

Review Article

The Role of Oxidative Stress in Atherosclerosis

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During the last decades several studies have examined the potential role of oxidative stress in atherogenesis.^{1,2} According to the theory of oxidative stress, atherosclerosis is the result of the oxidative modification of low density lipoproteins (LDL) in the arterial wall by reactive oxygen species (ROS). Evidence suggests that common risk factors for atherosclerosis increase the risk of the production of free ROS, not only from the endothelial cells, but also from the smooth muscle cells and the adventitial cells.³ Thus, hypercholesterolemia, diabetes mellitus (DM), arterial hypertension, smoking, age, and nitrate intolerance increase the production of free ROS. Importantly, several processes are triggered by those risk factors, including the expression of adhesion molecules, the proliferation and migration of smooth muscle cells, the apoptosis of endothelial cells, the oxidation of lipids, the activation of metalloproteinases and the alteration of vasomotor activity.^{4,5} This hypothesis raises questions about the understanding of the pathways that induce the oxidative process, as well as the molecular events in vasculature.⁶

Pathophysiologic mechanisms of oxidative stress (Figure 1)

Endothelial function is impaired in the earlier stages of atherogenesis and is strongly correlated with several risk factors. Endothelial dysfunction predisposes

to long-term atherosclerotic lesions and has been proposed as an important diagnostic and prognostic factor for coronary syndromes.⁷ The production of free oxidative radicals is believed to induce endothelial dysfunction, an initial step of atherogenesis. Oxidative stress leads to oxidation of LDL (ox-LDL), whose uptake by macrophages is easier compared to non-oxidized lipoproteins. It has been proven that the main sources of oxidative substances and ROS in atherosclerotic vessels are macrophages and smooth muscle cells.⁸ Indeed, hypercholesterolemia stimulates the production of superoxide anion radicals (O_2^-) from the smooth muscle cells of vessels, an event that leads to increased oxidation of LDL. Furthermore, the reduction of endothelial-produced NO and O_2^- is able to blunt normal endothelial dysfunction as a result of the decreased endothelial NO production. The increased production of ROS reduces the production and consequently the bioavailability of NO, leading to vasoconstriction, platelet aggregation and adhesion of neutrophils to the endothelium.⁹ In fact, oxidative stress by hydrogen peroxide (H_2O_2) increases phosphorylation of tyrosin kinases, which leads to stronger binding of neutrophil cells on endothelium and alteration of vessel permeability.¹⁰ Another mechanism through which oxidative stress (by H_2O_2) affects atherogenesis is the production of transcription factors such as nuclear factor κ B (NF- κ B) and activator protein 1 (AP-1), which participate in the expression of adhesion molecules,

such as vascular cellular adhesion molecules (VCAM-1), intracellular adhesion molecules (ICAM-1), E-selectin and other cytokines. It is well established that NF- κ B acts in smooth muscle cells of atherosclerotic vessels and is inactivated by antioxidants and anti-inflammatory agents such as salicylics and glucocorticoids.¹¹ Thus, it seems that atherosclerosis is an inflammatory process strongly affected by oxidative stress.

Interaction of NO and ROS in the vessel wall

The ROS group includes superoxide anion (O_2^-), hydroxyl radical (OH^\cdot) and peroxyntirite (NOO^\cdot). Although hydrogen peroxide (H_2O_2) and hypochlorous acid ($HOCl$) are not free radicals, they also have oxidative properties, especially in the presence of metal anions.¹² Hydrogen peroxide (H_2O_2), which is more stable, plays a principal role and can be diffused easily and converted into hydroxyl radicals of high efficacy in the presence of metal ions (e.g. Fe^{2+}). The interaction of O_2^- with NO leads to the production of peroxyntirite, a substance less effective for the activation of guanilyl cyclase. Therefore, NO bioavailability becomes remarkably reduced. In stages of advanced atherosclerosis, despite the fact that NO production remains the same, decomposition of NO from ROS is increased (Table 1).¹³

Table 1. Examples of free radicals in biological systems.

| Name | Formula |
|-----------------------------------|-------------|
| Superoxide anions | O_2^- |
| Alkoxy and peroxy radical | RO, RO_2 |
| Hydroxyl radical | $^\cdot OH$ |
| Nitric oxide and nitrogen dioxide | NO, NO_2 |

The role of enzyme systems in the oxidative process

The importance of ROS in vascular diseases is clear, and there has therefore been particular interest in the enzyme sources that contribute to the production of free radicals in the vessel wall. As shown in Figure 2, several enzyme systems, which use various substrates as sources of electrons, seem to be important in this process. A subsequent reduction of molecular oxygen (O_2) occurs in favor of a variety of ROS. Specifically one-electron reduction of molecular oxygen leads to the formation of superoxide, while two-electron reduction leads to hydrogen peroxide.

Nicotinamide-adenine dinucleotide phosphate-oxidase

NAD(P)H oxidase has been proved to be an important source of free oxygen species in vascular cells. It consists of five subunits: $p40^{PHOX}$ (PHOX= phagocyte oxidase), $p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$ and Nox.¹⁴ It is reg-

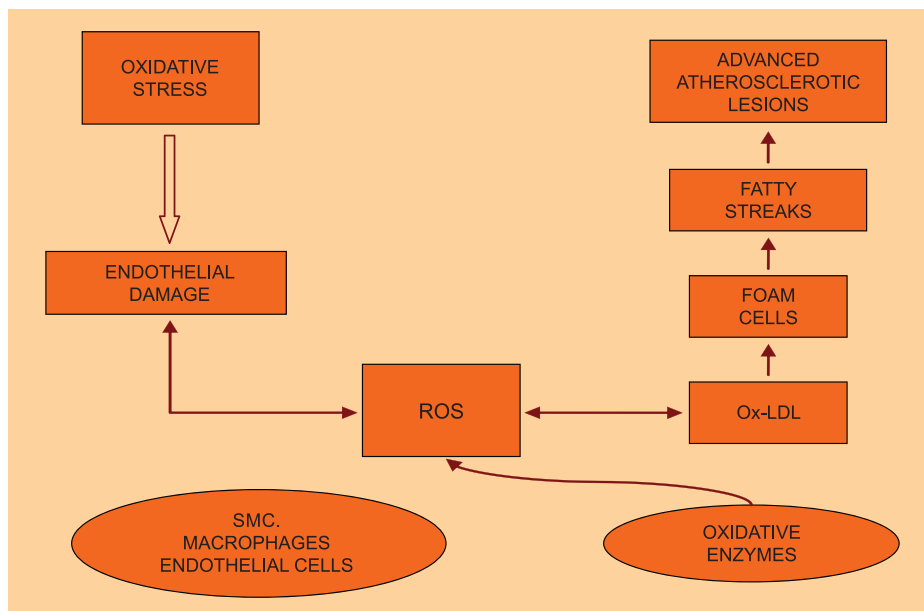


Figure Interaction of oxidative stress and atheromatosis. The main sources of oxidative substances and reactive oxygen species in atherosclerotic vessels are macrophages and smooth muscle cells (SMC). Reactive oxygen species (ROS) production, in turn, induces endothelial dysfunction. In addition, oxidative stress leads to oxidative modification of LDL (ox-LDL) and advanced atherosclerotic lesions.

ulated by a variety of pathophysiological stimulations relevant to atherosclerosis, such as angiotensin II, thrombin, platelet-derived growth factor, tumor necrosis factor- α , and natural forces such as wall stress. Activation of the type I receptor of angiotensin II leads to the stimulation of protein kinase C, provoking the increase of ROS from NAD(P)H, with a subsequent increase in blood pressure and disturbance of vasodilatation.¹⁵

Xanthine oxidase

This enzyme exists in plasma and endothelial cells but not in smooth muscle cells. (Xanthine dehydrogenase also exists, but superoxide radicals can be produced only by the oxidation of xanthine in uric acid by xanthine oxidase. Xanthine dehydrogenase is transformed quickly into oxidase through proteolysis.) In experimental animals with hypercholesterolemia it is capable of producing increased amounts of active radicals leading directly to reduced NO activity.¹⁶ It has been observed that in vessels of hypercholesterolemic patients, vasodilation is improved by the presence of allopurinol or oxypurinol, an inhibitor of the enzyme. Additional facts that support the role of xanthine oxidase in the process of atherogenesis are the following: 1) in patients with coronary syndrome the levels of this enzyme were found to be increased—the same applies to NAD(P)H; and 2) in young asymptomatic patients with familial hypercholesterolemia the increased activity of the enzyme is an early event.¹⁷

Nitric oxide synthase

Under normal conditions, binding to tetrahydrobiopterin (BH₄), which acts as cofactor and is used as a substrate for L-arginine, leads to NO formation.¹⁸ However, under certain circumstances, the uncoupling of this enzyme can lead to O₂ reduction and the production of ROS, with consequent production of peroxynitrite and oxidation of lipids and proteins. A series of pathological states is associated with the uncoupling of NO, such as hypercholesterolemia, atherosclerosis, DM and arterial hypertension. Under certain conditions, this event could be the result of reduced levels of BH₄.¹⁹⁻²¹ Production of NO in endothelial cells needs a certain amount of BH₄ and its administration restores the impaired, endothelium-dependent, vasodilation. In addition, L-arginine is also important for NO production and right endothelial function.²² In patients with hypercholesterolemia or

advanced atherosclerosis, L-arginine administration improves NO production and vasodilation. Finally, levels of asymmetric dimethylarginine (ADMA)—a substance which leads to the uncoupling of NO synthase^{23,24}—are elevated in various conditions, such as arterial hypertension, and are in a way related to endothelial dysfunction. Administration of L-arginine in patients with elevated levels of ADMA improves endothelial function, suggesting that L-arginine deficiency stimulates nitric oxide synthase to ROS production.

Myeloperoxidase

This is produced by activated phagocytes and uses H₂O₂ for the production of more powerful oxidative substances. This enzyme, through NAD(P)H, leads to the production of HOCl and its analogs (substances related to endothelial injuries due to the action of H₂O₂).²⁵ It is considered to participate in the process of atheromatosis by the induction of oxidative modifications in low and high density lipoproteins.²⁶ This hypothesis is consistent with the results of clinical trials, according to which the levels of this enzyme and its products are elevated in patients with coronary syndrome. In contrast to human lesions, these oxidative products are absent in experimental animals with apolipoprotein E and LDL-receptor deficiency. The three mechanisms through which myeloperoxidase participates in oxidative modifications are NO consumption, LDL oxidation, and reaction with L-arginine for the production of NO synthase inhibitors. All of these are dependent on H₂O₂. Immunohistochemical studies have proved the presence of myeloperoxidase and HOCl in atherosclerotic lesions.²⁷ Therefore, both these substances participate in the modification of LDL and in atherogenesis.

Lipoxygenases

These are enzymes that catalyze the intake of O₂ reaction from the polyunsaturated lipid acids, creating a family of biologically active lipids, such as prostaglandins, thromboxanes and leukotrienes, which participate in inflammatory reactions and increase the permeability of vessels. In experimental models, 15-lipoxygenase induces LDL oxidation by enzymatic and non-enzymatic reactions;²⁸ 15-lipoxygenase and 5-lipoxygenase are expressed in atherosclerotic lesions in humans and animals with apolipoprotein E deficiency. Experimental animals with an absence of the 15-lipoxygenase

gene or reduced expression of 5-lipoxygenase are protected from lesions like those found in animals with apolipoprotein E and LDL-receptor deficiency. Clinical data demonstrate that various genotypes of 5-lipoxygenase promoter are found in patients with atherosclerotic lesions or inflammation. Whether lipoxygenases participate in atherogenesis through lipid oxidation or defensive modifications is under investigation. The most important enzymes and the reactive species which are produced are summarized in Table 2.

Therapeutic approaches

The central role of oxidative stress in the atherosclerotic process has been studied in numerous epidemiological and experimental studies. Although a wealth of evidence exists to support the correlation between increased oxidative stress and various vascular diseases, the findings from antioxidant administration for the prevention of cardiovascular diseases are controversial.^{29,30} The initial hypothesis for the use of antioxidants is that since they interfere in the LDL oxidation they should reduce the level of atherosclerotic lesions at the clinical level. This hypothesis led to clinical trials for the examination of their action in cardiovascular diseases.^{31,32}

It is already known that there are numerous cardiovascular agents that have antioxidant properties, such as carvedilol (a non specific b-blocker that also acts as an a-blocker), nebivolol, which reduces oxidative stress in hypertensive patients and increases NO production, Ca⁺⁺ channel blockers, and aspirin, whose action is re-

lated to the improvement of endothelial function.³³ Furthermore, inhibition of the renin-angiotensin system by angiotensin converting enzyme inhibitors (ramipril-SECURE study) or angiotensin II receptor antagonists (losartan-LIFE study) has been proved to reduce the activity of NAD(P)H oxidase, improve endothelial dysfunction and lead to reduction of cardiovascular events in experimental models with arterial hypertension and hypercholesterolemia, as well as in high risk patients.^{34,35} According to experimental data, statins, through the increase of catalase and BH₄ levels, lead to an increase of NO production and inhibit LDL oxidation, while at the same time restoring vitamin C and E levels and endogenous antioxidants such as ubiquinone and glutathione. Vitamins C and E are also antioxidants that can inhibit the oxidative process for the prevention of atherosclerotic lesions. It has been proved in experimental models with atheromatosis that vitamin C stimulates the increase of BH₄ levels and the activity of NO synthase and improves endothelial dysfunction.³⁶ In addition, according to clinical studies, vitamin C administration in patients with coronary syndromes, arterial hypertension and hypercholesterolemia increases NO bioavailability.³⁷⁻³⁹ Similar data arise from vitamin E administration, which reduces LDL oxidation and improves NO bioactivity and endothelial dysfunction owing to malnutrition. Co-administration of vitamin C and E seems to improve endothelial function in hyperlipidemic patients.⁴⁰ Laboratory studies have shown that antioxidant vitamins can restore a deficiency of antioxidant enzymes, which are reduced in various cardiovascular diseases.⁴¹ Glu-

Table 2. Important antioxidants and their action.

| Antioxidants | Way of action |
|---|---|
| Vitamin C | Increased NO production by elevated levels of BH ₄ and NO synthase activity |
| Vitamin E | Reduced LDL oxidation Reduced MCP-1 production Improved NO activity |
| Flavonoid components | Reduced LDL oxidation Vasodilation of coronary arteries |
| ACE-inhibitors | Vasodilation of coronary and peripheral arteries through increased levels of plasma bradykinin |
| Angiotensin II type 1-receptor inhibitors | Improved endothelial function through increased activity of OH dismutase and inhibition of NAD(P)H oxidase |
| Lipid lowering: Statins | Increased NO bioavailability and expression of NO synthase Reduced production of ROS by angiotensin II in smooth muscle cells by the inhibition of Rac-1 – key molecule of NAD(P)H oxidase |

ACE – angiotensin converting enzyme; BH₄ – tetrahydrobiopterin; LDL – low density lipoprotein; MCP-1 – monocyte chemoattractant protein-1; NAD(P)H – nicotinamide-adenine dinucleotide phosphate-oxidase; NO – nitric oxide.

tathione superoxidase is significantly reduced in patients with coronary syndromes. Therefore, as the levels of this enzyme are negatively related to the risk of coronary syndromes, this could be used as a new marker of oxidative stress.⁴² In patients with coronary syndromes a reduction of superoxide dismutase has also been observed, an event that contributes to endothelial dysfunction. In addition, there are experimental studies showing that overexpression of this enzyme reduces LDL oxidation and apoptosis. Studies in human cells have proved that vitamin C can replace absent glutathione, while the combination of C and E increases paraoxonase activity, which is reduced in cardiovascular diseases. Natural antioxidants such as polyphenols, which are found in fruits and vegetables, seem to be extremely useful, can improve lipid metabolism and reduce ox-LDL.^{43,44} Their combination seems to be better than monotherapy. The most important antioxidants and their way of action are shown in Table 2.

Disadvantages of therapeutic approaches to oxidative stress

The disappointing results of antioxidant strategies, in both the prevention and limitation of atherogenesis and cardiovascular complications in humans, raise questions about the role of oxidative modification of LDL in atheromatosis. Out of the twelve studies that used antioxidant vitamins in varying concentrations, only five showed a benefit with regard to primary endpoints.⁴⁵ In the CHAOS study (Cambridge Heart AntiOxidant Study), the administration of a-tocopherol (vitamin E)

at a dose of either 400 or 800 IU/d caused a remarkable reduction in the combined primary endpoint of cardiovascular death and nonfatal myocardial infarction. In the SPACE study (Secondary Prevention with Antioxidants of Cardiovascular disease in End-stage renal disease), administering 800 IU/d a-tocopherol to hemodialysis patients with pre-existing cardiovascular disease significantly reduced the incidence of fatal and nonfatal myocardial infarction, ischemic stroke, peripheral vascular disease and unstable angina. In the ASAP study (Antioxidant Supplementation in Atherosclerosis Prevention) a combination of a-tocopherol (272 IU/d) and ascorbic acid (500 mg/d) decreased carotid intima-media thickness in hypercholesterolemic males.^{1,46,47} In contrast to these positive outcomes, the remaining antioxidant supplementation studies did not show any positive effect on primary endpoints related to cardiovascular events.⁴⁸ Among them was the CARET study, in which b-carotene (30 mg/d) was administered and was terminated early because of the increase in fatality due to cardiovascular events,⁴⁹ as well as the HPS⁵⁰ (Heart Protection Study) and HATS.⁵¹ The studies are summarized in Table 3. Numerous reasons could explain the failure of antioxidants in the prevention and treatment of cardiovascular diseases. There are points that need further examination, such as:

1. the fact that random generation of free radicals cannot explain the progression rate of atherosclerosis.
2. the level of oxidative modification of LDL.
3. in what way the oxidative process contributes to the creation of atherosclerotic plaque and why atherosclerotic lesions occur at specific sites on vessel wall.

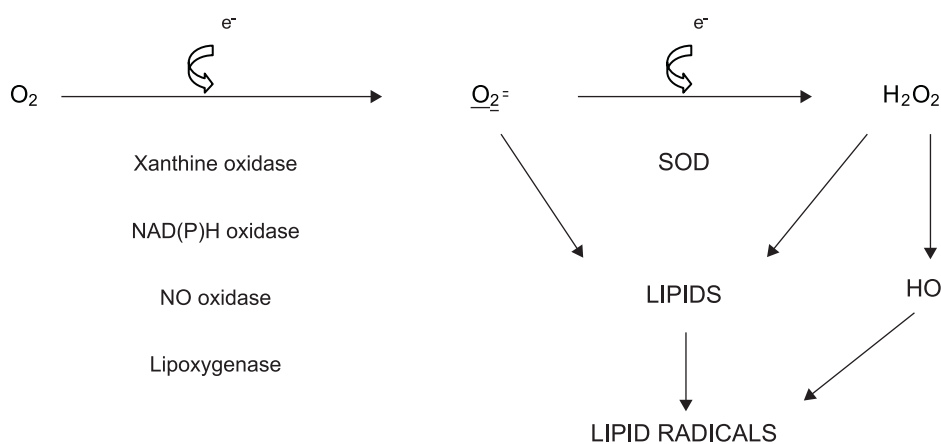


Figure 2. Reactive oxygen species formation. Several enzyme systems use various substrates as sources of electrons. A subsequent reduction of O_2 occurs in favor of a variety of reactive oxygen species. Specifically, a single-electron reduction of molecular oxygen leads to the formation of superoxide (SOD), while a two-electron reduction leads to the formation of hydrogen peroxide. In addition, reactive oxygen species react with lipid acids, creating a family of biologically active lipid radicals.

Table 3. Important trials involving antioxidant administration.

| Trials | Study type | Therapeutic intervention | Outcome |
|--------|---|--|--|
| CHAOS | 2002 patients with angiographically proven coronary artery disease, follow up for 1.5 years (UK). | vitamin E 800 mg/d or 400 mg/d | Significant reduction in cardiovascular events and non-fatal myocardial infarction. No significant reduction in mortality rate from cardiovascular events. |
| ASAP | 520 smoking and non-smoking males and postmenopausal female patients, age 45–69 with hypercholesterolemia. The extent of atherosclerosis in the common carotid arteries was assessed ultrasonographically. Follow up 6 years. | a-tocopherol 91 mg/d and vitamin C 250 mg/d | Significant reduction in the slope of the mean intima-media thickness in the common carotid arteries. |
| CARET | 4060 males, 45-74 years old, asbestos workers, and 14,254 men and women, heavy smokers (>20 pack-years) follow up for 5.5 years (US). | b-carotene 30 mg/d, +/- retinale (vitamin A ₁) 25,000 IU/d. | The trial was terminated early due to an increase in all-cause mortality in the supplement combination. |
| HPS | 20,536 high risk individuals, follow up 5 years. | vitamin E 600 IU/d, vitamin C 250 mg/d, b-carotene 20 mg/d | The combined antioxidant strategy failed to reduce the risk and mortality rate for cardiovascular events during the follow up. |
| HATS | 160 patients with clinical coronary syndrome, follow up for 3 years. | simvastatin, niacin and/or combination of vitamin E (800 IU/d), C (1 g/d), b-carotene (25 mg/d), selenium (100 mg/d) | The use of antioxidants did not reduce the relevant risk of new cardiovascular events. |
| ATBC | 27,271 male smokers, 50-69 years old, free personal history. | b-carotene 20 mg/d, vitamin E 50 mg/d | Vitamin E: important reduction in fatal myocardial infarction. B-carotene: no significant effect on fatal myocardial infarction. |

- which antioxidants best inhibit endothelial dysfunction, proliferation of smooth muscle cells, hypertrophy and lipid oxidation.
- which patient groups respond better to antioxidant treatment.
- what are the required doses of antioxidants and in what way may the side-effects, such as DNA damage after the high dose administration of vitamin C, be confronted.
- finally, it is remarkable that, at least experimentally, the formation of ox-LDL requires the total reduction of vitamin E, while as we know, neither vitamin C nor E are so much reduced, even in advanced atherosclerotic lesions.^{52,53}

According to the above observations, it becomes obvious that, at least in experimental models, the process of atherosclerosis should be separated from oxidative modifications, since the inhibition of LDL oxidation is neither necessary nor sufficient to limit atherosclerotic lesions. This conclusion comes in accordance with studies that have shown a minor effect of antioxi-

dant vitamins C and E on atherosclerotic lesions. The fact that more markers of oxidative stress, inflammation and antioxidant status could be studied does not seem to be sufficient to determine the risk of cardiovascular events and to identify the patient group that will benefit from an antioxidant strategy.⁵⁴⁻⁵⁷

Conclusions

There is a large body of evidence connecting the effects of oxidative stress with atherogenesis. However, so far we have not determined a specific causative relation between oxidative events in general and oxidative modification of LDL in particular with respect to atherosclerosis. The existing clinical studies of the role of antioxidants according to specific endpoints are quite disappointing. It is therefore important, with the contribution of molecular cardiology and pharmacogenetics, to elucidate the molecules as well as the inhibiting mechanisms that interfere in the oxidative process.

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