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Review

Antiatherosclerotic activity of drugs in relation to nitric oxide function

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Abstract

Many studies have shown that loss of endothelium-derived nitric oxide is a major factor of ischemic episodes in patients with coronary artery disease and there is increasing evidence to suggest that nitric oxide might exert antiatherosclerotic actions. Based on these concepts, the results of animal studies on the effects of lipid lowering drugs, antioxidants, angiotensin converting enzyme inhibitors, Ca^{2+} channel blockers, estrogens and agents which modulate nitric oxide bioavailability are presented and compared to the results of patient studies and clinical trials. In spite of encouraging results obtained with antioxidants in animals, clinical trials could only show a clear positive effect of vitamin E treatment on the outcome of cardiovascular disease. Angiotensin converting enzyme inhibitors can ameliorate endothelial dysfunction in coronary heart disease, but their impact on disease progression remains unclear. There is evidence that estrogen replacement therapy in post-menopausal women may increase the bioavailability of nitric oxide. Finally, improved endothelial function and plaque stability clearly contribute to the clinical benefits of lipid lowering interventions, statins in particular. Taken together, these studies lend support to the concept that improving endothelial function and nitric oxide release might serve as valuable elements in the prevention or therapy of cardiovascular disease.

Keywords: Vascular endothelium; Cardiovascular disease; Nitric oxide (NO)

1. Introduction

Recently it has become clear that oxidative processes are involved in the pathogenesis of atherosclerosis, that atherosclerosis is accompanied by profound alterations of the behavior of the endothelial cells and that mediators released by the endothelial cells can modulate atherogenesis. Nitric oxide is one of those endothelium-derived mediators, in addition to cytokines, prostacyclin, and an endothelium-derived hyperpolarizing factor, whose identity is still debated. This review focuses on the impact of drugs on atherosclerosis and on the alterations of nitric oxide signaling during atherosclerosis. After an introduction to current concepts about the pathogenesis of atherosclerosis, the main text is focused on antiatherosclerotic effects of lipid lowering drugs, antihypertensive drugs, antioxidants, estrogens and compounds which interfere with nitric oxide function. Local interventions, which are currently tested in syndromes of accelerated atherosclerosis, namely heart transplant atherosclerosis, coronary vein graft disease and restenosis after percutaneous transluminal coronary angioplasty are not addressed.

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2. Pathophysiological aspects of atherosclerosis

2.1. Intimal thickening and initiation of atherosclerosis

Focal or diffuse thickening of the tunica intima (inner coat) of the large conduit arteries (e.g., aorta, carotid, femoral, and coronary arteries) is required before atherosclerosis can develop. At birth, the intima of most arteries consists solely of endothelial cells, but soon after birth, focal and circumferential thickening occurs spontaneously and contains smooth muscle cells and connective tissue. The intimal thickening is not pathological, but it marks locations where atherosclerosis tends to develop later in life under the influence of atherogenic stimuli. Intimal thickening can be induced in species, which do not develop intimal cushions using balloon denudation with an embolectomy catheter or by placement of perivascular silastic collars (De Meyer and Bult, 1997). Both procedures create discrete injury of the media, which evokes smooth muscle cell proliferation, followed by their migration to the intima and an extended phase of proliferation and matrix deposition in the intima.

Atherosclerosis is characterized by the deposition of intracellular and extracellular lipids and by the appearance

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of macrophages and T lymphocytes in the intima. As macrophages and smooth muscle cells underneath the endothelial cells accumulate lipid, they acquire a 'foamy' appearance. Clusters of lipid-laden cells become macroscopically visible as fatty streaks (Davies and Woolf, 1993). These flat, fatty lesions may transform to a fibroatheroma, which has a very characteristic microanatomy with a core of extracellular lipid covered at the luminal side by a thick fibrous cap. Surrounding the core are lipid laden foam cells, while ischemia in the necrotic core initiates angiogenesis. This type of plaque may cause narrowing of the lumen once the compensatory vascular remodeling becomes exhausted.

Concepts on the initiation of atherosclerosis are mostly based on studies of animal models of hypercholesterolemia, a well-known risk factor for the human disease. The hypercholesterolemia increases oxidative stress in the vascular wall and this is associated with oxidation of low-density lipoprotein (LDL) and stimulation of redox-sensitive transcription factors, like nuclear factor κB (NF-κB) (Witztum, 1994; Diaz et al., 1997). NF-κB promotes transcription of genes such as vascular cell adhesion molecule-1 and monocyte chemotactic protein-1, resulting in the attraction of leukocytes by the endothelium. The recruited mononuclear cells produce an inflammatory response and the monocyte-derived macrophages internalize oxidized LDL through scavenger receptors. As these receptors are not down-regulated by the intracellular cholesterol level, massive cholesterol accumulation occurs and the cells transform to foam cells. The dominant localization of plaques at flow dividers may be explained by the absence of the normal shear stress exerted by laminar blood flow leading to down-regulation of certain shear stress-responsive endothelial genes involved in vessel homeostasis, such as endothelial nitric oxide synthase (eNOS) and the antioxidant enzyme superoxide dismutase (Traub and Berk, 1998).

2.2. Compensatory arterial remodeling

It is now appreciated that vascular remodeling, i.e., compensatory enlargement, inadequate compensatory enlargement or shrinkage at sites of atherosclerotic lesions, is a major determinant of vessel lumen size in atherosclerosis (Birnbaum et al., 1997). As a consequence, standard coronary angiography cannot visualize plaques, unless the compensatory vascular remodeling has become exhausted and lumen narrowing occurs. The intracellular and intercellular mechanisms of the compensatory enlargement, which proceeds until the plaque occupies more than 40% of the lumen, are largely unclear. One hypothesis proposes that the endothelium senses changes in shear forces as the atheroma expands and that local release of mediators is involved in the rearrangement of cells in media and adventitia leading to a compensatory enlargement (Birnbaum et al., 1997). Mouse and rabbit models of remodeling suggest that eNOS, whose activity and expression are raised by increased shear stress, provides a signal to promote adaptive enlargement of the vessel and its lumen (Tronc et al., 1996; Rudic et al., 1998).

2.3. Plaque stability

The thromboembolic events following plaque fissure are a major cause of clinically manifest acute ischemic syndromes (Lee and Libby, 1997). The vast majority of myocardial infarctions arise from atherosclerotic lesions that are minimal to moderate in severity as quantified by arteriography. The mechanisms leading to coronary thrombosis include: frank rupture of a plaque's fibrous cap, intra plaque hemorrhage and superficial erosion of the endothelium. Plaque rupture occurs when the mechanical stresses in the fibrous cap exceed a critical level that the tissue can withstand (Lee and Libby, 1997). Biological factors weakening the fibrous cap include infiltration of the shoulder region with inflammatory macrophages and T cells. The macrophages can promote local expression or activation of matrix metalloproteinases, which decrease the strength of the cap by degrading collagen and other matrix components.

Furthermore, vulnerable regions of advanced human plaques are characterized by a prominent apoptotic cell loss (Isner et al., 1995; Kockx et al., 1998a). The apoptosis occurred predominantly in smooth muscle cells, situated near macrophage-derived foam cells. Inflammatory cytokines and nitric oxide released by the macrophages could contribute to the smooth muscle cell death. The significance of apoptotic cell death of macrophages and smooth muscle cells is very different (Kockx et al., 1998a). Apoptotic death of macrophages, which are the main source of matrix metalloproteinase activity, could improve plaque stability, as it would decrease breakdown of collagen fibers in the fibrous cap. In contrast, the disappearance of smooth muscle cells could lead to plaque destabilization, since smooth muscle cells are central to the synthesis and maintenance of collagen fibers type I and III. Stabilization of atherosclerotic plaques could be achieved by inducing apoptosis in the macrophage population and by protecting the smooth muscle cells. Progress in our understanding of the biology of unstable atheroma is hampered by the limitations of current imaging techniques and the lack of representative animal models (Lee and Libby, 1997).

2.4. Dysfunction of nitric oxide signaling in atherosclerosis

Numerous studies demonstrated a defective nitric oxide signaling in response to acetylcholine, serotonin or increased flow in atherosclerotic arteries of rabbits and other species, and in patients with coronary artery disease (Har-

rison, 1996; Matthys and Bult, 1997). The deterioration is an early event, and can be observed in patients with typical angina or with cardiac risk factors but with angiographically smooth coronary arteries. Subsequent studies demonstrated impaired endothelium-dependent vasodilatation in the coronary and in the peripheral microcirculation, e.g., in the coronary and in resistance arteries which do not develop atherosclerosis. Also, the mere presence of cardiovascular risk factors was associated with dysfunctional microvascular endothelium, as seen in patients with hypertension, hypercholesterolemia, smoking or diabetes. The current weight of evidence suggests that impaired endothelium-dependent vasodilatation is the predominant mechanism underlying ischemic manifestations. It should be kept in mind, however, that the contribution of endothelium-derived hyperpolarizing factor to endothelium-dependent vasodilatation becomes more important in the peripheral circulation and that this pathway (Urakami Harasawa et al., 1997) and prostacyclin biosynthesis (Beetens et al., 1986) are disturbed by hypercholesterolemia as well. The mechanisms underlying the dysfunctional endothelial nitric oxide signaling are multifactorial and involve both defective biosynthesis and increased inactivation.

2.4.1. Increased degradation of nitric oxide

Hypercholesterolemia increases the production of superoxide anion by the intima and the media resulting in inactivation of nitric oxide, with concomitant formation of peroxynitrite (Harrison, 1996). Raising the antioxidant capacity in the vessel wall by the administration of superoxide dismutase, polyethylene-glycolated or liposome-entrapped (White et al., 1994) to ensure cell entrance, partly restored the endothelium-dependent relaxation in the aorta of cholesterol-fed rabbits. The disturbed balance between vascular superoxide and endothelial nitric oxide production may be compensated for by upregulation of endogenous superoxide dismutase. In rabbits (Matthys and Bult, 1997) and mice (Bonthu et al., 1997), but not in pigs or humans, the endothelial dysfunction is determined by the plaque size and hypercholesterolemia alone rarely impairs the endothelial dilator function. Apparently, the rabbit is capable of keeping the superoxide and nitric oxide production in balance, as long as lesions do not develop.

2.4.2. Reduced biosynthesis of nitric oxide

Vasodilator responses to acetylcholine and serotonin vanish early, before impairment of the dilation to other agonists, Ca^{2+} ionophore A-23178 or mechanical stimuli. The agonist specificity of the early dysfunction points to selective alterations in endothelial receptors, signal transduction, or increased translocation of eNOS to the caveolae, where the enzyme is resistant to activation by Ca^{2+}/calmodulin (Feron et al., 1999). It further indicates that the early defect is not due to shortages of the substrate L-arginine or the cofactor tetrahydrobiopterin. It also implies that the expression of eNOS in endothelial cells is not reduced (Kanazawa et al., 1996), though this could occur in endothelial cells covering advanced lesions (Wilcox et al., 1997). Paradoxically in human endothelial cells eNOS expression fell after cytokine treatment, but the overall eNOS activity increased due to enhanced biosynthesis of the cofactor tetrahydrobiopterin (Rosenkranz et al., 1994), and the transport of L-arginine into the cells may increase as well (Durante et al., 1996). These findings illustrate that besides the expression of eNOS protein or the function of endothelial cell receptors other factors may determine eNOS activity.

2.5. Upregulation of inducible nitric oxide synthase

Functional (Verbeuren et al., 1993) and histochemical studies demonstrated the expression of inducible nitric oxide synthase (iNOS) mRNA and protein in rabbit (Luoma et al., 1998) and human plaques, where it colocalizes with nitrotyrosine in macrophages (Buttery et al., 1996; Wilcox et al., 1997; Luoma et al., 1998). Despite the abundant expression of extracellular superoxide dismutase, the presence of epitopes characteristic of oxidized lipoproteins and nitrotyrosine residues implied that malondialdehyde, hydroxynonal and peroxynitrite are important mediators of oxidative damage in iNOS-positive macrophage-rich lesions (Luoma et al., 1998).

2.6. Impact of nitric oxide on atherosclerosis

Antiatherogenic properties of nitric oxide include inhibition of superoxide anion generating enzymes, inactivation of superoxide anion and inhibition of LDL oxidation, lipid peroxidation and cell damage by acting as terminator of radical chain propagation reactions (White et al., 1994; Harrison, 1996; Matthys and Bult, 1997). Furthermore, nitric oxide interferes with leukocyte recruitment by decreasing the expression of monocyte chemotactic protein-1 and cell adhesion molecules by suppressing NF-κB-like transcriptional regulators. The inhibitory effects on T-cell proliferation and on platelet adhesion and aggregation are often considered antiatherogenic effects of nitric oxide.

It is assumed that nitric oxide formed by eNOS protects against early atherogenesis, but in the atherosclerotic plaque the situation may change fundamentally since the high output isoform iNOS is expressed in an environment with oxidative stress. In this setting, nitric oxide or peroxynitrite could initiate lipid peroxidation in LDL, and cause cytotoxic effects in endothelial and smooth muscle cells (Matthys and Bult, 1997). Nitric oxide has also been reported to inhibit proliferation and to induce apoptotic cell death in smooth muscle cells, and to activate matrix metalloproteinases, whereas peroxynitrite inactivates the tissue inhibitor of metalloproteinase-1. This spectrum of activities

could favor expansion of the necrotic core and weakening of the tensile strength of the fibrous cap, thereby raising the risk of plaque fissure and atherothrombotic complications.

3. Cholesterol-lowering therapy

A logical strategy to prevent or treat atherosclerosis is to target the major risk factor hypercholesterolemia by diet and/or lipid-lowering drugs. The discontinuation of a cholesterol-rich diet in monkeys and rabbits improved the architecture of the lesions, which can occur in the absence of plaque regression (Harsch et al., 1997; Rosenson and Tangney, 1998). The morphological changes included increases in smooth muscle cells and collagen, and reductions in macrophage number, extracellular lipid, calcifications and neovascularization. The smooth muscle cells lost their lipid accumulation and showed strong reductions of the expression of the pro-apoptotic Bax and the frequency of apoptosis, indicating that they became less susceptible to undergo apoptosis (Kockx et al., 1998b). Together with the drastic increase of cross-banded collagen fibers these changes point to an increased tensile strength of the plaque.

A second clear advantage of reducing plasma cholesterol levels is the amelioration of the endothelial dilator function in experimental animals as well as in humans (Harrison, 1996). Treatment of cholesterol-fed diabetic mice with the bile acid-binding resin cholestyramine preserved the endothelium-dependent relaxation of aortic rings to acetylcholine (Kamata et al., 1996). Also in hypercholesterolemic patients with angiographically normal coronary arteries the impairment of endothelium-dependent dilation was reversed by cholestyramine coupled to a cholesterol-reducing diet (Leung et al., 1993). Even a single LDL apheresis improved endothelium-dependent vasomotion in hypercholesterolemic patients (Tamai et al., 1997), which would support a direct and fast beneficial effect of cholesterol lowering on endothelial function. The effects of statins and fibrates are discussed in more detail.

3.1. Statins

The major rate-limiting enzyme in cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which catalyzes the conversion of HMG-CoA to mevalonic acid. Several fungal metabolites, now termed statins, have been shown to be potent, competitive inhibitors of this enzyme. The decrease in cholesterol biosynthesis leads to an increase in LDL receptors and thus an increased clearance of LDL. Analysis of recent clinical studies suggests that the beneficial effects of statins cannot be fully explained on the basis of reductions in plasma cholesterol levels (Rosenson and Tangney, 1998). At a molecular level, mevalonate is not just the precursor of cholesterol, but also of several other end products such as farnesol and geranylgeraniol. The latter isoprenoids serve as lipophilic plasma membrane anchors of receptor proteins, that are vital for signal transduction, cell proliferation and oncogene function. Moreover, some statins display antioxidant activities, and fluvastatin is as potent as probucol in this respect (Mitani et al., 1996; Yamamoto et al., 1998).

At a cellular level, the beneficial effects of statins on clinical events therefore involve non-cholesterol mechanisms that modify cell migration and proliferation, inflammatory responses, plaque stability, oxidative stress, endothelial cell function, thrombus formation, platelet function and the fibrinolytic balance (Rosenson and Tangney, 1998). Moreover, upregulation of eNOS expression independent of lipid-lowering and prevention of the down regulation of eNOS by oxidized LDL have been observed in endothelial cell cultures treated with statins (Hernandez-Perera et al., 1998; Laufs et al., 1998).

3.1.1. Animal studies

In vivo studies confirmed the capability of statins to inhibit smooth muscle cell migration and proliferation independently of the plasma cholesterol levels in rabbit models of intimal thickening (Bellosta et al., 1998). The inhibition became more pronounced with lipophilic compounds and was prevented by local delivery of mevalonate, demonstrating that the statin effect was related to local inhibition of mevalonate biosynthesis in the artery. This result further proved that the statin concentration in the vessel wall may become sufficiently high to inhibit mevalonate biosynthesis, in spite of their pronounced pre-systemic hepatic clearance. Since smooth muscle cells are essential for the maintenance of the fibrous cap and its tensile strength, the inefficacy of pravastatin as inhibitor of smooth muscle replication could be considered beneficial. Indeed, pravastatin treatment has been shown to stabilize atherosclerotic plaques in rabbit and primate models (Harsch et al., 1997; Rosenson and Tangney, 1998). It remains to be determined whether other statins decrease plaque vulnerability as well, thereby contributing to their clinical benefit.

Animal studies have demonstrated beneficial effects of HMG CoA reductase inhibition on endothelial cell function. Lovastatin preserved endothelium-dependent relaxation and inhibited the accumulation of cholesterol in the aorta of cholesterol-fed rabbits (Senaratne et al., 1991), but failed to reverse bioavailability of nitric oxide in vivo, assessed as the urinary excretion of nitrate and cyclic GMP. This failure was presumably due to the stimulatory effect of lovastatin on vascular superoxide radical generation (Böger et al., 1997). Simvastatin slightly retarded the functional loss in young LDL receptor deficient rabbits, but could not inhibit the development of atheroma (Dowell et al., 1995), whereas pravastatin retarded lesion progression and preserved coronary vasomotion in this model (Kroon et al., 1993). Also in aortic rings of cholesterol-fed

rabbits pravastatin preserved the endothelial dilator function more effectively than simvastatin (Jorge et al., 1994).

3.1.2. Human studies

Recent clinical trials have demonstrated beyond doubt that statins are effective in reducing acute coronary events and overall mortality in primary and secondary prevention, including women and the elderly (Rosenson and Tangney, 1998). As the benefits of statin therapy cannot be fully explained on the basis of reductions in plasma cholesterol levels, it has been proposed that plaque stabilization or improved vasomotor control may have a greater impact on clinical events than the size of the stenosis. Clinical studies indeed confirmed that a reduction in serum cholesterol with pravastatin, lovastatin, or lovastatin in combination with the antioxidant probucol (Anderson et al., 1995) improved endothelium-dependent coronary vasomotion in patients with hypercholesterolemia and coronary artery disease. Lipid lowering therapy with simvastatin also augmented both the basal and the stimulated nitric oxide mediated flow responses in the peripheral circulation of patients with hypercholesterolemia (Stroes et al., 1995; Hayoz et al., 1995). The improvement occurred within 1 month of treatment (O'Driscoll et al., 1997) and was also seen upon lowering of desirable cholesterol levels in healthy middle-aged men (Vogel et al., 1996). In one study using human subcutaneous small arteries abnormalities of both endothelium-dependent and -independent relaxations were observed and both were normalized with effective lipid-lowering by simvastatin (Goode and Heagerty, 1995). LDL cholesterol lowering therapy with fluvastatin has also been shown to improve the disturbed acetylcholine induced forearm blood flow response in hypercholesterolemic patients by increasing the bioavailability of nitric oxide (John et al., 1998).

Cholesterol lowering with pravastatin was effective in eliminating the transient myocardial ischemic episodes during daily life in a significant proportion of patients with coronary artery disease (van Boven et al., 1996; Andrews et al., 1997). Furthermore, therapy with pravastatin during 2 years preserved the regional myocardial perfusion in patients with coronary artery disease, whereas patients on placebo deteriorated (Aengevaeren et al., 1997). Even a short-term treatment with fluvastatin led to an improved regional myocardial perfusion, especially in areas of ischemia (Eichstadt et al., 1995). This suggests that the amelioration was due to functional restoration of coronary endothelium, before anatomic regression of stenosis can occur following long-term treatment. Similarly, 3 weeks treatment with pravastatin reduced the diastolic blood pressure responses to angiotensin II and noradrenaline in patients with hypercholesterolemia and mild hypertension (Straznicky et al., 1995). These data point to a direct fast beneficial effect of cholesterol lowering on endothelial function, though the antioxidant properties of some statins may be involved as well (Rosenson and Tangney, 1998). These mechanisms could all contribute to the amelioration of endothelial function and the stabilization of plaques, thereby explaining the clinical benefits of statins in coronary artery disease.

3.2. Fibrates

Fibric acid derivatives ('fibrates') have marked effects in lowering triglyceride-rich lipoproteins and generally cause a modest reduction in LDL and usually about 10% increase in high density lipoprotein (HDL) (Staels et al., 1998a). Though the LDL lowering is often not very spectacular, fibrate treatment preferentially reduces the population of the small dense LDL particles with an equivalent increase in the intermediate subfraction. The dense particles are highly atherogenic because of a low binding affinity for the LDL receptor, a prolonged plasma half life and a low resistance to oxidative stress.

The molecular mode of action of the fibrates was poorly understood until recently. It is now appreciated that fibrates are synthetic ligands for a transcription factor, which coined the name peroxisome proliferator-activated receptor α (PPARα, Staels et al., 1998a). PPARs are members of the superfamily of nuclear hormone receptors that also includes steroid, retinoid and thyroid hormone receptors. They heterodimerize with the retinoid X receptor and bind to DNA at specific PPAR response elements (PPREs) to modify the transcription of genes involved in glucose (PPARγ) and lipid homeostasis (PPARα and PPARγ). PPARα activation by fibrates stimulates the gene expression of human apolipoprotein-A1, thereby increasing plasma HDL. The triglyceride lowering effect of fibrates involves both increased gene expression of lipoprotein lipase leading to enhanced catabolism of very low density lipoprotein, and effects on fatty acid synthesis and catabolism leading to reduced secretion of very low density lipoprotein (Staels et al., 1998a).

Furthermore, it has recently been shown that fibrates inhibit the interleukin-1-induced production of interleukin-6 and expression of cyclooxygenase 2 in human aortic smooth muscle cells via a PPARα mediated repression of NF-κB signaling at a transcriptional level. Accordingly, fenofibrate treatment of hyperlipidemic patients decreased the plasma concentrations of interleukin-6, fibrinogen and C-reactive protein. It thus appears that PPAR ligands, in addition to their beneficial effects on HDL, triglyceride and fatty acid metabolism, may suppress atherogenesis by inhibiting inflammatory responses of smooth muscle cells, and decreasing the plasma concentration of acute phase proteins (Staels et al., 1998b).

3.2.1. Human studies

Gemfibrozil has been documented to reduce coronary heart disease by approximately 30% in middle aged men with primary hyperlipidemia (Frick et al., 1987). Angiographic studies showed that gemfibrozil (Frick et al., 1997)

and bezafibrate (Ericsson et al., 1996) retard the progression of coronary and vein graft atherosclerosis and decrease the number of coronary events. Finally, fenofibrate improved vasomotor responses in the microcirculation in patients with hypercholesterolemia as measured by nailfold capillaroscopy (Haak et al., 1998). Whether this improvement was secondary to an improved lipid profile is not yet clear.

4. Antioxidants

Based on the evidence that increased oxidant stress in the vascular wall is causally related to atherogenesis and may impair endothelial nitric oxide function directly by oxidative breakdown of nitric oxide or indirectly through the formation of oxidized LDL (Witztum, 1994), a beneficial effect of antioxidants on cardiovascular disease can be anticipated. Indeed, epidemiological studies demonstrated that a diet high in antioxidant vitamins is associated with lower cardiovascular morbidity and mortality (Diaz et al., 1997). Antioxidants can slow the early atherogenic process in rabbit and primates, though atherogenesis may worsen upon treatment of mice with the antioxidants butylated hydroxytoluene (Munday et al., 1998) or probucol (Bird et al., 1998). From the fact that different antioxidant compounds such as probucol, β-carotene, butylated hydroxytoluene and vitamin E have been successfully used in animal studies, it may be concluded that these substances indeed act via the property they all share namely their antioxidant potential. By suppressing the excessive generation of reactive oxygen species in the vessel wall (Keaney et al., 1995), they could reduce the expression of vascular cell adhesion molecule-1 (Weber et al., 1994; Cominacini et al., 1997; Fruebis et al., 1997a,b) and other redox sensitive genes and preserve endothelial nitric oxide signaling. Finally, other types of drugs may suppress atherosclerosis by antioxidant properties as well, as suggested for the β-adrenoceptor antagonist carvedilol (Donetti et al., 1998), angiotensin converting enzyme inhibitors, Ca^{2+} channel blockers (vide infra) and statins (vide supra).

4.1. Probucol

Probucol is a cholesterol lowering drug with powerful antioxidant properties, which has however, the disadvantage that it reduces plasma HDL cholesterol (Olsson and Yuan, 1996).

4.1.1. Animal studies

Treatment with probucol reduced atherogenesis in several rabbit models (Carew et al., 1987; Mao et al., 1991; Delrio et al., 1996; Schwenke and Behr, 1998; Braesen et al., 1998; Donetti et al., 1998), as well as the intimal hyperplasia evoked by a perivascular collar or balloon denudation in hyper- and normocholesterolemic rabbits (Donetti et al., 1998; Tanaka et al., 1998). Moreover, not only the plaque volume, but also its composition with less foam cells and necrosis and more smooth muscle cells and extracellular matrix, and a thicker fibrous cap were improved by probucol (Braesen et al., 1998; Oshima et al., 1998). The antiatherosclerotic effects occurred independently of its cholesterol lowering effect (Carew et al., 1987; Oshima et al., 1998) and were accompanied by a reduction in plasma thiobarbituric acid reactive substances, a diminished vascular superoxide anion generation and improved endothelium dependent relaxations (Keaney et al., 1995; Delrio et al., 1996; Inoue et al., 1998). The preserved endothelial function was also independent of cholesterol lowering or lesion size, which strongly points to a direct protective effect of the antioxidant on nitric oxide (Keaney et al., 1995).

At least two studies showed that the antiatherosclerotic effects of antioxidants in rabbits are not simply related to the degree of antioxidant protection of plasma LDL, and that probucol reduces atherogenesis by mechanisms not shared by all antioxidants (Shaish et al., 1995; Fruebis et al., 1997a,b). This is further strengthened by the observation that probucol inhibited intimal hyperplasia evoked by balloon injury in normocholesterolemic rabbits. The suppression of smooth muscle cell proliferation was attributed to inhibition of mitogen-activated protein kinase and protein kinase C (Tanaka et al., 1998), though probucol failed to inhibit the increased protein kinase C activity in the media of cholesterol fed rabbits (Ozer et al., 1998). Furthermore, in LDL receptor deficient mice probucol did not attenuate (Benson et al., 1998) or even worsen (Bird et al., 1998) atherogenesis, in spite of the protection of LDL against oxidation and reductions of serum cholesterol and triglycerides in very low density lipoprotein and chylomicrons. This illustrates that there are important species differences in the intracellular effects of probucol or its metabolism, or that the decrease of HDL in these murine models has an overwhelming atherogenic effect (Bird et al., 1998).

4.1.2. Human studies

A study measuring forearm blood flow in hypercholesterolemic patients could not demonstrate functional improvement by probucol (McDowell et al., 1994), in contrast to the rabbit studies. The probucol quantitative regression Swedish trial (PQRST) was not encouraging either, as treatment with probucol in addition to cholestyramine did not influence the femoral artery lumen size (Walldius et al., 1994), in spite of an increased protection of LDL against oxidation and degradation by macrophages. The negative finding in this study was attributed to a decrease of large HDL particles, which carry most of the antiatherosclerotic effect of HDL. Furthermore, probucol had an unfavorable effect on diet-derived antioxidants and reduced serum vitamin E and carotenoids (Olsson and Yuan, 1996). In hypercholesterolemic patients with a com-

bined increase of LDL and HDL cholesterol, probucol could still be a valuable option. In a small population of those patients probucol arrested wall thickness of the carotid artery, whereas an increase, indicative of atherosclerosis progression, was seen in matched patients receiving other lipid lowering regimens (Baldassarre et al., 1997).

In contrast, pretreatment with probucol was active against the accelerated atherosclerosis evoked by percutaneous coronary balloon angioplasty. Probucol reduced the lumen loss with 70% and the rate of restenosis about 40% when compared with placebo treatment for 6 months (Tardif et al., 1997; Rodes et al., 1998). Multivitamins (beta carotene, vitamin C and vitamin E) either given alone, or in combination with probucol, were inactive in this setting.

4.2. Vitamin C

4.2.1. Animal studies

The antiatherosclerotic potential of vitamin C (ascorbic acid) has been recognized for some time. Administration of a low dose vitamin C (50 mg kg^{-1} day^{-1}) to cholesterol-fed rabbits resulted in decreased lipid infiltration and less intimal thickening, without influencing plasma cholesterol or the area of the aortic fatty streaks (Beetens et al., 1986). Also the combination of low dose vitamin C (about 75 mg kg^{-1} day^{-1}) and E (50 mg kg^{-1} day^{-1}) failed to influence the area of the lesions (Sun et al., 1998), but reduced their severity (Mahfouz et al., 1997). These data suggest that low dose vitamin C, alone or in combination with vitamin E, reduces lesion activity rather than reducing the area of the fatty streaks. However, the combination of high dose vitamins E and C (500 mg kg^{-1} each) reduced both cholesteryl ester accumulation and progression of the area of pre-existing cholesterol induced aortic fatty streaks, without influencing the lesions in the iliac femoral artery induced by balloon denudation 9 weeks prior to the start of the antioxidant treatment (Bocan et al., 1992).

With respect to endothelial function, a low dose vitamin C (50 mg kg^{-1} day^{-1}) failed to prevent the progressive fall of prostacyclin biosynthesis in cholesterol-fed rabbits, whereas a higher dose (200 mg kg^{-1} day^{-1}) maintained the endothelial prostacyclin production at normal levels for at least 8 weeks (Beetens et al., 1986). Treatment with vitamin C or butylated hydroxytoluene reversed the decrease in blood flow velocity in the microcirculation in hypercholesterolemic rabbits (Freyschuss et al., 1997).

4.2.2. Human studies

Recent reports provide some evidence for a protective role of vitamin C in human atherosclerosis. First, ascorbate and urate were the strongest determinants of the antioxidative capacity in plasma of smoking and non smoking men and the presence of circulating antibodies against oxidized LDL in cigarette smokers. Second, a 5-year prospective study of Finnish men suggests that vitamin C-deficient men are at an increased risk of myocardial infarction (Olsson and Yuan, 1996). Finally, in a multiple linear regression analysis of antioxidant markers in plasma samples obtained from patients undergoing cardiac catheterization none of the antioxidants was an independent predictor of the extent of atherosclerosis. However, lower plasma ascorbic acid concentrations predicted the presence of an unstable syndrome (Vita et al., 1998b). On the other hand, no effect of a self selected supplementary vitamin C intake was seen on the progression of early atherosclerosis in the common carotid artery in patients with coronary artery disease treated with placebo or colestipol plus nicotinic acid (Hodis et al., 1995; Azen et al., 1996), whereas the data of another study provided limited support for a protection against atherosclerosis in individuals more than 55 years old (Kritchevsky et al., 1995). Taken together, these results are consistent with the rabbit data and support the hypothesis that the beneficial effects of vitamin C may result in part from an influence on lesion activity or plaque vulnerability, rather than a reduction in the extent of atherosclerosis (Vita et al., 1998b).

Direct improvement of endothelial dysfunction has been reported from high doses of vitamin C ($\geq$ 1 g), in patients with coronary artery disease (Levine et al., 1996; Ito et al., 1998) which was at least in part due to prevention of the accelerated degradation of nitric oxide by radicals (Hornig et al., 1998). Also, the endothelial dysfunction in subjects with risk factors for atherosclerosis such as chronic smoking (Heitzer et al., 1996), insulin-dependent diabetes (Timimi et al., 1998), hypertension (Solzbach et al., 1997) or hypercholesterolemia (Ting et al., 1997) was ameliorated by high doses of vitamin C, but not by doses producing only a modest increase in plasma ascorbate (Gilligan et al., 1994b). In healthy subjects pretreatment with vitamin C prevented the rapid impairment of flow-mediated, endothelium dependent vasodilatation evoked by acute elevation of plasma homocysteine (Chambers et al., 1999) and the combination of vitamin C and E blocked the transient deterioration of endothelial function evoked by a single high-fat mea (Plotnick et al., 1997). These studies indicate that vitamin C supplementation can ameliorate endothelial dysfunction and plaque vulnerability.

4.3. Vitamin E

4.3.1. Animal studies

The effects of dietary vitamin E (α-tocopherol) supplementation on the development of plaques in rabbits are variable, ranging from no effect (Keaney et al., 1994; Shaish et al., 1995) or suppression (Schwenke and Behr, 1998) at low doses, to adverse effects at high doses (Godfried et al., 1989; Keaney et al., 1994). Combining vitamin E (146 IU day^{-1}) with selenium (22 μg day^{-1}), a cofactor of peroxidases, led to an improved antiatherogenic effect, whereas the inhibition of fatty streak formation by vitamin E was not influenced by vitamin C (0.8 g day^{-1})

(Schwenke and Behr, 1998). Vitamin E supplementation can reduce the cholesterol-induced formation of reactive oxygen species in the arterial wall, and this could explain the improved acetylcholine-induced relaxation in rabbit carotid artery (Stewart et al., 1994) and aorta (Keaney et al., 1994). In LDL receptor deficient mice receiving a high fat, high cholesterol diet, the combined dietary antioxidants 0.1% vitamin E, 0.5% β-carotene and 0.05% vitamin C inhibited LDL oxidation and fatty streak development (Crawford et al., 1998).

4.3.2. Human studies

In women, but not in men, there was a weak correlation between the intake of vitamin E, as assessed by a food questionnaire, and wall thickness of the carotid artery (Kritchevsky et al., 1995). Supplementary vitamin E intake appeared to be effective in reducing the progression of early atherosclerosis as demonstrated by serial coronary angiography (Hodis et al., 1995) or by measurement of the carotid artery wall thickness (Azen et al., 1996), but the effects were not seen in subjects treated with lipid-lowering drugs. The first prospective, double blind secondary prevention trial to test the hypothesis that antioxidants would oppose the progression of atherosclerosis was the Cambridge heart antioxidant study (CHAOS). In men and women with angiographically proven coronary atherosclerosis daily supplements of 800 or 400 IU vitamin E reduced the combined primary endpoint of cardiovascular death and non-fatal myocardial infarction by 30% (Stephens et al., 1996). The CHAOS study particularly showed a highly significant reduction of the frequency of non fatal myocardial infarctions after a shorter time than cholesterol lowering trials. This contradicts the view that studies of antioxidants would require much longer than those of cholesterol lowering, since the antioxidants would mainly retard the development of early atherosclerosis. The fast effect of vitamin E in the CHAOS study supports the concept that oxidative processes in vulnerable, macrophage-rich shoulder regions of the plaque could lead to destabilization and rupture.

4.4. β-Carotene

4.4.1. Animal studies

Though β-carotene did not protect LDL from oxidation, it reduced fatty streak development in hypercholesterolemic rabbits in one study (Shaish et al., 1995), but not in another study (Keaney et al., 1993). In spite of the lack of effect on plaque size and LDL susceptibility to oxidation, β-carotene preserved endothelium-dependent relaxation (Keaney et al., 1993).

4.4.2. Human studies

Three large scale clinical studies have been rather disappointing (Olsson and Yuan, 1996). Treatment with β-carotene, alone or in combination with retinol for several years did show beneficial effects on cardiovascular events, and was even accompanied by increases in cardiovascular mortality in two trials. It should be noted, however, that these large studies were not primarily designed to identify effects on atherosclerosis. In other words, the participants were not selected on grounds of a low intake of vitamins or an increased risk for coronary heart disease, which is a large difference with the cholesterol lowering trials.

4.5. Miscellaneous antioxidants

L-2-Oxothiazolidine-4-carboxylate, which augments the intracellular antioxidant glutathione by providing the substrate cysteine for glutathione synthesis, significantly improved the flow-mediated dilation in the brachial artery of patients with coronary artery disease (Vita et al., 1998a) and the iron chelator desferrioxamine ameliorated endothelial cell dysfunction in coronary arteries of patients with non-insulin dependent diabetes (Nitenberg et al., 1998).

5. Vasodilator drugs

5.1. Angiotensin-1 converting enzyme inhibitors

Inhibitors of angiotensin-1 converting enzyme attenuate the formation of angiotensin II and allow kinins, such as bradykinin to accumulate by inhibition of their degradation. In addition to its contractile activity, angiotensin II can promote superoxide anion generation and induce smooth muscle cell migration and proliferation through activation of angiotensin II type 1 (AT_1) receptors (Prescott and Sawyer, 1993; Linz et al., 1995). Angiotensin converting enzyme activity is slightly higher in atherosclerosis prone, hemodynamically stressed foci of the rabbit aorta as compared to lesion-resistant areas (Markle, 1989). Furthermore, cholesterol-rich diets increase angiotensin converting enzyme activity and angiotensin receptor expression in atherosclerotic plaques in rabbits (Mitani et al., 1996; Hoshida et al., 1997) and in the aorta of monkeys (Song et al., 1998). Therefore, it is conceivable that angiotensin converting enzyme activity in the vascular wall contributes to atherogenesis, a notion which is supported by studies demonstrating antiatherosclerotic effects of high doses of angiotensin converting enzyme inhibitors and angiotensin AT_1 receptor antagonists.

5.1.1. Animal studies

The intimal hyperplasia evoked by denudation of the rat carotid artery was reduced by angiotensin converting enzyme inhibitors and to a lesser extent by angiotensin AT_1 antagonists (Linz et al., 1995). The bradykinin B_2 receptor antagonist icatibant (HOE 140) opposed the effect of angiotensin converting enzyme inhibition, suggesting that the protective effect of angiotensin converting enzyme inhibitors was due to both the blockade of angiotensin II

formation and of kinin degradation. The fact that nitro-L-arginine methyl ester blocked the effect of ramipril in this model suggests that nitric oxide may play a major role in the inhibitory effect of the angiotensin converting enzyme-inhibitors (Farhy et al., 1993). However, reduction of the intimal thickening after collar placement in the rabbit was only observed for high doses of perindopril (Janiak et al., 1994; Hickey et al., 1996), but not for hypotensive doses of zabicipril, enalapril or moexipril (De Meyer et al., 1995). Therefore, the effect of angiotensin converting enzyme-inhibitors on intimal thickening differs according to the species, the experimental model (balloon denudation or collar) as well as to the particular inhibitor studied. Indeed, it has been suggested that suppression of intimal hyperplasia by angiotensin converting enzyme inhibitors is due to inhibition of smooth muscle cell migration at blood pressure lowering doses (Prescott and Sawyer, 1993), and this may explain the lack of effect in situations where smooth muscle cell migration is less important.

Therapy with high doses of various angiotensin converting enzyme inhibitors retarded the progression of cholesterol-induced plaques in rabbits and monkeys (Riezebos et al., 1994a; Shibutani et al., 1994; Linz et al., 1995; Song et al., 1998) without changing plasma lipids. However, the regression of the lesions after cholesterol withdrawal from the rabbit diet was not accelerated by ramipril (Riezebos et al., 1994b). Most investigators employed doses of drugs which are not in clinical use and cause significant reductions in arterial pressure. Low doses of trandolapril (Chobanian et al., 1995) and perindopril (Fennessy et al., 1996), which had little or no effect on blood pressure, failed to suppress atherogenesis in hyperlipidemic rabbits, though the low doses inhibited serum and aortic angiotensin converting enzyme activity to the same extent as the high doses. The latter suggests that the atheroprotection by these drugs was not directly related to inhibition of angiotensin converting enzyme activity in the arterial wall. Yet, the atheroprotection is not simply due to their hypotensive effect, since other blood pressure lowering drugs failed to retard atheroma progression. This is supported by the reduced plaque size and cholesterol content of the rabbit aorta upon treatment with a dose of enalapril which had no effect on blood pressure (Schuh et al., 1993).

Antiatherogenic activities did not occur when rabbits were given doses of angiotensin AT_1 receptor antagonists which blocked most pressure effects of infused angiotensin (Schuh et al., 1993; Fennessy et al., 1996). However, at higher doses angiotensin AT_1 receptor antagonists caused reductions in blood pressure as well as aortic atherosclerosis similar to those seen in studies with angiotensin converting enzyme inhibitors in rabbits (Sugano et al., 1996; Hope et al., 1999), pointing to the involvement of angiotensin AT_1 receptors. Inhibition of the breakdown of bradykinin could further contribute to the atheroprotection by angiotensin converting enzyme inhibitors, since blockade of bradykinin B_2 receptors eliminated the suppressive effect of quinapril on P-selectin expression in myocardial tissue of cholesterol fed rabbits (Hoshida et al., 1999). In contrast, the bradykinin B_2 receptor antagonist icatibant did not suppress the antiatherogenic effect of perindopril in the rabbit aorta (Fennessy et al., 1996). Regional differences in the expression of bradykinin B_2 receptors could form one explanation for this discrepancy.

At a cellular level, the amelioration coincides with a preservation of superoxide dismutase activity (Mitani et al., 1996), suppression of nuclear transcription factor κB and diminished expression of P-selectin, interleukin-8 and monocyte chemoattractant peptide 1 (Hernandez-Presa et al., 1998; Hoshida et al., 1999). This is in accordance with the activation of superoxide anion generating systems by angiotensin II. It further confirms the proposal that antihypertensive doses of angiotensin converting enzyme inhibitors and angiotensin AT_1 receptor antagonists inhibit early atherosclerotic lesion development via effects on macrophage recruitment rather than via angiotensin II-mediated effects on smooth muscle cell migration and proliferation (Prescott and Sawyer, 1993).

The antiatherosclerotic effects of angiotensin converting enzyme inhibition were accompanied by improved endothelium-dependent relaxations and increased nitric oxide-release (Riezebos et al., 1994a). As already mentioned before, it is difficult to assess whether the improved endothelial function contributes to, or is secondary to a reduction in the lesion formation. However, an improved endothelial cell function has been documented in hyperlipidemic rabbits treated with ramipril in the absence of an effect on the fatty streak formation, suggesting that the changes in the nitric oxide function occurred independently of morphological changes (Finta et al., 1993). Furthermore, upon cessation of the cholesterol rich diet, ramipril increased the bioavailability of spontaneously released nitric oxide, without influencing the receptor induced release or atheroma regression (Riezebos et al., 1994b). Also, the beneficial effects of quinapril on the expression of P-selectin in post-ischemic myocardial tissue in cholesterol fed rabbits were eliminated by blockade of bradykinin B2 receptors or inhibition of nitric oxide synthesis (Hoshida et al., 1999).

5.1.2. Human studies

Cardiac ischemic events have been prevented by angiotensin converting enzyme inhibition in patients with coronary artery disease and left ventricular dysfunction in a number of clinical studies (Cleland and Krikler, 1993; Linz et al., 1995). However, a recent quantitative coronary angiography study in patients with normal left ventricular function could not demonstrate an effect of quinapril on stenosis progression or new stenosis development as compared to placebo treatment (Cashin-Hemphill et al., 1999). Since standard coronary arteriography is inadequate to assess the severity of diffuse coronary artery disease, this disappointing result does not exclude that angiotensin con-

verting enzyme inhibition may retard plaque growth or ameliorate endothelial dysfunction. This is corroborated by the results obtained in a clinical trial in which improved endothelium-dependent relaxation was observed in normotensive patients undergoing a non-surgical revascularisation procedure and who were treated for 6 months with quinapril (Mancini et al., 1996). Though the improved endothelial cell function by angiotensin converting enzyme inhibition may help to explain the beneficial effects of angiotensin converting enzyme inhibitors in reducing the number of ischemic events observed previously in patients with left ventricular dysfunction (Cleland and Krikler, 1993), their clinical value as antiatherosclerotic agents remains an open question (Cashin-Hemphill et al., 1999).

Taken together, these studies indicate that angiotensin converting enzyme inhibition can ameliorate endothelial dysfunction and oppose early atherosclerosis due to attenuation of the superoxide anion generating effects of angiotensin II and due to an enhanced endothelial release of nitric oxide secondary to diminished breakdown of bradykinin or related kinins. Furthermore, a mild reduction of blood pressure induced by angiotensin converting enzyme inhibitors or angiotensin AT_1 receptor antagonists may contribute to their antiatherosclerotic effects (Hope et al., 1999). Finally, the increased expression of mast cell derived chymase with a specific angiotensin II-forming activity in atherosclerotic lesions may contribute to local angiotensin II accumulation (Kovanen, 1997; Takai et al., 1997). The resistance of chymases to angiotensin converting enzyme inhibitors could explain why the antiatherosclerotic activity of these drugs requires doses which are supramaximal with respect to the inhibition of plasma and vascular angiotensin converting enzyme activity.

5.2. Ca^{2+} antagonists

5.2.1. Animal studies

The data on antiatherosclerotic effects of Ca^{2+} channel blockers in rabbits are conflicting. The inhibition of early atherosclerosis reported for verapamil, nicardipine, nifedipine, isradipine and nilvadipine was not confirmed in other studies (Fisher and Grotta, 1993; Riezebos et al., 1994a; Hayashi et al., 1998). This discrepancy also exists among regression experiments: isradipine 0.25 mg kg^{-1} facilitated cholesterol removal and plaque regression in one study (Kunjara et al., 1994), while the same dose retarded regression in another (Riezebos et al., 1994b). Different dose regimens are an obvious explanation for the discordant results reported for the same Ca^{2+} antagonist and in some 'positive' studies the drug was administered in near lethal doses. Another explanation is the dietary cholesterol content, which was very high (2% or even more) in several studies demonstrating an antiatherosclerotic activity, while the same Ca^{2+}-antagonist was inactive when the diet contained less than 0.5% cholesterol or in LDL receptor deficient rabbits. The cholesterol load has a large impact on the cellular composition of the lesion. High cholesterol promotes formation of fatty streaks with an abundant presence of macrophage-derived foam cells and very few myocytes. The portion of smooth muscle cells increases at 0.5% cholesterol or less, and the lesion acquires a greater resemblance to human atheroma.

Few studies addressed the effects of Ca^{2+} entry blockers on endothelial nitric oxide release. The effects of isradipine on eNOS function in cholesterol-fed rabbits mirrored its effects on aortic plaque progression. Endothelium-dependent relaxation improved when the lesions were suppressed by isradipine (Habib et al., 1986; Kunjara et al., 1994), whereas the same dose failed to restore endothelial cell function when plaque size was not affected either (Riezebos et al., 1994a). The effects of other Ca^{2+} entry blockers on the deterioration of eNOS signaling remain to be investigated.

Cell culture experiments indicate that some Ca^{2+} blockers reduce LDL cholesterol accumulation by cells, inhibit monocyte migration and lipid peroxidation, and preserve superoxide dismutase activity (Chen et al., 1997; Hayashi et al., 1998). These atheroprotective activities are independent of the inhibition of Ca^{2+} channels. This is elegantly demonstrated by the observation that the (*R*)-enantiomer of lercanidipine with a very low affinity for Ca^{2+} channels, was as potent as lercanidipine in inhibiting collar-induced intimal smooth muscle cell proliferation and cholesterol-induced fatty streak formation (Soma et al., 1998). Hence, it is concluded that some of these drugs can inhibit cell proliferation and early atherosclerosis by their antioxidant activity or other properties, not related to the inhibition of Ca^{2+} fluxes or antihypertensive effects.

5.2.2. Human studies

Three major clinical trials addressing the effects of diltiazem and verapamil in patients after myocardial infarction indicated a reduction in recurrent infarctions, but did not show an overall effect on mortality. If patients with heart failure, which did worse on treatment, were excluded from analysis, mortality was reduced by verapamil in the remaining population and a similar trend was seen with diltiazem (Cleland and Krikler, 1993). In a prospective trial, nifedipine inhibited the angiographic progression of coronary artery disease and reduced the number of newly formed coronary lesions, confirming two other studies with dihydroperidine Ca^{2+} entry blockers (Jost et al., 1992). Addition of Ca^{2+} channel blockers to statin therapy may act synergistically in retarding the progression of established coronary atherosclerosis (Jukema et al., 1998). Furthermore, treatment of cardiac transplant patients with diltiazem prevented the usual decrease in mean coronary artery lumen diameter. This suggests that Ca^{2+} antagonists can prevent the early, accelerated intimal proliferation in response to chronic immune injury (Schroeder et al., 1993). These results warrant trials with a further follow up to

determine whether the suppression of coronary lesion formation by Ca^{2+} entry blockers, alone or in combination with statin therapy, may become a new strategy in the prevention of coronary artery disease.

6. Estrogens

The action of estrogens involves the binding to intracellular estrogen receptors in target tissues leading to changes in the expression of genes with estrogen response elements. In addition to these genomic activities, which may result from activation of the classic estrogen receptor α or the novel β receptor, some effects of estrogens in the vasculature involve fast, direct membrane interactions with ion channels (Farhat et al., 1996). Female hormones, particularly estradiol, have been suggested to exert antiatherosclerotic effects (Nathan and Chaudhuri, 1997). This assumption is based on results of the Framingham study, showing a lower incidence of cardiovascular events in premenopausal women than in age-matched man, and on the observation that postmenopausal women are more likely to develop coronary artery disease than premenopausal women of the same age.

The cardioprotective effect of estrogens appear to be mediated through a variety of mechanisms (Farhat et al., 1996; Nathan and Chaudhuri, 1997). Apart from increasing the plasma levels of HDL and decreasing those of LDL and preventing the oxidation of LDL, their beneficial effect also involves direct effects on the vascular wall. Estrogens have been shown to inhibit vascular smooth muscle cell proliferation in culture. Furthermore, it has been proposed that eNOS-derived nitric oxide contributes to the antiatherosclerotic effect of estrogens. Indeed, vascular reactivity studies pointed to a greater basal release of nitric oxide in normal arteries of female animals and upregulation of eNOS expression and activity by pregnancy and treatment with estradiol. The receptor-mediated upregulation of eNOS mRNA and activity was also seen in estrogen treated bovine and human endothelial cells in culture. However, others could not demonstrate upregulation of eNOS expression in endothelial cells in culture and a receptor-mediated decrease of the production of superoxide anion (Arnal et al., 1996) or a rapid decrease of the Ca^{2+} dependency (Caulin-Glaser et al., 1997) have been proposed as alternative explanations for the acute augmentation of nitric oxide bioavailability upon estrogen treatment.

6.1. Animal studies

Beneficial effects of estrogen therapy have been demonstrated in models of intimal thickening induced by denudation (Foegh et al., 1994; Chen et al., 1996) or perivascular collars (Akishita et al., 1997; Moroi et al., 1998) in rabbits and rodents, but not in primates (Geary et al., 1998). In the denudation models, this might be due to inhibition of medial vascular smooth muscle proliferation by the estrogen receptor β or another pathway (Iafrati et al., 1997), or due to acceleration of endothelial regrowth (Krasinski et al., 1997; White et al., 1997). The accelerated recovery of nitric oxide formation by eNOS could contribute to their effects on intimal thickening. However, the observation that collar-induced intimal proliferation in eNOS deficient mice is completely abolished by pregnancy points to the involvement of other factors besides alterations in eNOS expression or activity (Moroi et al., 1998). Whether the antiproliferative effects of estrogens are due to modulation of iNOS expression remains controversial. Physiological concentrations of 17-β-estradiol have been reported to decrease cytokine-induced iNOS expression and activity in macrophages (Hayashi et al., 1997) and in the rat aorta (Kauser et al., 1998), but pharmacological concentrations may raise iNOS expression in the rat aorta (Binko et al., 1998).

Estrogens ameliorate cholesterol-induced atherosclerosis (Nathan and Chaudhuri, 1997). Ovariectomy results in lesion formation similar to the male animals and replacement therapy with 17-β-estradiol in these ovariectomized animals significantly diminished cholesterol-induced lesion formation in mice (Bourassa et al., 1996; Elhage et al., 1997), rabbits (Holm et al., 1997a,b) and monkeys (Geary et al., 1998). The cardioprotective effect of the estrogen was completely lost after balloon denudation, suggesting the presence of endothelial cells as a prerequisite for the antiatherogenic activity of 17-β-estradiol (Holm et al., 1997b; Geary et al., 1998). Indeed, acute as well as chronic administration of 17-β-estradiol to ovariectomized animals fed an atherogenic diet improved the impaired endothelium-dependent relaxation. However, it should be noted that estrogens in relatively high doses can produce endothelium-independent relaxation, possibly by blocking Ca^{2+} channels (Farhat et al., 1996) or by elevating cyclic AMP and cyclic GMP levels in the vascular smooth muscle (Mügge et al., 1993).

Recent studies suggest that nitric oxide contributes to the antiatherogenic effects of estrogens in the rabbit, but not in the mouse. The antiatherogenic effect of 17-β-estradiol was reduced by long-term inhibition of nitric oxide synthesis in hypercholesterolemic rabbits (Holm et al., 1997a), but not in apolipoprotein E deficient mice (Elhage et al., 1997). The latter result corroborates the observation that female mice with a targeted disruption of the eNOS gene still developed less intimal thickening in response to collar-induced vascular injury (Moroi et al., 1998). It thus seems that the contribution of eNOS-derived nitric oxide to the antiatherogenic effects of estrogens is species dependent.

6.2. Human studies

Several studies have demonstrated benefits of estrogen replacement therapy on the incidence of coronary heart

disease and risk for cardiovascular mortality in postmenopausal women, without any change in the risk of stroke. With respect to vasomotor control, acute improvement of vasodilator responses of epicardial and microvascular coronary arteries (Herrington et al., 1994; Reis et al., 1994) and of peripheral arteries (Gilligan et al., 1994a) was obtained during estrogen infusion in postmenopausal women with risk factors for atherosclerosis. The very acute beneficial effect of estrogen on endothelial vasodilator function appears to involve both nitric oxide (Guetta et al., 1997) and non nitric oxide mediated mechanisms (Tagawa et al., 1997). Early after transdermal estrogen administration, the vasomotion was improved as well (Roqué et al., 1998), but the effect disappeared after three weeks of transdermal application, presumably because of lower plasma levels of estradiol achieved with chronic administration (Gilligan et al., 1995). Indeed, oral estrogen replacement therapy for 9 weeks improved the flow-mediated vasodilatation of the brachial artery (Lieberman et al., 1994) and estrogen replacement therapy in postmenopausal women up to two years increased the plasma levels of nitrite/nitrate (Rosselli et al., 1995). The latter results are compatible with a prolonged increase of the bioavailability of nitric oxide, either due to increased eNOS activity or due to antioxidant properties of estrogens.

It is concluded that estrogen replacement therapy improves endothelium-dependent vasomotion and exerts antiatherosclerotic effects in animal models and postmenopausal women. The animal data suggest that the antiatherosclerotic effects of these pleiotropic hormones are mediated by an enhanced bioavailability of nitric oxide as well as by other mechanisms.

7. Interventions related to nitric oxide

7.1. L-Arginine

L-Arginine, the substrate of nitric oxide synthases, enters cells by facilitated diffusion via the y^+ transporter protein, which colocalizes with eNOS in caveolae (McDonald et al., 1997) and endothelial cells can resynthesize L-arginine from L-citrulline (Hecker et al., 1990). Exogenous L-arginine addition fails to restore the output of nitric oxide (Bult et al., 1995) or the endothelium-dependent relaxation of isolated atherosclerotic rabbit arteries (Verbeuren et al., 1993; White et al., 1994; Harrison, 1996) and human arteries (Cooper and Heagerty, 1998). Hence, the intracellular arginine stores appear to be sufficient for maximal eNOS activity. However in vivo studies showed that L-arginine may improve endothelium-dependent vasodilatation, though the behavior of conduit arteries with overt atherosclerosis appears to be different from arterioles in the microcirculation which do not develop atherosclerosis.

7.1.1. Animal studies

Oral L-arginine supplementation caused a striking inhibition of fatty streak formation (Cooke et al., 1992) and the progression of preexisting atheromatous lesions (Böger et al., 1997) in male, but not in female (Jeremy et al., 1996) hypercholesterolemic rabbits. L-Arginine administration to hypercholesterolemic rabbits improved the ex vivo endothelium-dependent relaxation of the isolated aorta instantaneously. Whether this was due to supply of substrate for nitric oxide synthases is unclear, since the response to nitroglycerin was improved to a similar extent (Cooke et al., 1991). Also prolonged in vivo L-arginine treatment partly restored systemic nitric oxide formation, as assessed by the urinary excretion of nitrates and ameliorated endothelium-dependent relaxation of the isolated aorta to some extent (Singer et al., 1995; Böger et al., 1997), though the latter effect was not sustained in another study (Jeremy et al., 1996). As the endothelial dysfunction in the rabbit aorta is strictly dependent on plaque size, the antiatherogenic effect of L-arginine supplementation could contribute to the improved endothelium-dependent relaxation. However, two studies reported that the endothelium-dependent vasodilatation in the microcirculation in hypercholesterolemic rabbits (Girerd et al., 1990) and pigs (Kuo et al., 1992) was slightly greater during L-arginine infusion. As arterioles do not develop atherosclerosis, this points to additional mechanisms. It remains to be determined whether the antiatherosclerotic and antioxidant effects of L-arginine are mediated by increased elaboration of nitric oxide or other mechanisms (vide infra).

7.1.2. Human studies

In patients with coronary artery disease or peripheral artery occlusive disease, L-arginine failed to improve endothelium-dependent dilation of conduit arteries with atherosclerotic plaques (Drexler et al., 1991; Bode-Böger et al., 1996). However, several groups reported that L-arginine infusion improved or even restored endothelium-dependent vasodilatation in the coronary and peripheral arterioles without overt atherosclerosis in hypercholesterolemic patients (Drexler et al., 1991; Creager et al., 1992; Adams et al., 1997), though this could not be confirmed by others (Casino et al., 1994; Dakak et al., 1998; Nitenberg et al., 1998).

7.1.3. Possible explanations for the arginine paradox

In view of plasma levels of L-arginine in the range of 150 to 250 μM and a K_m of 5 to 10 μM for NOS, it is surprising that L-arginine availability can ever limit nitric oxide biosynthesis. However, it is possible that the concentration of L-arginine in microdomains of the cell, e.g., in the caveolae is not reflected in the total cellular concentration (McDonald et al., 1997). Another possibility is that hypercholesterolemia impairs endothelial L-arginine transport, thus eventually depleting the intracellular stores. The latter could also result from increased L-arginine consumption by iNOS, arginase I or arginase II.

However, reversal by L-arginine of hypercholesterolemic endothelial dysfunction may not simply reflect the replenishment of the substrate for nitric oxide production (Jeremy et al., 1996) and could be based on its abilities to scavenge superoxide anions (Wascher et al., 1997) or their generation (Böger et al., 1997), thereby protecting nitric oxide from degradation. Arginine might further antagonize the effect of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase which accumulates in cholesterol-fed rabbits (Böger et al., 1997). Moreover, as the results are only seen with L-arginine administration in vivo, other systemic effects of the amino acid, e.g., its secretagogue effects on the adrenals and other endocrine organs, may prevail (Barbul, 1990). Hence, part of the antiatherosclerotic effect of systemic arginine administration could relate to the release of insulin, glucocorticoids or other immunosuppressive hormones, which are known to suppress atherosclerosis. This is illustrated by observations in healthy persons, where L-arginine infusion stimulated basal and acetylcholine-induced relaxation in the peripheral circulation and decreased the systemic blood pressure. The concomitant increase in urinary nitrate and cyclic GMP could not simply be attributed to a direct stimulating effect of L-arginine on eNOS, as prostaglandin E_1-induced dilation also increased these parameters in the urine (Bode-Böger et al., 1996). Indeed, the vascular effects seen after systemic L-arginine infusion in normal human volunteers, are to a substantial part mediated by the release of insulin (Giugliano et al., 1997). Therefore, further studies are needed to explore the beneficial effects of L-arginine.

7.2. Tetrahydrobiopterin

Deficiency of the cofactor tetrahydrobiopterin caused uncoupling of the L-arginine-nitric oxide pathway and led to increased in vitro formation of oxygen radicals by NOS itself. In hypercholesterolemic patients, endothelial responses measured by forearm venous plethysmography were restored during infusion of tetrahydrobiopterin or the folate precursor 5-methyltetrahydrofolte. Neither treatment had an effect in control subjects (Stroes et al., 1997). However, tetrahydrobiopterin infusion may not be active by supplying NOS with its cofactor but also by acting as an antioxidant (Verhaar et al., 1998).

7.3. Inhibitors of inducible nitric oxide synthase

Assuming that the abundant nitric oxide generation from iNOS in advanced lesions may activate processes which could promote atherosclerosis or increase plaque vulnerability, inhibition of iNOS could be considered beneficial. This view is supported by the attenuated fatty streak upon treatment of cholesterol-fed rabbits with the iNOS inhibitor aminoguanidine (Panagiotopoulos et al., 1998). Yet, this evidence is circumstantial and suppression of the formation of advanced glycation end products or another activity could contribute to the atheroprotection by aminoguanidine.

7.4. Organic nitrates

Organic nitrates are widely used in the management of coronary artery disease and are prodrugs from which nitric oxide is liberated via mechanisms which remain controversial (Parker and Parker, 1998). They are given not only to patients with stable angina pectoris, but also to those with unstable angina, acute myocardial infarction, and heart failure. A major limitation of the use of nitrates is the rapid development of tolerance during sustained therapy. Several hypotheses have been proposed to explain this loss of hemodynamic and antianginal efficacy. The free radical hypothesis suggests that nitrate tolerance is caused by an increased production of superoxide anion by the endothelium during nitrate therapy (Münzel et al., 1995). Inactivation of the nitric oxide released from the organic nitrate by superoxide anion generated by the vasculature then results in the loss of responsiveness to nitrates. The demonstration that iNOS expressing macrophages become hypersensitive to the toxic effects of exogeneously added nitric oxide donors (Mohr et al., 1998) could point to a possibility to kill the macrophages, while saving the smooth muscle cells in advanced plaques.

7.4.1. Animals studies

Treatment with spermine-NONOate (Yin and Dusting, 1997) or the cysteine-containing nitric oxide donor *N*-nitratopivaloyl-*S*-(*N'*-acetylalanyl)-cysteine ethylester (SPM-5185), which are resistant to the development of tolerance, inhibited intimal hyperplasia in rats and rabbits (Matthys and Bult, 1997). In contrast, nitroglycerin treatment only decreased the early medial smooth muscle cell proliferation without affecting intimal thickness after 3 weeks in the rat denudation model. This could be related to the development of tolerance with this drug. In these models, the nitric oxide donors appear to exert their major effect on smooth muscle cell migration, which is a crucial event in intimal thickening. Whether this is relevant to human atherosclerosis is unlikely, as atherosclerosis develops in an existing intima and migration of smooth muscle cells from media to intima is not considered a major determinant in atherogenesis.

Pentaerythrityl tetranitrate, an organic nitrate has been documented to inhibit cholesterol-induced fatty streak formation in rabbits, but the effect was not seen with isosorbide mononitrate. This could be due to differences with respect to the development of tolerance or the nitric oxide releasing capacity between the two organic nitrates. Conversely, treatment with molsidomine, pro-drug of the spontaneous nitric oxide donor 3-morpholino-sydnonimine (SIN-1), actually enhanced lesion formation in the rabbit. This may relate to the generation of superoxide anion from SIN-1, which could abrogate the beneficial effects of the simultaneously released nitric oxide.

7.4.2. Human study

Data from one small clinical study gave no evidence to suggest that long-term treatment with isosorbide dinitrate retarded angiographic progression of coronary artery disease or reduced cardiovascular morbidity or mortality (Cleland and Krikler, 1993).

8. Summary

Animal studies addressing the oxidation hypothesis of atherosclerosis have tremendously improved the understanding of early atheroma formation. It is now also appreciated that the concomitant deterioration of the bioavailability of endothelial nitric oxide is a major determinant of ischemic episodes in patients with coronary artery disease, and that nitric oxide derived from eNOS exerts antiatherosclerotic activities as well. However, nitric oxide might act as a double edged sword and the expression of iNOS in advanced plaques could favor processes which decrease plaque stability. Much has still to be learned on the biology of plaque vulnerability and arterial remodeling, processes which may have a greater impact on respectively acute myocardial infarctions and clinical stenosis than the mere plaque size. Progress in these fields is seriously hampered by the lack of sophisticated visualization methods and relevant animal models. On the other hand, the notion that endothelium dependent vasodilatation in the periphery reflects the defect in the coronary circulation, has provided a powerful tool to monitor the response to antiatherosclerotic interventions in patients by means of noninvasive measurements of flow-mediated vasodilatation in the brachial artery.

Lipid lowering drugs remain the treatment of choice to retard progression of atherosclerosis and its sequelae. It has become clear that non-lipid activities may explain the benefit of statins and fibrates in reducing coronary events. Statins exert antiinflammatory effects and improve endothelial reactivity and plaque stability. The mode of action of fibrates has recently been unraveled, and involves activation of peroxisome proliferator-activated receptors, which regulate the expression of apolipoprotein A, and genes involved in fatty acid metabolism and inflammation. In spite of the encouraging results in animal studies, controlled clinical trials were in most cases unable to show a clear positive effect of antioxidant treatments on the outcome of cardiovascular disease. What has been measured in the human studies as opposed to the animal studies is the progression of existing disease and not the initiation of lesions, which may explain the disappointing results. Yet, vitamin E has been shown to decrease the rate of non-fatal myocardial infarction and the progression of coronary artery disease. Furthermore, the matter of dosage and the use of combinations of antioxidants is complicated by the often encountered pro-oxidant action of many antioxidants (Bast et al., 1991). Further, long-term studies are thus needed to define the position of vitamins C and E and other antioxidants in the prevention or treatment of atherosclerosis.

So far, clinical trails failed to demonstrate unequivocal effects of angiotensin converting enzyme inhibitors on the progression of coronary artery disease, though these drugs can improve coronary vasomotion. Clinical trials point to an antiatherosclerotic potential of certain Ca^{2+} channel blockers, which appears to be unrelated to blockade of Ca^{2+} fluxes and involves antioxidant or other effects. For both types of vasodilator drugs further clinical studies are required to test whether they exert additive or even synergistic beneficial effects in combination with lipid lowering treatments. In contrast, it has been well documented that estrogen replacement therapy retards the progression of atherosclerosis in post-menopausal women, in part via amelioration of vascular dysfunction by increasing the bioavailability of nitric oxide and by non-endothelium dependent mechanisms. The retardation of early atherogenesis in rabbits and the improved vasomotor responses in patients with atherosclerosis or risk factors upon L-arginine treatment warrants further studies of the atheroprotective mechanisms exerted by this basic amino acid. From these studies, the concept arises that improving endothelial nitric oxide release might serve as an important element in the prevention and therapy of cardiovascular disease. If this proves to be true, lipid-lowering therapy would be beneficial in patients with angina or hypertension in whom such interventions had not been considered useful previously.

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