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Nitric oxide function in atherosclerosis

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ATHEROSCLEROSIS is a chronic inflammatory process in the intima of conduit arteries, which disturbs the endothelium-dependent regulation of the vascular tone by the labile liposoluble radical nitric oxide (NO) formed by the constitutive endothelial nitric oxide synthase (eNOS). This defect predisposes to coronary vasospasm and cardiac ischaemia, with anginal pain as the typical clinical manifestation. It is now appreciated that endothelial dysfunction is an early event in atherogenesis and that it may also involve the microcirculation, in which atherosclerotic lesions do not develop. On the other hand, the inflammatory environment in atherosclerotic plaques may result in the expression of the inducible NO synthase (iNOS) isozyme. Whether the dysfunction in endothelial NO production is causal to, or the result of, atherosclerotic lesion formation is still highly debated. Most evidence supports the hypothesis that constitutive endothelial NO release protects against atherogenesis e.g. by preventing smooth muscle cell proliferation and leukocyte adhesion. Nitric oxide generated by the inducible isozyme may be beneficial by replacing the failing endothelial production but excessive release may damage the vascular wall cells, especially in combination with reactive oxygen intermediates.

Key words: Atherosclerosis, Endothelial cell, eNOS, iNOS, Intimal thickening, Nitric oxide, Peroxynitrite, Superoxide anion

Atherosclerotic Lesion Development

The intima is the soil for atherosclerosis

Atherosclerotic lesions develop in the inner coat (or tunica intima) of the aorta, the large elastic arteries e.g. the carotid arteries and the arteries supplying the lower extremities, and the medium-sized muscular arteries, such as the coronary arteries. At birth, the intima consists solely of endothelial cells, but soon after birth focal and circumferential thickening occurs.[1] This spontaneously developing intima consists of smooth muscle cells, connective tissue and isolated macrophages, and is considered an adaptation to mechanical wall stress.[2] Although not pathologic at this stage, the thickened intima marks locations where atherosclerosis tends to develop later in life under the influence of atherogenic stimuli e.g. hypercholesterolaemia.

Features of human atherosclerosis

Early atherosclerotic lesions are characterized by the deposition of lipids and the appearance of macrophages and T-lymphocytes in the intima. As macrophages and a few 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.[3] Progressively, these flat, fatty lesions transform to raised fibrolipid plaques, as intimal smooth muscle cells proliferate and deposit extracellular matrix, mainly collagen. In a subsequent stage, the advanced lesion has a characteristic microanatomy with a core of extracellular lipid separated from the media by smooth muscle cells and covered at the luminal side by a thick fibrous cap. Surrounding the lipid core are lipid-filled foam cells. The ischaemia in the necrotic core initiates angiogenesis. This type of plaque may cause narrowing of the lumen once the compensatory vascular remodelling process which

increases the external diameter of the vessel becomes exhausted. Only then, lesions become angiographically visible. The final stage, the complicated plaque, may arise either from fissure of the fibrous cap or from intra-plaque haemorrhage. The thromboembolic events following plaque fissure are a major cause of clinically manifest acute ischaemic syndromes. If the thrombus is not occlusive, it becomes incorporated into the plaque and is organized by invading macrophages and smooth muscle cells, thereby further compromising the lumen of the vessel. The sequence of fissure, thrombus formation, organization and incorporation into the plaque may occur repeatedly.

Models of atherosclerosis and intimal thickening

Models of atherosclerosis

Current knowledge of the initiation of the atherogenic process is largely based on rabbit or primate models of hypercholesterolaemia, which may be diet-induced or genetically determined as in Watanabe heritable hyperlipidaemic (WHHL) rabbits. Hypercholesterolaemia provokes intravascular lipid infiltration leading to the formation of fatty streaks, which resemble early human lesions.[3] Protracted cholesterol feeding eventually results in advanced fibrolipid plaques containing necrotic debris, as in advanced human disease.

Models of intimal thickening

Intimal thickening can be induced experimentally by creating a modest mechanical injury of the smooth muscle cells of the media. The most extensively investigated model involves balloon denudation of the intima of the rat carotid artery with an embolectomy catheter.[4] The discrete mechanical injury of the underlying media evokes smooth muscle cell proliferation in the media, followed by migration to the intima and an extended phase of intimal proliferation. The endothelial cells are completely removed by the initial insult and regrowth of the endothelial cells from the lesion edges is virtually absent. The removal of the endothelial cells is not essential nor sufficient for the process of intimal hyperplasia.

Placing a flexible collar around the rabbit carotid artery does not create direct endothelial injury, but induces smooth muscle cell proliferation in the media, followed by migration and prolonged proliferation in the intima.[5] Both models illustrate the three wave paradigm for the involvement of smooth muscle cells in the formation of intimal cushions.[4]

The inflation of an angioplasty balloon in arteries of rabbits, pigs or other experimental animals is used to mimic restenosis due to accelerated intimal thickening after percutaneous transluminal coronary angioplasty (PTCA). The vessel wall distension by the repeated inflation of a slightly oversized balloon creates a much more extensive injury of the media and the lamina elastica interna than the gentle passage of an embolectomy catheter. Unlike balloon denudation, the balloon angioplasty thus predisposes to thrombus formation. In accordance with restenosis after PTCA in humans, the incorporation and organization of the non-occlusive thrombus adds to the bulk of neointima formation.[4] A further difference with balloon denudation is the quick and often complete recovery of the endothelial cell layer through outgrowth from patches of cells which remained present after the angioplasty.

Atherosclerosis is a Chronic Inflammatory Process

The long-standing and continuously refined 'response-to-injury' hypothesis[6] considers the lesions as the result of an excessive inflammatory-fibroproliferative response to various forms of insults to the endothelium and smooth muscle. Moreover, the presence of T-lymphocytes in atherosclerotic lesions at all stages of development points to an important immunologic component in atherogenesis.[7] T-lymphocytes and macrophages are capable of producing numerous inflammatory mediators and growth factors, and have been demonstrated to be in an activated state in atherosclerotic lesions.

Pathogenetic mechanisms in hypercholesterolaemia-induced atherogenesis

Several different sources of injury to the endothelium can lead to endothelial dysfunction and initiate the disease process.[6] In hypercholesterolaemia-induced atherosclerosis, the major causal agent is now assumed to be oxidized LDL (oxLDL).[8,9] Oxidation of lipoproteins flooding the intima may result from the production of reactive oxygen intermediates or 15-lipoxygenase activity in the endothelial cells (Fig. 1). OxLDL in turn is cytotoxic to endothelial cells by the metal-catalysed production of free radicals from lipid hydroperoxides contained in the modified lipoprotein particle.[10] Furthermore, oxLDL is chemotactic for monocytes and T-lymphocytes. Newly formed epitopes in oxLDL elicit cell-mediated and humoral immune re-

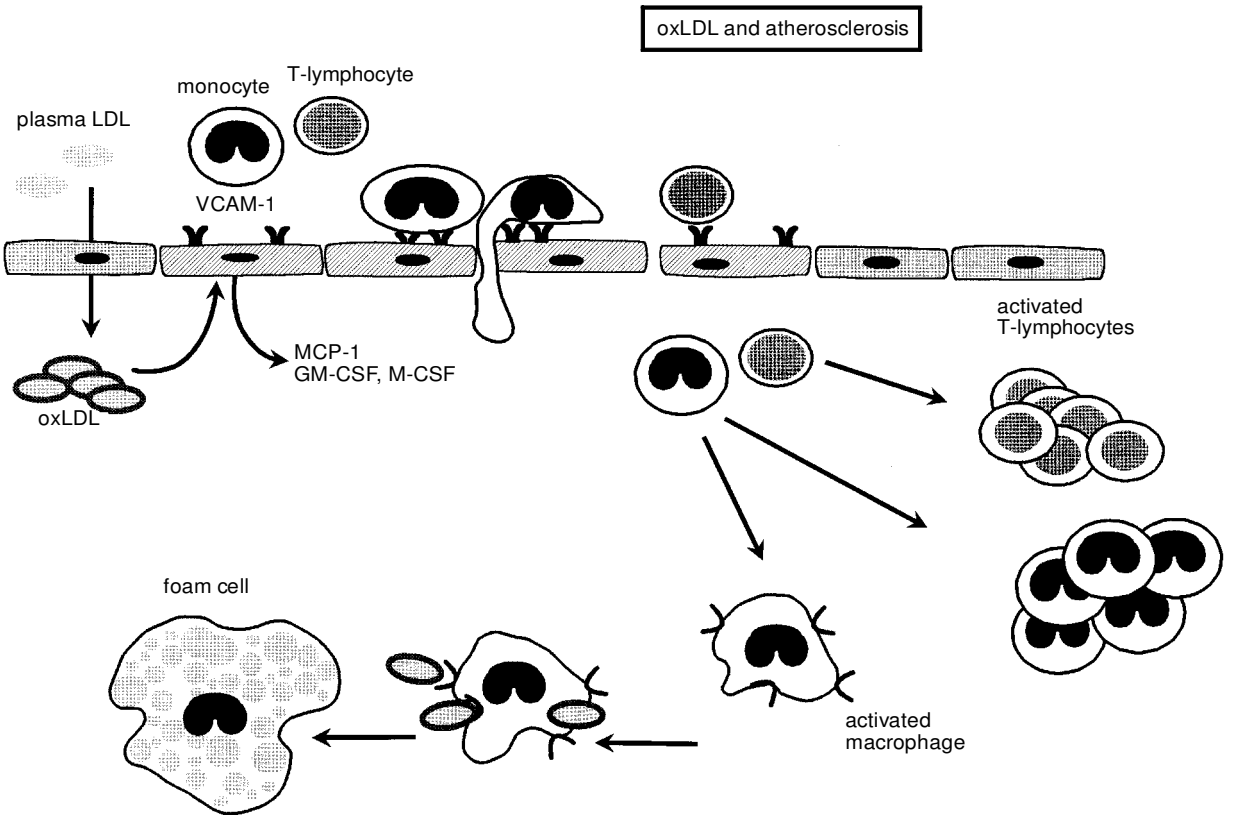

FIG. 1. Lipoproteins flooding the intima become oxidized. Oxidized LDL (oxLDL) is chemotactic for monocytes and T-lymphocytes. OxLDL promotes mononuclear cell adhesion, infiltration and proliferation by stimulating the endothelial cells to express adhesion molecules, e.g. VCAM-1, and to produce chemotactic factors, e.g. MCP-1, and growth factors. Uptake of oxLDL by macrophages leads to foam cell formation.

sponses.[7] Minimally oxidized LDL stimulates the endothelial cells and smooth muscle cells to secrete monocyte chemotactic protein-1 (MCP-1) and growth factors involved in the differentiation and proliferation of monocytes, and oxLDL may, synergistically with cytokines, promote mononuclear leukocyte adhesion to the endothelium through the induction of vascular cell adhesion molecule-1 (VCAM-1).[11,12] The oxidative stress-sensitive nuclear transcription factor κB (NF-κB) may be a crucial intermediate in the inflammatory activation of the endothelium.[13] Monocyte-derived macrophages internalize oxLDL through scavenger receptors. As these receptors are not down-regulated by the intracellular cholesterol level, massive cholesterol accumulation occurs and the macrophages transform to foam cells.

Thus, it appears that endothelial cells, through the oxidation of LDL, recruit macrophages to remove the invaded lipoprotein particles. This attractive hypothesis also implies that a chronic inflammatory response will develop if the macrophages are unable to eliminate oxLDL sufficiently.

Atherosclerosis and Nitric Oxide Signalling

The nitric oxide signalling pathway in normal arteries

A major step forward in the understanding of blood vessel physiology was the discovery by Furchgott and Zawadzki[14] of a factor released by the endothelium that relaxed the underlying smooth muscle. This endothelium-derived relaxing factor (EDRF) was later identified as nitric oxide (NO)[15–17] or a related nitrosylated compound e.g. S-nitrosocysteine.[18,19] Nitric oxide is formed by a five-electron oxidation of a terminal guanidino nitrogen atom of the amino acid L-arginine, with concomitant formation of L-citrulline, by an enzyme known as nitric oxide synthase (NOS).[20] There are two major classes of NO synthases: constitutive and inducible (iNOS) enzymes. Constitutive isoforms are expressed in endothelial cells (eNOS), in neuronal cells (nNOS) and in certain other cell types. The activity of these isoforms is strictly calcium-calmodulin dependent and is present both in the cytosol and associated with membranes.

Stimulation of the appropriate receptors on the endothelial cell by physical (shear stress resulting from increased flow, mechanical deformation) or chemical (acetylcholine, bradykinin, substance P, ATP) stimuli raises the cytoplasmic calcium levels, with concomitant eNOS activation and formation of NO.[21] Serotonin (5-hydroxytryptamine, 5-HT), although a potent vasoconstrictor through activation of 5-HT_2 receptors on the vascular smooth muscle cells, also mediates dilatation by the EDRF/NO-dependent mechanism, through activation of 5-HT_1-like receptors on the endothelium.[22] In biological systems, the dominant reactions of NO will be with another free radical such as superoxide anion, transition metals such as haem iron, or oxygen.[20] In the vessel wall, NO diffuses into the underlying smooth muscle cells to react with the haem group of a cytoplasmic guanylate cyclase. The formation of cyclic GMP then causes vasodilatation.[23–25]

Nitric oxide also raises cyclic GMP in the endothelial cells themselves, which inhibits the production of the potent endothelium-derived contracting factor endothelin.[26,27] Inhibitors of NOS and guanylyl cyclase revealed an inhibitory role of NO but not of cyclic GMP in endothelin secretion by porcine aortic endothelial cells.[28]

Nitric oxide signalling in atherosclerosis

It has been recognized for a long time that atherosclerotic blood vessels are very susceptible to the development of vasospasm *in vivo*[29–32] and are hyperreactive to contractile agonists *in vitro*.[32–35] Because coronary vasospasm can be provoked by several stimuli with different mechanisms of action, it has been proposed that dysfunction or denudation of the endothelium in atherosclerosis may contribute to that phenomenon by leaving constrictor responses unopposed.[36–38] Even before it was realized that NO accounts for the biological activity of endothelium-derived relaxing factor, it was indeed demonstrated that artery segments obtained from atherosclerotic animals showed a loss of endothelium-dependent relaxation in organ bath experiments.[35,39–42] From then on, numerous *in vitro* studies confirmed the defect in the NO signalling pathway in isolated atherosclerotic blood vessels in rabbits,[43–49] pigs,[50–53] rats,[54] primates[55] and humans.[43,56–58] Basal as well as stimulated NO release appeared to be affected.[58–60]

Urinary nitrate, an index metabolite for NO formation *in vivo*,[61,62] is decreased in cholesterol-fed rabbits.[63] Catheterization-based studies in patients with coronary artery disease also demonstrated the impairment of endothelium-dependent coronary vasodilatation to acetylcholine[64–69] or increased flow,[70–72] particularly at atherosclerosis-prone branch points.[73] The deterioration of endothelium-dependent vasodilatation is an early event, as it can be observed in patients with typical angina or cardiac risk factors but with angiographically smooth coronary arteries.[73–80] The current weight of evidence suggests that impaired endothelium-dependent vasodilatation is the predominant mechanism underlying inappropriate constriction leading to ischaemic manifestations.[75,81,82] The instantaneous relief of the ischaemic attacks by the NO donor nitroglycerin points to a defect in the endogenous NO pathway. Unopposed vasoconstrictor responses in general, but also the loss of the EDRF-component in the net reaction to some agonists, e.g. serotonin and norepinephrine in the pig and dog,[37] and increased endothelin release in the absence of EDRF may contribute to the occurrence of vasospastic events in atherosclerotic vessels.

The systemic nature of the defect in NO signalling

The EDRF/NO pathway is also active in the small vessels determining the resistance of the vascular tree,[83,84] thus contributing to blood pressure regulation.[85] Several studies (reviewed by Anderson *et al.*[86]) demonstrated that atherosclerosis in conduit vessels is accompanied by impaired endothelium-dependent vasodilatation in the microcirculation e.g. in the coronary[76,87–89] and peripheral resistance vessels.[90–93] Also the mere presence of cardiovascular risk factors was associated with dysfunctional microvascular endothelium.[94,95] While the expected hypertensive effect might contribute to the progression of cardiovascular disease, this also implies that, besides a dysfunctional endothelial NO pathway, other factors are involved in the initiation and progression of atherosclerotic plaques, since lesions do not develop in these microvessels. The systemic nature of the endothelial dysfunction could be of use in the non-invasive evaluation of endothelial function in readily accessible arteries.[96]

In summary, established atherosclerosis or the presence of risk factors e.g. hypertension, hypercholesterolaemia, and even male gender,[97,98] decrease the activity of the EDRF/NO pathway. Endothelium-dependent dilatation is

lost progressively as atherogenesis continues. Conduit vessels with lesions as well as resistance vessels in which lesions do not develop are affected, the latter apparently to a lesser extent.[65,93]

Explanations for the Defective NO Signalling Pathway

The mechanisms underlying the dysfunctional endothelial NO signalling pathway in atherosclerosis and hypercholesterolaemia are multifactorial (reviewed in Refs 99 and 100). Atherosclerotic arteries demonstrating disturbed endothelium-dependent relaxation are still capable of dilating to the NO donor nitroglycerin which provides the smooth muscle with NO upon enzymatic or thiol-dependent bioconversion.[101] As this demonstrates that the smooth muscle is still responsive to the dilatory action of NO, defective endothelial EDRF/NO release or increased NO inactivation after release appear to be involved. This is supported by the decreased release of bioactive NO from isolated perfused atherosclerotic arteries as assessed by a superfusion bioassay.[41,44,50,102,103]

Endothelial receptor dysfunction

Endothelium-dependent dilatation is lost in a progressive, hierarchical fashion.[82] Vasodilator responses to acetylcholine and serotonin are lost early, before impairment of the dilatation to other receptor agonists e.g. substance P, to the receptor-independent stimulus calcium ionophore A-23178 or to mechanical stimuli. The agonist specificity of the early dysfunction suggests that it is not caused by a nonspecific impairment of the ability of the endothelial cells to produce NO, but points to selective alterations in endothelial receptor function or post-receptor effector pathways. This view is strengthened by the observation that the receptor-induced release of other endothelial products, such as prostacyclin, is attenuated as well.[104,105] In this respect, it has been demonstrated that the pertussis toxin-sensitive G_i protein signalling pathway, which is employed by serotonin to elicit endothelium-dependent relaxation in the pig coronary artery, is impaired in the early stages of the atherosclerotic process.[106] Several studies have demonstrated that incubation of vessel segments with lipoproteins, and in particular with oxLDL, inhibited endothelium-dependent relaxation in a way similar to hypercholesterolaemia *in vivo* (reviewed by Flavahan[99]). Lysophosphatidylcholine, a component of oxLDL, mimicked the effects of the whole particle,[107–109] inhibited agonist-stimulated calcium signalling in cultured endothelial cells[110] and selectively inhibited G_i protein-dependent signalling in porcine endothelial cells.[111]

Expression of eNOS activity

The receptor selectivity (see above) argues against a reduced expression of eNOS activity in endothelial cells overlying atherosclerotic lesions. This assumption has recently been confirmed by *in situ* hybridization of eNOS mRNA and immunohistochemistry of eNOS protein in the aorta of hypercholesterolaemic rabbits. The results suggested that the expression of eNOS mRNA and protein was even increased in endothelial cells overlying fibrofatty plaques.[112] Studies of the expression and the activity of eNOS after *in vitro* exposure of endothelial cells to LDL, oxLDL or cholesterol yielded contradictory results, ranging from initial upregulation, via no effect to downregulation of eNOS expression. After acute exposure of the isolated rabbit carotid artery to cholesterol-rich liposomes, acetylcholine-induced release of EDRF/NO was evaluated functionally in a superfusion bioassay and appeared to be enhanced.[113] This could be due to augmentation of the release and/or prolongation of the half-life of NO. Exposure of endothelial cell membranes to liposomal cholesterol raised the activity of plasma membrane bound eNOS at low cholesterol concentrations, but had the opposite effect at higher concentrations.[114] The effects were attributed to modulations of the lipid environment of the membrane bound eNOS. Cholesterol was without effect on the activity of eNOS in the cytosol of endothelial cells. Interestingly, the increased activity of particulate eNOS was accompanied by a concentration-dependent increase in superoxide anion production, but the authors did not investigate whether eNOS was the source (see below). Low concentrations of oxLDL have been reported both to increase[115] and to decrease[116] the expression of mRNA, protein and activity of eNOS in cultured endothelial cells. The upregulation of eNOS mRNA was mimicked by lysophosphatidylcholine, one of the many constituents of oxLDL. This discrepancy between both reports could be due to large variability among different preparations of oxLDL with respect to biological activities. Downregulation of the expression of mRNA of eNOS by higher concentrations of oxLDL appears to be a more consistent finding.[115,116]

Native LDL was without effect on eNOS mRNA levels and eNOS activity,[115,116] although exposure of cultured endothelial cells to LDL may promote superoxide anion formation by eNOS[117] (see below).

Arginine availability

Although arginine availability seems to be sufficient initially in view of the receptor-selectivity of the endothelial dysfunction (see above), studies on endothelium-dependent vasodilatation suggest that L-arginine depletion may occur. It should be noted, however, that the studies addressing the effect of the NO precursor show discordant results. In this respect, the behaviour of conduit arteries with overt atherosclerosis appears to be different from arterioles in the microcirculation, in which atherosclerosis does not develop.

Conduit arteries with atherosclerosis

Most authors agree that *in vitro* L-arginine addition fails to restore the endothelium-dependent relaxations in the aorta[118–120] or femoral artery[121] with cholesterol-induced atherosclerotic lesions. One report showed that acute *in vivo* L-arginine administration to hypercholesterolaemic rabbits improved the endothelium-dependent relaxations in isolated large vessels *in vitro*, but it should be noted that responses to nitroglycerin were affected to a very similar extent.[122] Also prolonged *in vivo* L-arginine treatment ameliorated endothelium-dependent relaxations of isolated segments only marginally.[123] As the endothelial dysfunction is strictly dependent on the size of the lesions in rabbit conduit arteries,[35,49,124,125] the marked antiatherogenic effect of prolonged L-arginine supplementation[123] (see below) most likely explains the improved endothelium-dependent relaxations. In patients with coronary or peripheral artery occlusive disease, a positive effect of L-arginine on endothelium-dependent dilatation of the conduit arteries was lacking.[126,127]

Conduit arteries without overt atherosclerosis

Although the rabbit basilar artery develops neither atherosclerotic lesions nor a clear endothelial dysfunction after prolonged hypercholesterolaemia,[49] an improvement of the endothelium-dependent relaxations has been reported after *in vitro* exposure to L-arginine.[128] In addition, L-arginine also attenuated the augmented vasoconstrictor responses to potassium chloride, serotonin and endothelin. The authors suggested that the normalization by L-arginine of both the endothelium-dependent relaxation and the constrictor responses was the result of increased EDRF production. However, as cyclic GMP-mediated relaxation induced by endothelium-independent agonists was not studied, and as the contraction to a depolarizing potassium chloride solution is not affected by basal EDRF release,[37] it is not entirely clear whether the actions of L-arginine can be attributed solely to enhanced endothelial NO production. Lefer and Ma measured constrictions evoked by the NOS inhibitor L-NAME as an index of basal NO release by the endothelial cells of rabbit coronary arteries isolated after three weeks of cholesterol diet.[60] A reciprocal relationship existed between L-NAME evoked contractions and plasma cholesterol, suggesting that basal NO release by the segments became compromised in the absence of overt atherosclerosis. *In vitro* addition of L-arginine almost totally restored this index of basal NO production. However, as non-endothelial iNOS may be induced in arteries of cholesterol-fed rabbits,[129] it cannot be excluded that the vasoconstrictor responses to L-NAME resulted from inhibition of iNOS rather than eNOS.

Arterioles without overt atherosclerosis

The results obtained in conduit arteries suggest that L-arginine may upregulate the impaired eNOS activity only if the cholesterol-exposed arteries are still lesion-free. Accordingly, all studies, except one,[130] reported that L-arginine infusion resulted in marked improvement to complete restoration of endothelium-dependent vasodilatation in the coronary and peripheral microcirculation in hypercholesterolaemic rabbits,[131] pigs[88] and humans.[126,132]

The mechanism of the amelioration of endothelium-dependent relaxations by L-arginine is not yet clear, and could be due to an interaction with smooth muscle cells or other effects. 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 isoforms, it is indeed surprising that L-arginine availability can ever limit NO biosynthesis.[20] L-arginine enters cells by facilitated diffusion via the y^+ transporter.[133] As exogenous L-arginine addition neither induces endothelium-dependent relaxations by itself, nor enhances agonist-induced endothelium-dependent relaxations in normal isolated vessel rings,[120,129,134] the intracellular stores appear to be sufficient for maximal eNOS activity in physiological circumstances. The increase in membrane cholesterol associated with hypercholesterolaemia might impair endothelial L-arginine transport, thus eventually depleting the intracellular stores. The latter may also result

from the increased output of inactive nitrogen oxides, as demonstrated in hypercholesterolaemic rabbit aorta.[103] However, reversal by L-arginine of hypercholesterolaemic endothelial dysfunction may not simply reflect the replenishment of the substrate for NO production. The observation that the effect of L-arginine administration to hypercholesterolaemic rabbits is not sustained and depends on the anatomic site and sex[135] indeed supports a more complex mechanism of action.

As the best results are obtained in the microcirculation after L-arginine treatment *in vivo*, other less well characterized systemic effects of the amino acid e.g. its secretagogue effects on the adrenals and pituitary gland, may prevail.[133] This is illustrated by observations in healthy persons, where L-arginine infusion stimulated basal and acetylcholine-induced relaxation in the peripheral circulation[130,136,137] and decreased the systemic blood pressure.[127,138] 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 dilatation also increased these parameters in the urine.[127] Furthermore, intravenous L-arginine administration increased urinary flow, which by itself resulted in enhanced excretion of nitrate and CGMP, in the absence of elevated nitrate plasma levels.[138]

Endothelial NO synthase inhibition

L-arginine may be effective in conditions where endogenous NOS inhibitors are formed. N^G,N^G dimethylarginine (DMA) has been found in the urine and plasma of humans and inhibited macrophage and vascular NO synthesis *in vitro* and *in vivo* in animals and humans,[139] suggesting the existence of endogenous mechanisms to regulate NO synthesis. Recently, DMA was reported to be increased in the serum of cholesterol-fed rabbits.[140,141] All classes of NO synthases are liable to feedback inhibition by NO,[142] probably by the interaction of NO with the haem prosthetic group.[143] Hence, high output NO production by iNOS (see below) might downregulate eNOS activity. This is supported by the observation that chronic *in vivo* administration of large doses of an NO donor to rabbits depressed the *ex vivo* output of EDRF/NO in response to acetylcholine, as assessed by means of bioassay.[135] Endothelial NOS may also be suppressed by other locally produced inflammatory mediators e.g. the T-lymphocyte-derived mediator interferon-γ.[144]

Inactivation of NO by superoxide anion

Superoxide anion is known to inactivate EDRF/NO.[145,146] Generation of superoxide anion *in situ* in normal vessels reduced endothelium-dependent relaxation.[147,148] Under normal conditions, inactivation of EDRF by superoxide radicals is prevented by cytosolic CuZn superoxide dismutase (SOD)[149] and by extracellular SOD type C associated with heparan sulphate proteoglycans on the endothelial cell surface and in the interstitium.[148]

Hypercholesterolaemia in the rabbit increased the intimal[103,150] production of reactive oxygen species, resulting in increased degradation of NO (reviewed by Harrison and Ohara[100]). The tunica media beneath the atheromatous plaque in WHHL rabbits also inactivated EDRF/NO by an SOD-sensitive mechanism.[44] Increased vascular production of reactive oxygen species may result from enhanced xanthine oxidase activity in the endothelium[150] or from production by infiltrated monocytes.[151] In addition to direct inactivation of EDRF/NO by oxLDL and lysophosphatidylcholine,[152] oxLDL has been shown to stimulate the respiratory burst in neutrophils,[153] and lysophosphatidylcholine induced superoxide production in vascular smooth muscle cells via protein kinase C activation.[154] Endothelial NADPH oxidase systems,[155] activated by protein kinase C,[156] may also be involved. Protracted endothelial cell exposure to atherogenic native LDL concentrations increased superoxide anion production by three independent oxidative systems—cyclooxygenase, P450 isozyme and eNOS—of which the latter appeared to be the greatest source.[117] Nitrotyrosines, hallmarks of peroxynitrite formation from superoxide and NO, were detected intracellularly.

Furthermore, a striking feature of NOS is its ability to generate superoxide anion when either L-arginine or the cofactor tetrahydrobiopterin is limiting.[20] Under these circumstances, NADPH oxidation is uncoupled from synthesis of NO, and oxygen becomes the electron acceptor, resulting in superoxide formation. This has been demonstrated to occur in the constitutive NOS of the brain. Whether the low arginine levels needed for superoxide biosynthesis occur in intact endothelial cells *in vivo* is unclear. Arginine depletion of eNOS might occur from high local L-arginine consumption by iNOS (see below). Arginine availability may also be reduced by impediment of cellular uptake or delivery to eNOS, as has been suggested to occur in endothelial cell cultures

treated with native LDL.[117] In the latter experiments, LDL-exposed cells produced significantly more superoxide anion than untreated cells, which was reversed by arginine supplementation. Inducible NOS did not seem to be involved, as Ca^{2+}-independent arginine-to-citrulline conversion under apparent V_{max} conditions was low. Nevertheless, in conditions where iNOS, which is much more demanding for substrate then eNOS, is induced, insufficient L-arginine might result in superoxide anion release. This would also explain the benefit of providing L-arginine, i.e. to promote re-coupling, thus reducing vascular superoxide production and prolonging the half-life of EDRF/NO. These findings provide new insight into the mechanisms by which hypercholesterolaemia might both stimulate superoxide production and decrease functional NO levels.

The disturbed balance between vascular superoxide and endothelial nitric oxide production, resulting in the loss of functional NO, may be compensated for by iNOS activity in the vascular wall (see below) and/or by upregulation of endogenous SOD.[157–159] Addition in the organ bath of CuZn SOD, which does not penetrate cells, or preincubation with extracellular SOD type C, which binds extracellularly to vascular structures, also protected against the detrimental effects of superoxide radicals on endothelium-dependent relaxation.[148] Conversely, exhaustion of these protective mechanisms, which may be time-, species-, or vessel-dependent, may tip over the balance towards a net decrease in functional EDRF/NO.

In rabbits, but not in pigs, hypercholesterolaemia alone did not impair the endothelial dilator function in large vessels, but only occurred in arteries with intimal plaques,[35,44,46,48,49,125] with the exception of the coronary arteries.[60,160] Apparently, the rabbit is capable of keeping the superoxide and nitric oxide production in balance, as long as lesions do not develop. Superoxide production in the media beneath the plaque[44] or the presence of fatty streaks containing large amounts of macrophages and lipids, may disturb the balance by the high local superoxide production and the trapping of the lipophilic NO molecule.

Raising the antioxidant capacity in the vessel wall by the administration of CuZn superoxide dismutase, polyethylene-glycolated[161] or liposome-entrapped[121] to ensure cell entrance, partly restored the endothelium-dependent relaxation in the isolated aorta of the cholesterol-fed rabbit.[121,161] In keeping with these findings, it has been shown that addition of antioxidant vitamins in the diet of cholesterol-fed rabbits preserved the endothelium-dependent dilatation in the absence of an effect on lesion formation.[162,163] Also, dietary correction of hypercholesterolaemia in the rabbit normalized both the endothelial superoxide production and dramatically improved the vasodilator response to acetylcholine.[164] Oral administration of 2 g ascorbic acid produced marked improvement in the forearm vascular response to hyperaemia in patients with coronary artery disease.[165] However, short-term treatment with antioxidants of patients with hypercholesterolaemia did not improve the forearm vascular responses to acetylcholine.[166,167]

Eventually, atherosclerotic plaques, in particular when lipid-rich, may trap NO and may also mechanically disturb the normal dilatation of the medial smooth muscle. At this stage, the relaxation to exogenous NO donors e.g. nitroglycerin, and to endothelium-independent dilator substances e.g. atrial natriuretic peptide also becomes impaired.[102]

Atherosclerosis and Inducible Nitric Oxide Synthase Expression

Animal and human macrophages,[168,169] smooth muscle cells[170–172] and endothelial cells[173,174] are capable of expressing iNOS after stimulation with endotoxin or cytokines. In contrast to eNOS, iNOS produces high amounts of NO for a sustained period.[20] In early reports, the presence of a constitutive NOS in vascular smooth muscle cells has been suggested,[175,176] but these observations were probably related to the induction of iNOS during the isolation and manipulation of the cells or tissue.[172,177,178]

Atherosclerosis and iNOS

Only recently, functional and biochemical evidence suggested that cholesterol feeding of rabbits induced iNOS expression in the aorta[129] and in the lungs.[179] The addition of NOS inhibiting L-arginine analogues caused endothelium-independent contractions in the isolated atherosclerotic rabbit aorta, pointing to the continuous formation of NO by subendothelial iNOS.[129] The observation that the NOS inhibitors nitro-L-arginine methyl ester (L-NAME) and monomethyl-L-arginine (L-NMMA) were equipotent in this respect further supported the involvement of iNOS.[129] The expression of iNOS may account for the increased output of nitrogen oxides in arteries of cholesterol-fed rabbits.[103] Histochemical studies in WHHL rabbits confirmed the expression of iNOS in medial and intimal smooth muscle cells, and showed

significant enhancement of endotoxin-induced iNOS expression in atherosclerotic rabbits compared with normal New Zealand White rabbits.[180] In a chronic rejection model of transplant atherosclerosis in the rat, both macrophages and smooth muscle cells stained positive for iNOS.[181] More recently, it has been reported that iNOS is present within human atherosclerotic lesions and co-localizes with nitrotyrosine in macrophages and smooth muscle cells.[182] Induction of iNOS was also observed in the endothelium and smooth muscle of intramyocardial vessels of patients with ischaemic heart disease.[183]

The observation that iNOS induction in vascular smooth muscle cells, as in macrophages, is accompanied by upregulation of L-arginine transport,[184] may contribute to the stimulating effect of L-arginine on vessel relaxation in some experimental settings.

Mechanical injury and iNOS

The hypocontractility to several agonists observed after balloon denudation of rat[185,186] or balloon angioplasty of rabbit arteries[187] was also attributed to the induction of iNOS in the vessel wall and was already noticeable 6 h post-injury.[186] Unlike the case in normal arteries, L-arginine evoked significant relaxation in deendothelialized balloon-injured vessel segments, which was reversed by the NOS inhibitor L-NAME.[187] In the balloon-injured rat carotid artery, reverse transcription and polymerase chain reaction amplification showed the appearance of iNOS mRNA already 24 h post surgery, and *in situ* hybridization located iNOS mRNA in neointimal smooth muscle cells, particularly at the luminal side of the vessel, conferring a nonthrombogenic surface.[188]

Cytokines introduced in the affected vessel by infiltrating monocytes and T-lymphocytes may provide the stimulus for iNOS induction in the smooth muscle cells. Also, oxLDL[189] and LDL[190,191] have been shown to upregulate iNOS activity in macrophages and vascular smooth muscle cells under certain conditions. On the other hand, mediators that inhibit iNOS induction e.g. heat shock proteins[192] or NO itself,[193] may determine the final output of NO.

NO: A Radical with Anti-atherogenic Properties

Since the impairment in the EDRF/NO pathway occurs early or even precedes the development of visible lesions in the process of atherosclerosis, many authors have speculated on a causal role of this functional defect. This view is supported by a number of *in vitro* studies demonstrating the suppression by NO, produced endogenously or derived from NO donors, of several key processes involved in atherogenesis (Table 1).

In vitro studies

Interference with oxidative processes

Since superoxide anion contributes to oxidative stress, LDL modification[194] and inflammatory gene transcription via the activation of NF-κB,[13] the decreased formation or inactivation of superoxide by NO may be considered protective. In this respect, it has been shown that the NO derived from iNOS inhibits xanthine oxidase in interferon-γ-stimulated macrophages[195] and that authentic exogenous NO inhibits xanthine oxidase in a cell-free system,[196] possibly by reversible alteration of the flavin prosthetic site.[197] Nitric oxide also inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase.[196] The NO donors known as NONOates abrogate the cytotoxic effects of superoxide on Chinese hamster lung fibroblasts.[198] Moreover, NO also protected against cellular damage by other reactive oxygen species e.g. hydrogen peroxide and alkyl peroxides, by several mechanisms such as prevention of haem oxidation, inhibition of Fenton-type oxidation of DNA, and abatement of lipid

Table 1. Anti-atherogenic properties of nitric oxide in vitro

	Reference
Interference with oxidative processes	
Cytoprotection against oxidative stress	198–200
Inhibition of cell-mediated LDL oxidation	201–205, 210
Inhibition of lipoxygenase activity	207
Inhibition of oxLDL cytotoxicity	211
Inactivation of xanthine oxidase	195–197
Inhibition of NADPH oxidase	196
Reduction of endothelial hyperpermeability	212
Interference with leukocyte recruitment	
Suppression endothelial adhesion molecules	214, 217
Inhibition of monocyte chemotaxis	219
Inhibition of monocyte adhesion	217, 219, 220
Inhibition of neutrophil adhesion	60, 213–216
Inhibition of MCP-1 expression	218
Inhibition of NF-κB activation	217, 218
Antiproliferative actions	
Inhibition of smooth muscle cell proliferation	221–227
Inhibition of smooth muscle cell migration	228, 229
Inhibition of T-cell proliferation	230–232
Stimulation of endothelial repair	233
Inhibition of platelet activation	234–236

peroxidation.[199] Activation of the stress protein haem oxygenase by NO may contribute to its cytoprotective effect.[200] Furthermore, NO reduced the oxidative modification of LDL by macrophages,[201–204] endothelial cells[205] and lipoxygenase[206] by acting as a potent terminator of radical chain propagation reactions.[206] Also, as 15-lipoxygenase has been implicated in LDL oxidation,[8] NO might protect LDL by inhibiting lipoxygenase activity.[207] Conversely, the decreased expression of iNOS activity in oxLDL-laden foam cells[208,209] has been implicated in the accelerated oxidation of LDL by these macrophages.[210] Furthermore, NO released by donor compounds inhibited the cellular toxicity of the lipid hydroperoxides contained in oxLDL, presumably by scavenging the propagatory free radicals generated during peroxidation of the endothelial cell membranes.[211] NO donors also blocked the hydrogen peroxide-related increase in endothelial permeability by a cyclic GMP-mediated mechanism.[212]

Interference with leukocyte recruitment

Inhibition of NO synthase in endothelial cells by L-NAME increased the intracellular oxidative stress, resulting in enhanced adhesion of neutrophils via CD18/ICAM-1 interaction[213] or the upregulation of P-selectin on the endothelium.[214] Neutrophil adhesion to the endothelium was augmented by hypercholesterolaemia[60,215] and this increase was prevented by the NO donor SPM 5185.[216] Inhibition of NO biosynthesis also induced the expression of VCAM-1[217] and upregulated MCP-1 mRNA and protein in cultured human endothelial cells, whereas addition of the NO donor SIN-1 dose-dependently decreased MCP-1 mRNA expression and secretion, presumably by suppressing a NF-κB-like transcriptional regulator.[218] Authentic NO gas inhibited monocyte adhesion and chemotaxis[219] and exposure to shear stress inhibited monocyte adhesion by an NO-dependent mechanism.[220] Furthermore, NO donors decreased the cytokine-induced expression of the endothelial adhesion molecules VCAM-1, ICAM-1 and E-selectin.[217]

Antiproliferative action of nitric oxide

As the proliferation of vascular smooth muscle cells, macrophages and T-lymphocytes contributes to the progression of intimal lesions, cell growth inhibition by NO could significantly reduce lesion formation. Nitric oxide has been shown by several investigators to inhibit smooth muscle cell proliferation[221–227] as well as migration[228,229] *in vitro*. Both effects were cyclic GMP-mediated. T-cell proliferation is also reduced by NO.[230–232]

Interestingly, the effect of NO appeared to be quite different in endothelial cells, in that it induced endothelial cell growth and motility *in vitro* and mediated the mitogenic effect of vascular endothelial growth factor.[233]

Antiplatelet effects of nitric oxide

Although the inhibitory effect of NO on platelet adhesion and aggregation[234,235] (reviewed by Bassenge[236]) is often considered an anti-atherogenic effect of NO, platelets are minimally involved during the early stages of the atherogenic process. However, the endothelial surface over advanced human plaques often shows focal loss of cells[15] and platelet adhesion may promote the progression of those lesions, eventually leading to plaque fissuring and thrombosis. Also following gross mechanical injury evoked by balloon angioplasty, platelet-derived products have been proposed to contribute to neointima formation.[4]

Inhibition of atherosclerosis by NO in vivo

Several *in vivo* studies (Table 2) support the concept that NO may suppress both atherosclerosis and intimal thickening. Oral L-arginine

Table 2. Anti-atherogenic properties of nitric oxide in vivo

	Reference
Modulation of leukocyte-endothelial interaction	
attenuation by L-arginine	238
attenuation by NO donors	240, 241
increase by NOS inhibitors	239
Modulation of fatty streak formation in cholesterol-fed rabbits	
reduction by L-arginine	123, 237
reduction by NO donor	242
increase by NOS inhibitors	243, 244
Modulation of intimal thickening evoked by different stimuli	
balloon denudation	
reduction by L-arginine	134, 250, 251
reduction by EDRF/NO	252–254
reduction by eNOS transfection	259
reduction by exogenous NO	255, 256, 258
increase by NOS inhibitors	134, 250, 251
balloon angioplasty	
reduction by L-arginine	134
collar	
reduction by NO donors	260
vein graft	
reduction by L-arginine	249

supplementation caused a striking inhibition of fatty streak formation in hypercholesterolaemic rabbits.[237] Since the adhesion of monocytes to endothelial cells is imperative to lesion formation in this model, the authors further addressed the effects of the NO precursor on the adhesion of a murine monocytic cell line to the aortic endothelium of cholesterol-fed rabbits *ex vivo*.[238] The enhanced endothelial adhesiveness for monocytes in hypercholesterolaemic aortas was significantly reduced if the rabbits had received supplemental dietary arginine and significantly increased in rabbits treated with L-NAME. This was associated with, respectively, increased or decreased elaboration of vascular nitrogen oxides as measured by chemiluminescence. The observation that hypercholesterolaemia-induced impairment in endothelium-dependent relaxation was only marginally improved by L-arginine treatment while lesion formation was significantly reduced,[123] suggests that the beneficial effect of L-arginine may primarily infer from the increased activity of iNOS. However, it should be noted again that arginine and other basic amino acids are potent hormonal secretagogues in adrenals and many other endocrine organs.[133] Hence, part of the anti-atherosclerotic effect of systemic arginine administration might be related to the release of glucocorticoids or other immunosuppressive hormones which are known to suppress intimal thickening and experimental atherosclerosis.

The ambiguity of results obtained with L-arginine is avoided in studies with NO donor compounds or NOS inhibitors. L-NMMA and L-NAME increased leukocyte adhesion *in vivo* by a CD11/CD18-dependent mechanism.[239] Conversely, the NO donor SIN-1 prevented leukocyte adhesion. The observation that both SOD and SIN-1 inhibited leukocyte adhesion only under conditions associated with superoxide formation suggests that the anti-adhesive properties of NO may relate to its ability to inactivate the superoxide anion.[240] Another NO donor attenuated leukocyte endothelial interaction in an *in vivo* model of ischaemia-reperfusion, and this appeared to be in part mediated through a decreased expression of endothelial P-selectin.[241] Pentaerythrityl tetranitrate, an organic nitrate, has been documented to inhibit cholesterol-induced fatty streak formation in rabbits, but the beneficial effect was not seen with isosorbide mononitrate, another organic nitrate.[242] This could be due to differences with respect to the development of tolerance or the NO releasing capacity between the two organic nitrates. Conversely, treatment with molsidomine, whose active metabolite is the spontaneous NO donor SIN-1, actually enhanced lesion formation in the hypercholesterolaemic rabbit.[125] This may relate to the generation of superoxide anion from SIN-1, which could abrogate the beneficial effects of the simultaneously released NO.

Moreover, oral or parenteral treatment with the NO synthase inhibitor L-NAME for 4 to 12 weeks[243,244] enhanced fatty streak formation significantly. Therefore, the data suggest that vascular NO, produced by eNOS or iNOS, inhibits *de novo* formation of intimal lesions. However, this conclusion is somewhat confounded by the observation that L-NAME augmented plasma cholesterol levels in these rabbits, particularly after prolonged treatment. Since hypercholesterolaemia is the ultimate driving force for the lesions in this model, it is conceivable that this contributed to the accelerated atherosclerosis.

The inhibitory effect of NO on atherosclerosis may result from the above described *in vitro* actions, but NO-mediated decrease of endothelin production[28,245] may also be involved. Endothelin is a potent mitogen[246] and inducer of collagen synthesis[247] in vascular smooth muscle cell cultures, and its production may be increased in atherosclerosis.[248]

Inhibition of intimal thickening by NO

The interferences with cholesterol absorption or metabolism are circumvented in studies of intimal thickening in animals with normal plasma cholesterol levels. Oral L-arginine supplementation suppressed intimal hyperplasia in experimental vein grafts[249] and after balloon denudation of the rat carotid artery.[250,251] The NOS inhibitor L-NAME reversed the effect of arginine,[250] indicating that the attenuation of the intimal hyperplasia was mediated by NO. The NO is presumably formed by iNOS,[185,186,188] since regrowth of the endothelial cells is virtually absent, and eNOS activity does not recover. Moreover, the endogenous biosynthesis of NO appears to modulate the process, since systemic[250] or local, perivascular[251] administration of L-NAME aggravated intimal thickening in response to balloon denudation. Increasing the flow in the injured carotid artery by ligating the contralateral artery significantly reduced intimal thickening, and this effect was in part mediated by endogenous NO.[252] Likewise, the protective effect of angiotensin converting enzyme (ACE) inhibitors, which also block kinin degradation, may be mediated in part by stimulation of the endogenous production of NO by bradykinin.[253,254]

Conversely, treatments with exogenous NO by oral administration of the cysteine-containing NO donor SPM-5185[255] or by chronic inhalation of NO[256] were effective in reducing the size of intimal lesions after injury of the rat carotid artery. Nitroglycerin treatment only decreased the initial medial smooth muscle cell proliferation without affecting the thickness of the neointima after 3 weeks.[257] This may be due to insufficient NO formation as a result of the well known development of tolerance associated with this class of nitrovasodilators.[101] A single local treatment of the denuded rabbit femoral artery with a protein adduct of NO inhibited platelet deposition and neointimal proliferation in the injured rabbit femoral artery.[258] *In vivo* eNOS gene transfer in the vessel wall after denudation of the rat carotid artery provided further evidence for the inhibition of smooth muscle cell accumulation by NO. Transfection of the eNOS gene in the media not only restored the calcium-dependent NO production and concomitant relaxations of the denuded artery, it also inhibited neointima formation at day 14 after balloon injury by 70%.[259] This experiment provides direct evidence that NO is an endogenous inhibitor of vascular lesion formation *in vivo*. Furthermore, these experiments suggest the possibility of eNOS transfection or local delivery of long-lived NO adducts as potential therapeutic approaches to treat neointimal hyperplasia.

The inhibition of intimal thickening by NO is not restricted to models characterized by endothelial denudation, but is also seen when intimal thickening is induced in rabbit arteries by the perivascular placement of a collar. Oral treatment with the NO-donor SPM-5185 reduced the collar-induced intimal thickening, whereas only a tendency towards inhibition was observed by treatment with molsidomine, whose active metabolite is the NO donor SIN-1.[260] It is not clear whether the difference between the two drugs was related to the dose, or different characteristics of the NO donors, i.e. the presence of sulphydryl groups in SPM-5185 or the release of superoxide anion from SIN-1.

Finally, there are indications that NO inhibits neointima formation induced by balloon angioplasty of lesion-free animal arteries. The vessel wall distension by the balloon creates a much more extensive injury of the media, predisposition to thrombus formation and accelerated intimal thickening. Although the endothelial cells regenerate quickly, vascular reactivity studies show that the eNOS pathway remains dysfunctional,[134,261] whereas iNOS is induced in non-endothelial vascular cells.[187] Oral L-arginine supplementation improved the endothelium-dependent vasorelaxation and suppressed the intimal hyperplasia after balloon angioplasty of rabbit iliac arteries.[134] In accordance with the finding in the collar model, treatment with the NO donor SIN-1 did not influence intimal thickening following porcine carotid angioplasty, although the compound was effective in inhibiting medial smooth muscle cell proliferation.[262]

Also in two other models of intimal thickening a clear relationship between inhibition of smooth muscle cell mitosis and neointima formation is lacking. Smooth muscle cell mitosis was influenced less than intimal thickening after eNOS gene transfer in denuded rat arteries[259] and after NO donor treatment of rabbit collared arteries.[260] This suggests that NO exerts its major effect on smooth muscle cell migration,[228,229] which is a crucial event in intimal thickening. Whether inhibition of migration is of importance to human atherosclerosis remains to be determined, as atherosclerosis develops in an existing intima[1,3] and migration of smooth muscle cells from media to intima is not considered a major determinant in atherogenesis.[4]

NO: A Radical Promoter of Atherosclerosis (Table 3)

LDL oxidation

Nitric oxide is a nitrogen-free radical and can initiate lipid peroxidation in LDL in the absence[204,263] or presence[201,264–266] of superoxide anion. In the former case, LDL modification appeared to be restricted to an increase in lipid hydroperoxide content[201] without further evolution to a high-uptake form recognized by the scavenger receptor.[264] Nitrite- and NO-oxidized LDL demonstrated the biological properties of minimally oxidized LDL.[263] More extensive LDL oxidation occurred if superoxide anion was present e.g. during the decomposition of SIN-1.[264] Superoxide combines with nitric oxide to form the stronger oxidants peroxynitrite and its decomposition product the hydroxyl radical.[267–269]

Table 3. Pro-atherogenic properties of NO

	Reference
Oxidation of LDL	201, 204, 263–267
Cytotoxic effects	20, 268, 272–274
Induction of apoptosis	276, 277, 286
Increased matrix breakdown	282–284

Cytotoxic effects

The concept that peroxynitrite formation occurs in atherosclerosis is strongly supported by the immunohistochemical demonstration of extensive nitration of protein tyrosines in advanced human lesions.[270] The presence of 3-nitro-L-tyrosine, quantified in the human brain by high-performance liquid chromatography, is also considered indicative of oxidative stress induced by reactive oxygen intermediates and nitric oxide.[271] Excessive NO synthesis and peroxynitrite formation have been implicated in cytotoxic effects in endothelial cells,[268,272] smooth muscle cells[273] and macrophages.[274] Cell damage results from the inhibition of mitochondrial respiration, aconitase activity and DNA synthesis, as well as from iron loss.[20] On the other hand, nitric oxide-induced p53 accumulation safeguarded against DNA damage through p53-mediated suppression of iNOS gene expression, thus reducing the potential for NO-induced DNA damage.[275] The release of basic fibroblast growth factor from damaged vascular smooth muscle cells may counteract the toxic effects on the endothelium by stimulating endothelial cell proliferation.[273] In view of these findings, the protective effects of antioxidants in several models of atherosclerosis may in part derive from the prevention of NO breakdown by oxygen radicals.

Induction of apoptosis

Nitric oxide has also been reported to cause apoptosis or programmed cell death in macrophages[276,277] and smooth muscle cells.[278] Apoptosis participates in the regulation of the cellularity of intimal lesions in balloon-injured arteries[279] and human atherosclerosis.[280] Theoretically, augmentation of apoptosis by NO could retard plaque growth, which may be considered beneficial. However, an imbalance between proliferation and apoptosis has been suggested to underlie the development of the cell-poor, necrotic core.[281] The size of the core determines the stability of the plaque. Stimulation of apoptotic cell death by NO or other molecules may thus increase the risk of plaque fissure and thromboembolic complications.

Matrix breakdown

Enhanced matrix breakdown by the activation of matrix metalloproteinases by NO[282,283] or the inactivation of the tissue inhibitor of metalloproteinase-1 by peroxynitrite[284] may contribute to the destabilization of the lesions and may promote the development of a necrotic core in advanced plaques.

In summary, it has been known for a decade that the loss of endothelial NO production impairs endothelium-dependent dilatation and promotes vasospasm in atherosclerotic arteries. More recent evidence indicates that dysfunction of the endothelial NO pathway may promote atherosclerosis in view of the described protective effects of NO against leukocyte adhesion, oxidative processes, smooth muscle cell migration and proliferation. On the other hand, there is ample evidence to consider NO as a molecular aggressor in chronic inflammatory processes like atherosclerosis.[285]

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