REVIEW ARTICLE

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Oxidative Stress and Cardiovascular Disease

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Contents

Abstract

The endothelium is one of the most important, and certainly the most extensive, organs involved in cardiovascular homeostasis. The endothelium-derived vasoactive factors inhibiting smooth muscular cells contraction and proliferation, and platelet function, include nitric oxide (NO), prostacyclin and endothelial-derived hyperpolarizing factor. However, endothelial cells can also produce vasoconstrictive, proaggregant, promitogen mediators, such as thromboxane A_2, prostaglandin H_2, endothelin 1, and angiotensin II. Therefore, any impairment of endothelial function may trigger the typical chain of events of atherogenesis, characterised by vasoconstriction, cellular proliferation and thrombosis. In this regard, the biological link between endothelial dysfunction and atherosclerosis is a reduced bioavailability of NO. However, the precise mechanisms by which the endothelial dysfunction occurs remain still unclear.

A decreased bioavailability of NO can be caused by its enhanced reactive oxygen species (ROS) breakdown. Oxidative stress may represent a common mechanism by which different cardiovascular risk factors cause endothelial dysfunction and trigger atherothrombotic process.

1. Reactive Oxygen Species

The aerobic organisms, deriving their energy from oxygen reduction, inevitably produce oxygen free radicals that have an unpaired electron, associated with the atom of oxygen, in their external orbital.[1] Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and oxidrilic radical (OH^-), and other unstable intermediates of lipid peroxidation, are known as reactive oxygen species (ROS). O_2^- is generated by transferring of one electron to oxygen. O_2^- formation triggers the generation of other ROS. O_2^- is indeed converted into $H_2O_2 + O_2$. This reaction is fast, and it occurs spontaneously or it is catalysed by the superoxide dismutase (SOD). Clearly, the O_2^- is more toxic than H_2O_2, so its rapid elimination is important. In turn, the H_2O_2 can be scavenged by several mechanisms: (i) two enzymes, catalase and glutathione (GSH) peroxidase catalyse the conversion from H_2O_2 to $H_2O + O_2$; and (ii) the H_2O_2 can be converted by a spontaneously reaction (Fenton's reaction) in presence of Fe^{2+} into oxidrilic radical (OH^-). This radical reacts immediately with any biological compound (lipids, proteins, nucleic acids, or carbohydrates), starting the classical chain reaction that leads to the production of other ROS. The unsaturated fatty acids are the main targets of the lipid peroxidation reaction.

The cell possesses some scavenging mechanisms to detoxify ROS. The different scavengers of ROS include SOD, catalase, and GSH peroxidase. Since the reaction catalysed by GSH peroxidase requires the presence of GSH as substrate, the ratio between oxidised and reduced glutathione (GSSG : GSH) reflects the redox state of the cells, and it is crucial for ROS scavenging.

In contrast, the concentration of metal ions, such as Fe^{+2}, contributes to the generation of ROS.[1] Their action is reduced by the presence of binding proteins, including ferritin, transferrin, and lactoferrin.

Any imbalance between ROS producing and scavenging systems is defined as 'oxidative stress'. Recent evidence suggests that ROS act as second messengers for the activation of several intracellular redox processes involved in some physiologic or pathophysiological cellular events (growth/hypertrophy, migration/replication, survival/apoptosis, modulation of the endothelial function, pro-inflammatory changes). As all these events are clearly involved in the pathogenesis of cardiovascular diseases, the putative sources of ROS and their intracellular signalling are investigated by many disciplines, ranging from vascular biology to clinical medicine.

2. Oxidative Stress and Endothelial Dysfunction

The endothelium is one of the most important, and certainly the most extensive, organs involved in cardiovascular homeostasis. The endothelium-derived vasoactive factors, which inhibit smooth muscular cells contraction and proliferation and platelet function, include nitric oxide (NO), prostacyclin, and endothelial-derived hyperpolarising factor. However, endothelial cells can also produce vasoconstrictive, proaggregant, promitogen mediators, such as thromboxane A_2 (TxA_2), prostaglandin H_2 (PGH_2), endothelin 1, and angiotensin II. Therefore, any impairment of endothelial function may trigger the typical chain of events of atherogenesis, characterised by vasoconstriction, cellular proliferation, and thrombosis.[2] In this regard, the biological link between endothelial dysfunction and atherosclerosis is a reduced bioavailability of NO. However, the precise mechanism by which the endothelial dysfunction occurs remains unclear. A decreased bioavailability of NO can be caused by its enhanced ROS breakdown. Then, endothelial function is much more than the simple control of vascular tone, and releasing of vasodilatating mediators clearly reflects only one aspect of its protective role. The increase generation of ROS, by reducing NO availability, changes the 'milieu' of the vessel wall, triggering lipid peroxidation, inflammatory processes, and thrombus generation.

In agreement with the oxidative theory of atherosclerosis, one of the initial steps of the atherosclerotic process is the oxidation of low-density lipoproteins (oxLDL). Their scavenger receptors, expressed on the membrane of the endothelial cells, are selectively activated by oxLDL. The oxLDL may inhibit NO biosynthesis through different mechanisms: impairment of G-protein receptors, reduced availability of intracellular L-arginine; inactivation of NO through enhanced generation of O_2^-.[2] In addition, oxLDLs up-regulate the expression of gene encoding for endothelin-1, and hence increase production of endothelin-1 via protein-kinase C.[2] It is important to highlight that the reduced bioavailability of NO favours platelet activation, resulting in an impairment of endothelial anti-aggregant and anticoagulant properties, with enhanced expression of adhesion molecules, cytokines, and growth hormones, that play a key role in monocyte recruitment, pro-inflammatory infiltration, and proliferative processes.

3. Oxidative Stress and Cardiovascular Risk Factors

Cardiovascular risk factors, including diabetes mellitus, hypertension, hypercholesterolaemia, cigarette smoking, and hyperomocysteinaemia, are associated with enhanced ROS generation and endothelial dysfunction.

The relationship between diabetes mellitus and premature cardiovascular disease is well established.[3,4] Atherosclerosis occurs earlier in diabetic patients than in patients without diabetes and is more severe and diffuse in the former group.[5] Diabetic microvascular disease contributes to common complications, such as retinopathy and nephropathy. Although the link between hyperglycaemia and cardiovascular disease is not understood, the loss of the modulatory role of endothelium may be implicated in the pathogenesis of diabetic vascular disease.

Several studies have shown the impairment of endothelium-dependent relaxations to various receptor-mediated vasodilators in different vascular beds of diabetic animals.[6,7] Abnormal endothelial cell function appears to be associated specifically with hyperglycaemia rather than with any other potential metabolic disturbance. Indeed substantive evidence linking hyperglycaemia and endothelial cell dysfunction comes from *in vitro* incubation studies in which the exposure of arteries to elevated glucose levels caused endothelial dysfunction similar to that observed in diabetic animals.[8,9]

Although the mechanism by which diabetes contributes to endothelial dysfunction is not fully understood, hyperglycaemia is clearly recognised as a primary culprit in the pathogenesis of diabetic complications. A substantial body of evidence implicates oxidative stress as an important pathogenetic element in hyperglycaemia-induced endothelial dysfunction. Indeed, in animals models of diabetes, endothelium-dependent relaxations can be restored by treatment with antioxidants.[10,11] Accordingly, several studies reported that the short-term administration of ascorbic acid (vitamin C) improves endothelium-dependent vasodilatation in patients with type 1 and 2 diabetes mellitus.[12,13]

Our recent study reports increased NO inactivation by O_2^- as the major mechanism for impairment of endothelium-dependent relaxation in arteries exposed to high glucose levels.[14] We demonstrated that in human aortic endothelial cells, prolonged exposure to high glucose levels paradoxically increases expression of endothelial NO synthase (*NOS*) gene and protein. However, the up-regulation of endothelial NOS was associated with marked concomitant increase of O_2^- production.[14]

Indeed, isoprostanes (8-epi-prostaglandin $F_{2\alpha}$), an endothelial marker of oxidative stress,[15] have been detected in patients with diabetes mellitus. Isoprostanes are derived from arachidonic acid metabolism through cyclo-oxygenase (COX)-independent reactions promoted by ROS. As they are released from membranes or circulating low-density lipoprotein (LDL), isoprostanes circulate in the peripheral blood flow, and their urinary excretion is a well-documented in humans.[16]

In the presence of hypertension the endothelium can exert a compensatory mechanism that reduces peripheral vascular resistances, or, viceversa, may favour their increase through the release of vasoconstrictive agents. Certainly, an altered endothelial function contributes to maintain hypertensive condition and promote hypertension-related complications. By using intra-arterial infusion of L-NMMA, it was demonstrated that basal NO production was reduced in the different vascular beds of patients with hypertension.[17-19] In addition, endothelium-dependent vascular reactivity, stimulated by several agonists, is also impaired in patients with essential hypertension. At the same time, NO plasma levels are reduced.[20] Indomethacin (a nonselective inhibitor of COX) improves altered vasodilating response to acetylcholine, indicating that vasoconstrictive prostanoids contribute to endothelial dysfunction in patients with hypertension.[21]

Moreover, besides endothelium-derived contraction factors, such as TxA_2 and PGH_2, oxygen free radicals can play an important role in endothelial dysfunction in hypertension. Indeed, the antioxidant ascorbic acid has been found to improve endothelial vasodilator function in the forearm and coronary circulation by an NO-dependent mechanism in patients with essential hypertension.[22]

In addition, enhanced isoprostanes plasma and urinary levels have been demonstrated in smokers,[23] and in the presence of hypercholesterolaemia. Patients with hyperomocystenaemia showing enhanced levels of 8-epi-prostaglandin $F_{2\alpha}$, present with a higher incidence of major cardiovascular events.[24]

In conclusion, NO degradation by O_2^- represents a common mechanism by which different cardiovascular risk factors cause endothelial dysfunction and trigger atherothrombotic process.

4. The Two Faces of Endothelial Nitric Oxide Synthase

NO is synthesised from L-arginine by NOS through a 5 electron oxidation.[25] The activity of this pathway is a balance between the synthesis and breakdown of NO. Under physiologic conditions, NO production is not significantly affected by O_2^-. Hence, endothelium-derived NO can exert its vascular protective effects promoting an antiatherosclerotic environment. By contrast, in the presence of proatherogenic cardiovascular risk factors, enhanced O_2^- production occurs, inducing NO inactivation and, hence, the increased formation of a very powerful oxidant peroxinitrite ($ONOO^-$).[26]

By products of $ONOO^-$ are some of the most dangerous reactive species in biology. Recent evidences suggest that high concentrations of $ONOO^-$ are associated with endothelial dysfunction, as a consequence of increased O_2^- generation from different sources, such as COX, xanthine oxidase (XO), NADPH oxidase, and mitochondria.[27-29] In this regard, a series of studies from our group demonstrated that the reduced bioavailability of tetraiodiobyopterin (BH_4) causes an impairment of endothelial NOS (eNOS) catalytic activity. A dysfunctional eNOS becomes a source of ROS, such as O_2^- and H_2O_2, in place of NO[30-33] (see figure 1).

Tetrahydrobiopterin is a cofactor of the aromatic amino acids mono-oxygenases, which are regarded as key enzymes in the biosynthesis of several neurotransmitters, including catecholamines and serotonin.[34] Although it is well established that inherent errors in the metabolism of tetrahydrobiopterin lead to cofactor deficiency, hyperphenylalaninaemia, and neurological impairment,[35] it has only recently been recognised that tetrahydrobiopterin has an important role in the cardiovascular sys-

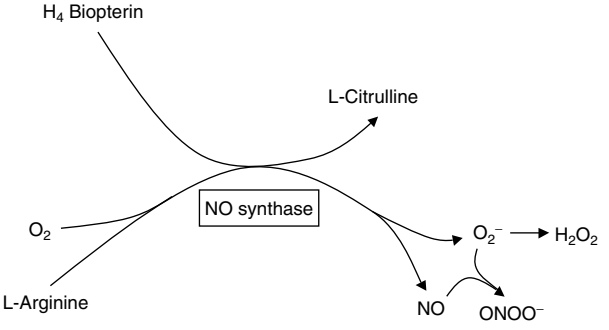

Fig. 1. NO synthase (NOS) activation in presence of not optimal concentrations of tetraiodiobyopterin (BH_4) provokes generation of superoxide anion (O_2^-), and then production of hydrogen peroxide (H_2O_2) and/or peroxinitrite ($ONOO^-$).

tem. The bioavailability of tetrahydrobiopterin is essential for catalytic activity of all NO synthases (figure 1).[25,26]

In order to understand the link between the availability of tetrahydrobiopterin and synthesis of NO, one has to take a closer look at NOS biochemistry. All NO synthases exhibit unique complexity and requirements for cofactors.[36-38] The NOS-catalysed conversion of L-arginine to L-citrulline and NO is a two-step redox process. L-arginine is first hydroxylated to the intermediate N^G-hydroxil-L-arginine, which undergoes oxidative cleavage to yield NO and L-citrulline.[39,40] Both steps require molecular oxygen, reduced nicotinamide-adenine dinucleotide phosphate (NADPH), and tetrahydrobiopterin.[36,39,40] The required reducing equivalents are derived from NADPH and are shuttled through the reduced flavins, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), to the haeme group.[41-43] All NOS consist of a flavin-containing reductase domain and a haeme-containing oxidase domain.[44] This built-in electron transport system is used to oxidise L-arginine to NO and L-citrulline.

The precise function of tetrahydrobiopterin in regulating the catalytic activity of NOS is still not completely understood.[45] However, tetrahydrobiopterin may play an important role in determining whether the electron flow in the enzyme can be directed to L-arginine. Several biochemical studies demonstrated that activation of purified constitutive NOS in the presence of suboptimal levels of tetrahydrobiopterin results in the uncoupling of oxygen reduction and arginine oxidation, thereby generating superoxide anions and subsequently hydrogen peroxide.[42,46,47] In agreement with these results, we have also shown that in isolated canine coronary arteries depleted of tetrahydrobiopterin, endothelial NOS may become a source of oxygen free radicals.[29]

In this regard, Pritchard et al.[48] found that the increased generation of superoxide anions in cultured endothelial cells exposed to LDL can be inhibited by L-NAME. These data further support hypothesis that NOS may be an important source of endothelial production of oxygen free radicals.

In isolated aortas from pre-hypertensive spontaneously hypertensive rats, tetrahydrobiopterin supplementation diminished the NOS-dependent generation of superoxide and its dismutase product hydrogen peroxide, while it increased the net production of NO.[49]

We demonstrated that the infusion of tetrahydrobiopterin into the brachial artery of patients with hypercholesterolaemia restores endothelial function by increasing the production of NO.[31] Therefore, the increased breakdown of NO could be explained by the decreased availability of tetrahydrobiopterin. During the decreased availability of its cofactor, endothelial NOS has been shown to produce, in addition to NO, an excessive amount of oxygen radicals.[29] Tetrahydrobiopterin supplementation may restore NO activity by decreasing the formation of oxygen radicals. The administration of tetrahydrobiopterin also improves abnormal endothelium-dependent coronary vasomotion in response to acetylcholine in patients with coronary artery disease.[32] Several other reports suggest that tetrahydrobiopterin may play a role in endothelial dysfunction during ischaemia reperfusion,[50] smoking,[51] and experimental diabetes.[52]

On the basis of these observations, we propose that NOS has a dual role in the pathogenesis of atherosclerosis: under physiological conditions, it generates low concentrations of NO, and probably peroxynitrite, which favours an antiatherosclerotic environment. However, during hyperlipidaemia and atherosclerosis, it may contribute to the formation of oxidative stress by a reduction in BH_4-dependent NO formation and unopposed superoxide formation by the enzyme. This concept further emphasises the role of redox state as a determinant of vascular integrity in atherosclerosis, and provides the background to investigate new therapeutic strategies to reduce the development and progression of cardiovascular disease.

5. Oxidative Stress as a Marker of Cardiovascular Events

Endothelial-dependent vasodilatation is impaired in patients with atherosclerosis or with cardiovascular risk factors. In experimental models of atherosclerosis, hypercholesterolaemia, hypertension, and diabetes mellitus, a significant correlation between oxidative stress and endothelial dysfunction was found. The reduced NO bioavailability, due to increased O_2^- formation, seems to be a common denominator of these conditions. Recent data have confirmed these modifications even in patients with risk factors or coronary disease, indicating a strong correlation between enhanced vascular O_2^- production and impaired vascular reactivity.

Several studies[53-55] demonstrated the predictive value of endothelial dysfunction on progression of atherosclerotic disease and cardiovascular events. The most recent of this study has further clarified the relationship among endothelial function, cardiovascular prognosis, and oxidative stress.[55] The authors evaluated forearm blood flow in response to acetylcholine in patients with coronary artery disease. These patients were then followed for a mean follow-up of 4.5 years. In addition, in a subgroup of these patients the testing of acetylcholine response was repeated during intra-arterial infusion of ascorbic acid. In agreement with previous studies, this study also found that the degree of increase in forearm blood flow in response to acetylcholine represented an excellent prognostic indicator for cardiovascular events. In-

terestingly, this study[55] shows for the first time that abnormal responses to acetylcholine could be improved by the administration of ascorbic acid only in the group of patients with cardiovascular events. In other terms, when the response to ascorbic acid is large, a large amount of oxidative stress must be present. In conclusion, elevated levels of oxidative stress predispose a poor cardiovascular prognosis.

A large number of experimental data support this hypothesis. ROS, as previously reported,[26] promote lipid peroxidation, stimulate smooth muscle cells proliferation, and trigger the expression of pro-inflammatory genes. In addition, ROS activate matrix metalloproteinases, promoting atherosclerotic plaque, instability, and rupture.

Hence, a therapeutic approach based on antioxidant agents would be beneficial in improving outcome in patients with atherosclerotic disease. Although several observational trials suggest that high doses of fruit and vegetables lower the cardiovascular risk profile, the results of the most recent large interventional trials are not promising. With the exception of the CHAOS study,[56] several trials with tocopherol (vitamin E), such as HOPE,[57] GISSI-Prevenzione,[58] and SECURE,[59] did not demonstrate any significant benefit in term of cardiovascular events. However, it is important to emphasise that treatment with tocopherol alone is perhaps not the best approach for reducing oxidative stress because: (i) the rate constant for reactions between tocopherol and O_2^- is several orders of magnitude less than the rate constant between NO and O_2^-. It is possible that oral administration of tocopherol, achieving low concentrations in peripheral tissues, has no effect on many important intracellular reactions; (ii) tocopherol is concentrated in lipid membrane or lipoproteins. Evidence indicates that the main oxidative reactions occur in the cytoplasm and intracellular space and would not be affected by lipid-soluble antioxidant; and (iii) after scavenging a radical tocopherol becomes a radical, which can enhance lipid peroxidation. For these reasons, it is normal to use cocktails of antioxidants, for example, tocopherol, ascorbic acid, and β-carotene together. However, even this approach was unsuccessful, as shown in the recent Heart Protection Study,[60] in which high-risk patients were randomised, not only to treatment with simvastatin versus placebo, but also to antioxidant vitamins versus placebo, in accordance with the 2×2 factorial design.

Another important issue is the extreme variability in the degree of oxidative stress. Several patients showed larger lipid peroxidation than others. Are these patients at higher cardiovascular risk level? Would this subset of individuals profit more from antioxidant treatment? These kind of questions should be answered before embarking on more clinical trials with antioxidant.

6. Conclusions

Given the above considerations and the contrasting results obtained so far, it is quite possible that the use of antioxidant vitamins will never prove to be the best approach to limit oxidative stress. In this regard, it is important to underline that two widely established pharmacological strategies to prevent the development of atherosclerosis and its complications have been shown to inhibit the production of ROS by vascular cells. Lipid lowering therapy decreases O_2^- production. Among the pleiotropic effects of statins there is the inhibiting effect of G protein, Rac-1,[61] which is a critical component of NADPH oxidase, a major vascular source of ROS. In addition, angiotensin II activates this enzyme. Indeed, in experimental models and in humans,[62] the pharmacological blockade of renin-angiotensin system at different levels is able to inhibit ROS production and improve endothelial function. Hence, the statins, ACE inhibitors, and angiotensin II receptor antagonists have well defined antioxidant effects not because they scavenge radicals, but because they block the production of radicals.

Future research should focus on identifying the markers of oxidative stress that could be helpful in the early identification of patients at high cardiovascular risk. At this time oxidative stress, inflammation, and reduced NO bioavailability, must be considered crucial target of a modern therapeutic approach against atherothrombotic disease.

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