosidine, and pyrroline. The formation of such compounds correlate directly with the vascular and renal complications of diabetes mellitus [152]. In cultured bovine aortic endothelial cells, hyperglycemia was shown to cause increased ROS production at the mitochondrial complex II [153]. Du et al. reported that basal NO production is significantly lower in endothelial cells cultured under high-glucose conditions [154]. Interestingly treatment of diabetic mice deficient for apolipoprotein E, with the soluble extracellular domain of the receptor for advanced glycation endproducts (RAGEs) completely suppressed diabetic atherosclerosis in a glycemia- and lipid-independent manner [155].

The increase of oxidative stress in diabetes mellitus may contribute to the reduce of vascular nitric oxide (NO) bioactivity and the endothelial dysfunction [156]. A recent study reported significantly increased superoxide production in human blood vessels from patients with type 2 diabetes. This increase was found to be associated with upregulated NAD(P)H oxidase activity and an increase in endothelial superoxide production mediated by eNOS [157].

Insulin has been found to induce NO-dependent vasodilation in human skeletal muscle [158, 159]. This vasodilator activity was blunted in insulin resistance states, such as obesity, hypertension and type 2 diabetes [160, 161]. Baron et al reported a relation between insulin levels and blood flow in legs under different conditions [161]. Insulin-dependent vasodilation was completely abrogated by L-NMMA infusion. Other studies suggest that insulin resistance is associated with blunted endothelium-dependent but not endothelium-independent vasodilation [162]. The possibility has
been discussed that the insulin vasodilator effect might be involved with kinase-mediated phosphorylation of NOS [163, 164], but other factors like ET-1 may also be involved [165, 166].

Evaluation of endothelial dysfunction

Endothelial dysfunction refers to several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and deregulation of vascular remodeling. Endothelial dysfunction may also be accompanied by decreased production and/or local bioavailability of NO. In the presence of increased oxidative stress, this may be caused by excessive production of superoxide anions, with an oxidation of NO before it can reach its target tissues. The interactions between NO and O$_2^-$ occur at an extremely rapid rate, three times faster than the reaction rate for O$_2^-$ with SOD [167, 168].

Ideally, testing for endothelial dysfunction should involve methods that are safe, simple, noninvasive, and reproducible. Endothelial dysfunction may appear at an early stage in the development of atherosclerosis. In advanced cases of atherosclerosis the endothelial dysfunction may be a secondary phenomenon. The dysfunction may or may not correlate with existence of subclinical atherosclerosis. Endothelial dysfunction is however not a discrete entity, nor does a gold standard exist for evaluation of it.

Circulating endothelium-derived regulatory proteins

Some circulating markers are generally used for evaluation endothelial dysfunction, such as asymmetric dimethylarginine [169-171], endothelin-1 [172-174], tissue plasminogen activator (tPA) [175-177], plasminogen activator inhibitor –1 (PAI-1) [178-180], vascular cell adhesion molecule-1 (VCAM-1) [181-183], inter-cellular adhesion molecule-1 (ICAM-1) [184, 185-187], von Willebrand factor (vWF) [177, 185-187], thrombomodulin [188, 189], soluble E-selectin [190, 191].

NO and NO metabolic production

Decreased availability of biologically active NO in the vascular wall may be one of the earliest detectable findings during atherogenesis. Possible causes include decreased expression of eNOS, lack of substrate or cofactors for eNOS, lack of appropriate activation of eNOS, or increased degradation of NO by ROS due to oxidative stress. NO production can be estimated by direct measurement of the amount of NO in plasma samples, or by assay of its metabolites nitrite and nitrate, by using chemiluminescence methods [192] or gas chromatography mass spectrometry [193, 194]. Urinary NO$_3^-$ [194-196] and cGMP [197] can also be used to evaluate the production of NO. Such measures are, however, extremely
dependent on dietary intake and may change with day-to-day alterations in nutrient intake [198], and are not practicable to use in the clinical investigations.

**Functional methods**

Functional methods are often used in clinical work to test the ability of endothelium to cause NO-mediated vasodilation in response to the pharmacological and physiological stimuli. In much of the literature, the term endothelial dysfunction has been used to refer to an impairment of endothelium-dependent vasodilation caused by a loss of NO bioactivity in the vessel wall. Several human studies have demonstrated endothelial dysfunction in connnected with atherosclerosis, diabetes, smoking, hypercholesterolemia, and hypertension. The techniques used include angiography, positron emission tomography, ultrasound, plethysmograph and Laser Doppler Flowmetry methods as well as direct capillaroscopic evaluation of microcirculation.

**Quantitative angiography** assessment of coronary endothelial function in humans in vivo was first reported by Ludmer et al in 1986 [199]. Coronary artery diameter was measured by quantitative angiography before and after intracoronary infusion of acetylcholine. In normal arteries, acetylcholine stimulates the endothelial release of NO, resulting in vasodilation [200]. Acetylcholine-induced vasoconstriction is one of the earliest manifestations of endothelial dysfunction [201]. The direct smooth muscle constrictor effect of acetylcholine and the lack of the NO-effect may explain the effect. L-NMMA can be selectively infused to assess basal NO activity in the coronary circulation [202].

**Intracoronary Doppler wires** can measure coronary blood flow and, thus assess the resistance coronary function. Coronary blood flow increases in response to agonists, such as acetylcholine. This increase is now used as a quantitative measure of endothelial function[203].

The disadvantage of the above two methods is their invasive nature and unsuitability for use in children or adults with advanced atherosclerosis.

**Positron emission tomography** (PET) is a unique method for noninvasive quantitation of myocardial blood flow [204, 205]. By measuring blood flow at rest and after pharmacological stimulation, it is possible to calculate the coronary blood flow reserve, which is an early marker of subclinical coronary atherosclerosis and endothelial function. The major disadvantages of PET are its very high cost and the radiation exposure.
The **brachial artery ultrasound method** was first described by Celermajer et al in 1992 [94], as a noninvasive assessment of flow-mediated vasodilation in the brachial or femoral artery. After occlusion of the upper arm for 5 min by a blood pressure cuff, releasing the occlusion induces a reactive hyperemia. As a consequence, the flow velocity in the brachial artery increases greatly (5-7-fold). This increase in shear stress results in an endothelial-mediated dilation (FMD) of the artery. The FMD has been shown to be mainly caused by endothelial release of NO[19], and to correlate with invasive testing of the coronary endothelial function [206]. There is also a correlation to the extent and severity of coronary atherosclerosis [207]. Being noninvasive, the ultrasound method has been applied widely to asymptomatic subjects groups, including children and young adults with risk factors, such as hypercholesterolemia [208, 209], diabetes [210], hypertension [211], and cigarette smoking [93, 212]. Among the merits of this technique is the possibility to repeat multiple tests in the same patient and to study a large number of patients. Among the disadvantages is that it is relatively difficult to perform and that there is a need for a skilled sonographer.

Most of the above estimates of endothelial dysfunction presumably reflect dysfunction at the level of conduit arteries. The microvascular endothelium with its very large surface area and synthetic capacity should be the most important determinant of plasma levels of endothelium-derived mediators. It is at the microcirculation level where the fine control of the blood flow takes place, and where tissue ischemia starts.

The methods generally used for clinical investigations on microcirculation have all their limitations. The location where most microcirculation observations are performed are: skin [213], lip [214], gingival tissue [215], tongue [216], conjunctiva [217-219] and retina [220]. As a consequence of recent technology advancements, it is now possible to study the abnormal microcirculation in brain tumors during surgery [221]. Clinical investigations of the cutaneous microcirculation are most often made no invasively, mainly based on Laser Doppler and Capillary Microscopy [222-225].

Hokanson et al [226] described an electrically calibrated **plethysmographic method** for measuring the forearm microcirculation after intra-arterial infusion of endothelium-dependent and – independent vasodilator compounds [227-229]. In this relatively simple and inexpensive method, venous occlusion plethysmography reflects resistance vessel function in the forearm blood flow. There is some concern about the day-to-day variability. It is ideally suited for one-time measurements.
**Laser Doppler Flowmetry** [225, 230] involves measurement of the Doppler effects of red blood cells occurring when they traverse the surface microvessels. A beam of low-power laser light is scattered in the nonmoving tissue and the moving blood cells. This results in a measurable Doppler effect, with a shift in frequency. The frequency shift is recorded and can be used to evaluate the amounts of blood moving through the tissue volume. In practice, a sensor affixed to the skin is used to sample the circulating speeds of all the red cells located within a semi-sphere with a diameter of about 1 mm. The Doppler frequencies are filtered to give the recorded signal. The latter is proportional both to the mean speed and to the number of red cells moving through the sampled tissue volume. The signal may be considered as a semi-quantitative index of tissue perfusion [231, 232]. The high sensitivity of this method allows ready recording of small changes in rhythmical vasomotion over time, as well as dynamic physiological tests, investigations on post reactive hyperemia, and evaluation of the reactivity after administration of vasoactive substances [223].

**Capillaroscopy** is a method used to visualize surface microvessels with use of in vivo optical microscopy. Examination of the nailfold region is the best validated method [233]. The aim of capillaroscopy is not to visualize the capillary wall but to study the flux of erythrocytes in the lumen. The capillaries of the nailfold appear as hair pin-like loops measuring 6-20\( \mu \text{m} \) in diameter [234]. The loops are more or less rectilinear and parallel with each other; they are aligned in rows and oriented toward the tip of the finger. By using high-quality capillaroscopic images and computerized systems, direct homodynamic assessment can be achieved of different parameters such as the CBV [235-238]. The technique can be used for both pathophysiological and pharmacological applications [239]. As have been mentioned above, the nailfold capillary loop has no smooth muscles. Its homodynamic characteristics, mainly represented by the CBV, are regulated by the precapillary artery tone. Thus it is a practicable method for registration of the microvascular endothelial response to physiological stimuli such as post-occlusive reactive hyperemia, and vasoactive compounds. It has been used for studies under pathological conditions such as smoking [240], diabetes [241, 242], hypertension [243], hypercholesterolemia [244]. The limitation of this technique is the need of extensive experience [245, 246]. The disadvantage of this method is that about 10-15% of the subjects are not possible to study, because of low skin transparency, very short loops, low image contrast, or difficulties for the patient to keep in the extreme steady condition, necessary during the patient examination [247].
Aims of the study

In previous work from this laboratory, vital microscopy was used to study effects of hypercholesterolemia in a rabbit model and the possibility to affect the cholesterol-induced microcirculatory changes by treatment with a water soluble antioxidant (vitamin C) or a lipophilic antioxidant (butylated hydroxytoluene) [248-250]. The capillaroscopic method used was found to be suitable for this type of study and it was clearly shown that the cholesterolemia causes a marked depression of the blood flow in the conjunctival microvessels of the rabbits. In accordance with the hypothesis that oxidative reactions are of importance for endothelial dependent regulatory effects on the endothelium, antioxidants were shown to have a clear preventive effect on the cholesterol-induced changes in the microcirculation.

It was considered to be of interest to study the effects of changes in lipid levels and antioxidants also on the human microcirculation with use of a similar noninvasive technique as in the animal experiments.

As a first step in this project, we wanted to know if a heavy oxidative stress causes microcirculatory changes. Smoking a cigarette is known to cause such a stress, and thus we started by studying the effect of smoking on the microcirculation in the nailfold of healthy volunteers.

Diabetes mellitus is a pathological condition characterized by its deteriorative complications in the microcirculation, and the possibility must be considered that oxidative stress is involved as a partial causative factor. We wanted to investigate if an oral supplement of an antioxidant could improve the microvascular reactivity in such patients.

Hyperlipidemia is another disease where involvement of oxidative processes have been strongly implicated, supported by our animal studies. As acute infusion of lipids results in a depressed vascular reactivity, we wanted to use the opposite situation with LDL apheresis to study vascular reactivity before and after rapid lipid lowering.

**Hypothesis:** “The influence of reactive oxidative processes are important factors for the function of the microcirculation. Any change in the burden of these would affect microvascular reactivity in a positive or a negative direction.”

**The specific subprojects of the present work are the following:**

- To define a suitable provocation of vascular reactivity using a capillaroscopic method for our purposes to evaluate effects on microcirculation in humans (**Papers I and IV**)
- To study if it is possible to affect smoking-induced effects on microcirculation by antioxidants: vitamin C (**Paper I**) and N-acetylcysteine (**Paper II**)
- To study if a rapid marked reduction in cholesterol levels affects the microcirculation (**Paper III**)
- To study if vascular reactivity can be affected in a positive direction by vitamin C treatment of patients with chronically disturbed glucose metabolism (**Paper V**).
Methods

Subjects

In study I and II, 24 and 37 healthy volunteers were registered. They were not allowed to have coffee, tea or smoking 2 hours before the examinations. In study I, vitamin C (C-vitamin Apelsin, ACO AB, Stockholm, Sweden) was given 1g or 2g the second day after the first examination. The subjects were allowed to smoke a cigarette, during 3-5 minutes. Microcirculation was recorded before and 1, 5, 10, 15, 20, 30 minutes after the smoking. In study II, the examination was performed before and after 2 weeks of treatment with 200mg t.i.d. N-acetylcysteine (TIKA Läkemedel AB, Lund, Sweden). The effect was recorded as in the first study.

In study III, 4 patients with familial hypercholesterolemia regularly treated with LDL-apheresis every third week were studied. The patients were examined immediately before apheresis treatment and 2 days after. Three of the patients were smokers, and the effect of the smoking was also examined.

In study IV, 10 healthy volunteers were studied. Four of them were analyzed according to a symmetric scheme with the use of single capillaries from two different fingers with three repeated measurements in each finger with six minutes between. After a clip-release rest for 3 minutes, another three measurements were performed. The capillary microscopy was repeated at three occasions with at least one week in between. A total of 144 recordings were performed.

In study V, 20 patients with diabetes type 2 were studied in a double-blind design. The patients were randomized to treatment with vitamin C 1g three times a day during a 2 weeks period and the other half to the same regimen with placebo tablets. All patients were examined by capillaroscopy and blood samples were collected before and after the intervention with vitamin C/placebo. After 4 weeks wash out period the groups were crossed over and the same protocol repeated. Three patients discontinued the participation in the study after the first treatment period.

Evaluation of microcirculation

The microcirculatory images were noninvasively obtained from the nailfold, using a video capillary microscopy (WX, multilocation capillary microscope, Xuzhou Optical, China; HS-164D CCD camera, China). The patient was seated in a comfortable chair with the arm placed at the height of the patient’s heart in a stable position, with room temperature at 22-24 °C. After 10 minutes rest, nail fold of the forth finger of the left hand was placed under the
microscope. During microcirculation imaging, the nailfold surface was covered with a drop of cedar wood oil to improve skin transparency. An optical fiber-equipped with cold light source (WX Light Source, Xuzhou Optical) was used to avoid confounding heating by the lights. The first row of nail-fold capillaries was selected. The images were magnified 450X and 780X through the video capillaroscope. The images were continuously recorded on a videotape recorder (NV-SH1000, Panasonic). Skin temperature of the observed finger was measured by Digital laboratory thermometer (Model BAT-12, Physitemp InstrumentInc, USA)(Fig1).

Fig 1. Set up of the capillaroscopy system
The capillary blood cell velocity (CBV) was measured by using computerized image analysis systems. Two different systems were used: the MCIP (Beijing) in paper I and II, and the Capiflow (Stockholm) in paper III and IV. The MCIP imaging system generates flying spots, which are adjusted to move in parallel with the movement of the blood cells along the capillary. When completely coinciding with the movement of the blood cells, the flying-spot velocity is accepted as the CBV. The frame-by-frame analysis was also used during some of the examinations. In this case four video frames of the microcirculatory images are continuously sampled (40-ms intervals) and directly stored on the hard disk of the system. The moving distance of the blood cells is measured off-line to calculate the CBV. In most cases the CBV was determined by the flying-spot method and then confirmed by the frame-by-frame analysis.

The Capiflow system uses the dual-window technique. In this technique the video signals are passed through a photometric analyzer that generates two photometric windows onto the TV monitor. The position of the windows can be manually adjusted both horizontally and vertically to a desired location along a suitable capillary. The video windows are sensitive to variations in the light intensity within the windows. Consequently, the passage of erythrocytes, leucocytes, and plasma gap through the investigated capillary will give rise to variations in optical density, which are quantified and converted to electronic equivalents. If the distance between the windows is known, the interwindow transit time, the time delay between similar events in the upstream and downstream windows, the velocity of blood cells or plasma gaps can be determined continuously. By applying a small cuff in the root of the finger, after the release of one- minute occlusion, a post occlusion reactive hyperemia is induced. This hyperemia is usually followed by a sharp increase to a peak blood flow velocity. The time from release to the peak can be calculated from the recording velocity curve. The result can be recorded both online and offline. All of the analyses were repeatedly performed offline to avoid possible variations.

**Brachial artery flow-mediated dilatation**

All ultrasound examinations were performed in a semi-darkened room, with subjects in supine position. The ECG was monitored continuously. The subject’s left arm was comfortably immobilized in the extended horizontal position to allow consistent imaging of the brachial artery. Artery diameter was imaged using a 10-MHz compact linear ultrasound transducer. B-mode ultrasound images were continuously recorded and frozen images were obtained by gating from the R wave of the ECG. Images were recorded on videotape and also digitally acquired. The digitized
images were subsequently analyzed on the same instrument [251]. Flow-mediated dilatation was measured after a release of 5 minutes forearm occlusion.

**LDL-apheresis**

LDL-apheresis was performed using the DALI system (Fresenius 4008 ADS, St. Wendel, Germany). This is a kind of hemoperfusion using polyacrylate-coated polyacrylamide gel, which is able to eliminate LDL from whole blood without prior plasma separation.

**Laboratory assessments**

Hemoglobin (Hb), erythrocyte volume fraction (EVF), leukocyte particle count (LPC), fibrinogen (Fbg), total CO₂ (ctCO₂) was assessed in the general health screening and HbA1c was used as an indicator of stable metabolic control in diabetic patients. Cholesterol, HDL and triglycerides levels were assessed by standard enzymatic assays (Boehringer Mannheim GmbH, Mannheim, Germany). High sensitivity CRP was determined by standard procedures, interleukin-1 ra and interleukin-6 by immunoassay (Quantikine HS IL-6 Immunoassay, R&D Systems, Minneapolis, USA). Oxidized LDL was determined using a commercial enzyme-linked immunosorbent assay utilizing the murine monoclonal antibody mAB.4E6 (Mercodia AB, Uppsala, Sweden). Ascorbate levels in plasma were determined after precipitation with metaphosphoric acid as described by Kallner et al [252].

**Ethical Permission**

Ethics Committee at Huddinge University Hospital (Huddinge, Sweden), approved the studies presented in this thesis.
Results and Discussion

Capillaroscopy for the evaluation of microcirculation

In study I and II, we used the MCIP system, a computer-generated flying-spot technique. The blood flow velocity in all the studies was measured in a specific arteriolar limb of a suitable capillary loop, with good contrast and visible signals. As in our previous animal study, the reproducibility of this method was found to be about 10% at velocities ranging from 0 to 1000µm/s [248]. In order to control the accuracy the CBV was confirmed by the frame-by-frame analysis in most cases. This method allows a quick and reliable CBV measurement with a reasonable reproducibility [253, 254]. Using this technique, a CBV range from zero to a maximum 1300µm/s can be measured [255]. As the CBV in human skin capillaries sometimes may reach up to about 3000µm/s, this technique has its limitation.

In study III, IV and V we used the Capiflow system [246]. The principle behind this highly computerized technique is the cross-correlation method, allowing detection of fast fluctuations of CBV. It is possible to assess the PRH of the skin blood flow directly, both online and offline. This method has previously been shown to have good reproducibility when used for evaluation of microcirculation in toes of diabetic patients [256]. We considered it important to select the most reliable parameter and define its reproducibility. In study IV, we collected data from 144 PRH recordings from 10 healthy volunteers. Each of them was analyzed offline 3 times by the Capiflow system. The time to peak mean value was found to be 6.3 ± 0.9 s (95% confidence). The correlation between repeated measurements in a single session was r>0.91 (coefficient of variation CV 6%). The between finger CV was 8% (r = 0.84). The CV of measurements between different days was about 20%, when single measurements were compared. However, the CV decreased to less than 13% when the mean value of at least two Ttp assessments at each occasion was used. The reproducibility was thus in agreement with previous reports. It is evident that the use of mean of at least two repeated PRH in every session improves the accuracy of the evaluation.

Microcirculation affected by smoking

Before our study two previous studies reported that nailfold microcirculation was affected by smoking [240, 257]. In study I, 23 of 24 healthy subjects were found to have an average CBV decrease of 40-50% to a nadir at 1 to 5 minutes after smoking. This decrease was followed by a slow increase in the velocity during the next 30-60 minutes. In study II all the 37 healthy volunteers exhibited a similar acute CBV decrease after smoking a single cigarette, with a
decrease of 37±7%. There was no significant difference between smokers and nonsmokers in the basal CBV. This acute effect is consistent with the two previous nailfold CBV and smoking studies [240, 257]. Some other studies with other techniques have demonstrated a decrease in skin blood flow immediately after smoking: laser Doppler flowmetry [257-262], Doppler ultrasound of the digits [263], venous plethysmography of the forearm and finger [264, 265]. Although the information provided is slightly different, they all suggest similar acute effects on skin capillary blood flow. Acute smoking has also been demonstrated to affect heart microcirculation, resulting in coronary flow reverse (by PET [266] and Doppler guidewire [267]). Acute smoking can affect the cerebral microcirculation, and smoking three cigarettes with two hr gap, caused a fall in cerebral blood flow (CBF) of about 40% [268]. Cigarette smoking also injures the gastric mucosal blood flow and in particular the microcirculation [269]. Furthermore cigarette smoking elicits the aggregation and adhesion by leukocytes and/or platelets to the endothelium in venules and arterioles [270].

The exact mechanisms behind the acute smoking effects on human microcirculatory flow are not very well understood, and may differ in different vascular beds. It is believed that nicotine-provoked sympathetic nervous activity (SNA) may be one of the causes [271]. Increased skin SNA leads to the release of vasoconstrictive factors (eg. NE), which in turn can induce a reduction in capillary flow. According to one publication smoking-reduced nailfold capillary blood flow is not related to the nicotine content of the cigarettes [240].

By using an echo-tracking system allowing continuous measurements of radial diameter (300 radings/s), Giannattasio C. et al found that acute smoking induces a sustained reduction in resting radial artery diameter and compliance [272, 273]. This adverse hemodynamic effect of smoking seemed to be related to SNA. Smoking-induced manifold CBV decrease might thus be affected by SNA both by direct inhibition at the level of the skin microvascular bed, and by the constriction of the radial artery.

Cigarette smoking is a strong oxidative stress to the vascular system, and may therefore reduce endothelium derived NO availability. A great number of studies have demonstrated smoking-impaired flow-mediated dilation (FMD) [93, 274, 275], which is reported to be mainly NO dependent[19]. Lekaki J. et al measured the acute smoking effect on endothelium-dependent arterial dilatation by use of B-mode ultrasound, on healthy subjects. After smoking one single cigarette the percent change in brachial artery diameter during reactive hyperemia was immediately decreased. Flow-mediated dilation remained depressed after 30 and 60 minutes and returned to baseline value after 90
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minutes. The provided data shows that acute smoking is associated with impairment of endothelium-dependent arterial dilatation, which is maintained for at least 60 minutes [276]. This is in accordance with our results of the effects of smoking on capillary CBV.

Effects of vitamin C and N-acetylcysteine on smoking-induced flow reduction

In study I, a total of 24 subjects were pretreated with 2g of vitamin C. In the first study we treated 17 subjects with this dose. This treatment significantly attenuated smoking-induced flow reduction 1 minute after smoking (p < 0.0001). This preventive effect could be demonstrated at all time point. There was no difference in basal CBV between smokers and non-smokers (p>0.05). In the next study, 1g of vitamin C or 2g of aspirin were shown to have no significant effect on the smoking-induced flow reduction (p>0.05).

In study II, N-acetylcysteine was used for pre-treatment of 37 subjects 200 mg three times per day. The basal CBV was not significantly different between smokers and non-smokers (597 ± 31µm/s and 551 ± 27 µm/s, respectively, NS). The heart rate, skin temperature, blood pressure and age of smokers and non-smokers showed no statistical difference. In the combined group (n=37) pre-treated with NAC, smoking-induced flow reduction decreased from 37 ± 7 % to 26 ± 8% (p = 0.0016). Further analyses were performed to investigate the effects in the smoking and non-smoking group. In the smokers (n = 22), NAC affected the smoking-induced flow reduction significantly from 37% to 21%, p = 0.0029). In the non-smokers group (n = 15), NAC had no significant impact on the smoking-induced flow reduction (from 34 ± 5 % to 39 ± 4 %), There was however a statistically significant change in the absolute levels (from 349 ± 33µm/s to 417 ± 41 µm/s, before and after pretreatment respectively, p = 0.012).

It is noteworthy that a very high dose of vitamin C dose was required to get a preventive effect on the smoking-induced flow reduction. A lower dose of vitamin C was without effect in most subjects tested. Heitzer et al used venous occlusion plethysmography to study the effect of intra-arterial administration of vitamin C to chronic smokers. It was shown that the response of the forearm blood flow to acetylcholine was markedly improved by vitamin C in the smokers but not in the control subjects [277]. Kaufmann et al used PET scanning, to study smokers´ coronary microcirculatory function after intravenous infusion of 3 g vitamin C. The baseline of the microcirculatory blood flow in controls and smokers was similar before vitamin C infusion, but significantly increased in the smokers after vitamin C infusion. Motoyama et al used B-ultrasound for detecting the brachial artery response, after
intravenous administration of vitamin C after smoking for 3-5 minutes. They found that FMD was reduced in smokers compared with nonsmokers, and that cigarette smoking acutely worsened the impairment of FMD. The treatment with vitamin C prevented the acute impairment by cigarette smoking [278].

All these results suggest that endothelial dysfunction exists in chronic smokers both in conduit artery and microcirculation, and that a high dose of vitamin C has a preventive effect. Our present results are similar to these studies. Furthermore the two different types of antioxidants both exhibited the same preventive effect in peripheral microcirculation, under acute smoking condition, both in smokers and nonsmokers. Comparing the results of study I and II, the preventive effects of N-acetylcysteine was however considerably lower than the effect of the high dose of vitamin C. N-acetylcysteine seems to be more beneficial in smokers than in nonsmokers. Thus vitamin C seems to be a more efficient antioxidant under the conditions employed. In view of the very high potential of vitamin C to inactivate superoxide ions, and in view of the high levels of \( \text{H}_2\text{O}_2 \) in the breath of smokers [103], it is tempting to suggest that part of the effect of vitamin C is due to this effect on superoxide ions.

In both of our studies, the acute smoking-induced flow reduction was not completely reversed, consistent with the possibility that part of the smoking-induced effects is not only related to an imbalance between prooxidants and antioxidants. Nicotine can increase the sympathetic nervous activity (SNA), and in animal experiments there is an impairment in endothelial-dependent vasodilation of the arterioles by unknown reasons [279]. We could not exclude the possibility that part of the effects of smoking seen in our acute smoking study may be mediated by nicotine.

Treatment with vitamin C has been shown to have a positive effect on aggregation and adhesion of leukocytes from hamsters exposed to cigarette smoke [108]. In humans smoking-induced adhesion of monocytes to endothelium can be prevented by vitamin C [280]. It was also shown that dietary supplement with vitamin C blocks accumulation of PAF-like inflammatory lipids induced by cigarette smoke in hamster [281]. All these findings led us to test the hypothesis that some of the effects of vitamin C demonstrated here may be mediated by the cyclooxygenase system. However, no significant effect of aspirin could be found in study I, suggesting that the cyclooxygenase system is not of critical importance.
Lipid lowering and smoking effects on microcirculation and brachial artery FMD

In study IV, 4 familial hyperlipidemia patients were treated with LDL-apheresis, 3 were smokers, and one an ex-smoker. They were treated every third week. After one session of treatment, cholesterol and triglycerides were reduced in all patients, from 10.5 ± 1.0mmol/l to 5.4 ± 0.6 mmol/l (p = 0.003) and from 2.7 ± 0.5mmol/l to 1.3 ± 0.3 mmol/l (p = 0.02) respectively. Microvascular reactivity was measured as the time to peak at PRH (Ttp). Before LDL-apheresis, at high lipid levels, the Ttp was 12.9 ± 1.55 s. Two days after LDL-apheresis it was 10.3 ± 0.76 s. As a reference, the Ttp average value in healthy subjects in study IV, was 6.3 s. Ttp of the familial hyperlipidemia patient’s was thus higher. The reduction in microcirculatory CBV induced by a single cigarette at its minimum level was 93 ± 38 µm/s before, and 80 ± 76 µm/s two days after LDL-apheresis ie no difference.

Endothelial function was also evaluated in the brachial artery by the ultrasound method. By releasing the forearm after 5 minutes occlusion, a post reactive hyperemia dilatation of the artery (FMD) can be recorded. The relative changes in diameter before LDL-apheresis were 1.0 ± 0.8 %, and 1.1 ± 0.9 % at the second day after treatment. (as a reference, in healthy subjects the corresponding figure is 3.9 ± 2.6%[251]). Three of the patients were treated with nitroglycerin. This treatment resulted in a increase in FMD both before and after LDL-apheresis (by 10.33 ± 3.3% and 12.6 ± 1.25% respectively). One patient had only a slight response to nitroglycerin 4.5 % and 4.6% (before and after LDL-apheresis respectively). Smoking effects on FMD were never studied due to technical problems.

Familial hyperlipidemia may sometime be resistant towards maximal dietary and pharmacological therapy [282] or even surgical diversion [283-285]. Direct elimination of LDL from the circulation by plasma apheresis may sometimes therefore be the most effective choice of therapy. Hypercholesterolemia is associated with endothelial dysfunction[124], believed to be related to the decrease of NO production and the increase of oxidation [286]. It has been indicated that endothelial dysfunction can be improved by lipid lowering therapy, both by statins and LDL-apheresis [287-290].

In this study, the microcirculation of the patients studied had a very low CBV and a prolonged Ttp compared with the control group. Using the flying-spot method Senn B. et al showed that hypercholesterolemic subjects have lower nailfold CBV immediately after local cooling. Lipid lowering did not alter this difference [244].
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Using nailfold microscopy Haak et al reported an improvement of Ttp after fluvastatin treatment in 12 weeks [291]. Brachial artery measurements exhibited a lower FMD in hypercholesterolemic patients, compared with healthy subjects [251]. It was suggested that the microcirculatory changes are correlated to the conduit artery endothelial dysfunction.

The lack of improvement of lipid lowering in the present study may have several possible explanations. Unfortunately, the number of patients was very small due to the rarity of this type of treatment. Because of the severe endothelial dysfunction of the patients, as shown in our results both in the microcirculation and in the conduit artery, the patients may have lost their ability to respond to the lipid lowering. In particular this may be the case with the patients who have combined risk factors — both severe familial hyperlipidemia and smoking. It seems possible that at some stage of the endothelial dysfunction changes may occur that are irreversible or at least not reversible by a transient reduction in the levels of serum lipids.

To our knowledge there is only one previous publication in which the effect of LDL-apheresis on endothelial function was studied [289]. In this work a total of 7 patients were studied and a more marked reduction of LDL-cholesterol levels were achieved than in our study. The endothelium-dependent vasodilation response to acetylcholine was significantly augmented after a single treatment with apheresis without changes in the endothelium-independent vasodilation response to sodium nitroprusside.

**Vascular reactivity in type 2 diabetes and effect of vitamin C**

The vascular reactivity in type 2 diabetes patients was found to be markedly reduced as compared to healthy subjects (11.5 ± 0.7 s before placebo and 12.0 ± 0.8 s before vitamin C as compared to 6.2 ± 0.9 s in healthy subjects measured under similar conditions). This finding is in accordance with a number of previous studies. The vasodilatory effect of acetylcholine and bradykinin was reported to be reduced in subcutaneous small resistance arteries in diabetic patients [292]. Forearm venous occlusion plethysmography on diabetic patients has demonstrated nitric oxide induced vasodilatation to be impaired [293-296]. Laser Doppler studies on forearm skin micro-circulation showed that the microvascular reactivity is reduced in diabetic patients [293, 297, 298]. Prolonged time to peak CBV has been reported to be present both in long and short duration of the disease [299]. According to one study metabolic control seems to be of greater importance for the microvascular dysfunction in diabetes type 2 than in diabetes.
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type1 [299]. A prolonged time to peak has been demonstrated in patients with severe late diabetic conditions [300]. With use of a method similar to that used here, Jorneskog et al [301] showed that the time to peak CBV (s) in the toe nailfold capillary was markedly prolonged during periods of bad metabolic control in diabetic patients (good control 12.8 ± 4.4s; bad control 36.2 ± 21.6s; healthy subjects 14.8 ± 5.7s).

In our study treatment of the diabetic patients (n=17) with vitamin C, 1g × 3 daily for 2 weeks, did not significantly affect vascular reactivity (vitamin C 11.2 ± 0.9s, placebo 10.6 ± 0.7s). In contrast Ting et al reported improvement effect of vitamin C on endothelial dysfunction in forearm resistance vessels of patients with non-insulin dependent diabetes [294]. Heitzer et al recently showed that endothelial function was improved by high levels of ascorbic acid [302]. In addition ascorbate seems to restore the endothelium-dependent vasodilation impaired by acute hyperglycemia in experiments on human volunteers [303].

The duration of the treatment in our study may have been too short to restore the disturbed microvascular reactivity in our patients. In most of previous human studies in which a positive effect of vitamin C has been demonstrated [294, 303, 304], intra-arterial infusion has been used, resulting in very high and probably unphysiological levels of the vitamin. Several previous studies using oral administration have not shown a significant change in vessel reactivity [305-308].

In this connection it is of interest that the only situation in which we observed a high significant effect of vitamin C on microcirculation was after acute administration of a very high dose of the vitamin (2g) (Paper 1) [309]. When a lower dose was used (1g), no effects on the smoking-induced reduction in CBV were observed. Whether or not “normal” oral supplements of vitamin C to a normal diet improve the microcirculation has not yet been clarified.

Our finding that rCBV was slightly higher after vitamin C, should be interpreted with caution in view of the small number of subjects and the day-to-day variation. It was recently pointed out that rCBV seems to be of limited value in the investigation of long-term effects on micro-circulation [310]. In any case further investigations on larger-scale might be needed to confirm our results.
General conclusions

It is concluded that it is possible to use capillary microscopy for evaluation of effects of oxidative stress on the nail-fold microcirculation, provided that highly standardized conditions are used. Due to the biological and methodological variations, the effects must however be relatively great in order to be measured with sufficient accuracy. Measurement of time to peak after a brief arterial occlusion seems to be the method of choice, provided that an equipment is available that allows accurate registration of the fast changes in the microcirculatory flow. The results obtained with this method in a population of patients with severe endothelial dysfunction was similar to that obtained with the considerably more complicated method used for measurements of the change in diameter of the brachial artery after arterial occlusion and exposure to nitroglycerin or acetylcholine. In view of its simplicity, evaluation of the nailfold microcirculation seems to have some merits.

![Figure 2 Suggested mechanisms for the effects of hypercholesterolemia and cigarette smoking on microcirculation.](image-url)
Smoking caused a very dramatic decrease in the microcirculatory blood flow, easily demonstrated by the method used. Since more than 50% of this reduction could be eliminated by a high dose of the antioxidant vitamin C, it is concluded that more than 50% of the smoking-induced changes are due to an imbalance between prooxidants and antioxidants caused by the smoking. A possible model is presented in Fig 2. Pretreatment with another antioxidant, N-acetylcysteine also had some preventive effect on the smoking-induced changes. This preventive effect was however considerably lower than that caused by an acute high dose of vitamin C. It is concluded that antioxidants have a beneficial effect on the endothelial function in connection with smoking-induced oxidative stress. Whether or not this effect is an advantage in relation to development of atherosclerosis, can however not be assessed at the present state of knowledge.

In view of the well documented negative effect of hypercholesterolemia on microcirculation, in both experimental animals and in patients, a drastic reduction of levels of cholesterol in the circulation would be expected to cause an increased blood flow in the microcirculation. In four hypercholesterolemic patients regularly treated with LDL apheresis, a lowering of plasma cholesterol by about 50% gave no obvious effect on the microcirculation or the smoking-induced effects on this microcirculation. The basal microcirculatory flow and the time to peak after brief arterial occlusion were however markedly different from healthy subjects, and the possibility must be considered that a chronic endothelial dysfunction may cause irreversible changes with impairment of the normal responses to various stimuli.

Patients with diabetes type 2 were found to have a markedly reduced vascular reactivity. In a double blind crossover study on 17 patients fail to demonstrate a significant effect on this reactivity by treatment with vitamin C (1g × 3 daily for 2 weeks).

To summarize; intravital microscopy of the microcirculation in the nailfold seems to be valuable for evaluation of endothelial function in connection with a marked acute oxidative stress. It was only under such conditions that clear effects of antioxidants on microcirculation could be demonstrated. In conditions associated with increased oxidative stress as diabetes and hyperlipidemia, we failed to improve microvascular reactivity by oral antioxidant supplement or by rapid lipid lowering. Due to the biological and methodological variations, the effects must however be relatively great to allow long-term studies with different interventions. In the latter type of studies, measurement of the time to peak is likely to be a better parameter than blood cell flow velocity.
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References


REFERENCES


132. Blair, A., et al., *Oxidized low density lipoprotein displaces endothelial nitric-oxide synthase (eNOS) from plasmalemmal...*


REFERENCES


