

## THE INTERACTION BETWEEN OXIDATIVE STRESS AND BIOMARKERS OF INFLAMMATION IN ATHEROSCLEROSIS

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**Summary:** A number of data demonstrated that there is a close relation between inflammation, oxidative stress and atherosclerosis. The initial event in atherogenesis is some form of endothelial dysfunction or activation. It can be triggered by mechanical, chemical, infectious or immunological insults, indicating that almost all risk factors for atherosclerosis could promote endothelial dysfunction. This triggers a cascade of inflammatory reactions, in which monocytes, macrophages, T lymphocytes and smooth muscle cells participate. These cells and the endothelium produce adhesion molecules, cytokines, growth factors and metalloproteinases, thus prolonging atherogenesis. An activation of oxidative producing enzymes such as NADPH oxidases and nitric oxide synthase (NOS) leads to oxidative stress and subsequent oxidative modification of LDL, and oxLDL can in turn activate the endothelial cells. Oxidatively modified LDL is uptaken by macrophages via scavenger receptors. This results in the accumulation of cholesterol within the macrophages and the formation of foam cells, a hallmark of atherosclerosis. The process continues with repeated cycles leading to the characteristic advanced lesions with the core of lipids and necrotic tissue which is covered by a fibrous cap.

**Key words:** atherosclerosis, inflammation, oxidative stress

### Introduction

Atherogenesis is a complex process currently considered as a chronic inflammatory response of the vascular wall to increasing cellular oxidative stress. The inflammatory characteristics of atherosclerotic lesions were observed 200 years ago. However, during the 20<sup>th</sup> century, the lipid theory dominated the field of atherogenesis. In 1976, R. Ross (1) turned our attention back to the inflammatory nature of atherosclerosis with his first significant review on atherosclerotic plaque formation. Almost all traditional risk factors for atherosclerosis are associated with and participate in the inflammatory process. Central to the initiation of atherogenesis is some form of localized endothelial dysfunction (2). Endothelial dysfunction or activation can occur in response to a variety of stimuli, such as oxidized LDL, free radicals caused by smoking, hypertension, diabetes, genetic alterations, elevated plasma homocysteine concentrations, infectious microorganisms, as well as antibodies.

### Inflammation in atherosclerosis

Healthy or quiescent endothelium serves as an important autocrine and paracrine organ with regulatory and control functions capable of maintaining vascular homeostasis. It mediates vasodilation, actively inhibits leukocyte adhesion and migration, platelet adhesion and aggregation, vascular smooth muscle proliferation and migration. Also, it inhibits coagulation, promotes fibrinolysis and participates in immune and inflammatory reactions (3). Quiescent endothelium does not normally bind white blood cells. But, soon after exposure to atherogenic stimuli, endothelial cells (ECs) are activated and initially acquire a proinflammatory and procoagulant phenotype. The dramatic changes in endothelial functional properties are mediated by the induced expression of several genes by nuclear factor-kappa B (NF- $\kappa$ B), commonly a proinflammatory transcription factor. Through the activation of NF- $\kappa$ B, ECs begin to express on their surface adhesion molecules (selectins, intercellular adhesion molecules – ICAM<sub>s</sub>, vascular cell adhesion molecules – VCAM<sub>s</sub>), that act as receptors for integrines present on monocytes and T-cells. VCAM-1 binds monocytes, T lymphocytes and the types of leukocytes found in early human

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and experimental atheroma (4). It seems that the expression of VCAM-1 directly depends on the nitric oxide (NO) production (5). So, the reduction of local NO production can block the expression of VCAM-1 (5), while the abnormal shear stress can increase the production of ICAM-1 (6), as well as the production of proteoglycans in smooth muscle cells, that can bind and retain lipoprotein particles which are, after their oxidation, capable of promoting an inflammatory response at the sites of plaque formation (7).

Apart from the adhesion molecules, ECs express chemotactic cytokines, such as monocyte chemoattractant protein-1 (MCP) responsible for the migration of monocyte into the intima, whilst CXL chemokines (8) help lymphocytes to penetrate the arterial wall. Under the influence of the macrophage-colony stimulating factor (M-CSF), monocytes are transformed into macrophages and begin to express scavenger receptors for modified lipoproteins on their surface (9). ACE inhibitors, aspirin, antioxidants, and  $H_2O_2$  scavengers exhibit opposite effects. This suggests that  $H_2O_2$  may function as a second messenger in macrophage NF- $\kappa$ B activation (10). During the ingestion of lipids, macrophage foam cells are formed. These cells together with T-cells located under a monolayer of endothelial cells represent the first lesion of atherosclerosis, the so-called fatty-streak. In this location, T-cells are activated and, together with native vascular wall cells, secrete cytokines, fibrogenic mediators and growth factors that can induce the migration and proliferation of smooth muscle cells (SMCs) which can, due to the degradation of arterial extracellular matrix by specialized enzymes expressed in medial SMCs, penetrate the subintimal area (11). They also secrete factors that further stimulate the recruitment of monocytes (12).

Inflammatory cytokines and C-reactive protein (CRP) induce the expression of cellular adhesion molecules, which mediate the adhesion of leukocytes to the endothelium. The process continues with repeated cycles leading from the first lesion of atherosclerosis through the fatty streak and intermediate lesions to the advanced lesions with the characteristic core of lipids and necrotic tissue which is covered by a fibrous cap. The necrotic core is formed from the apoptosis and necrosis of macrophages which empty ingested lipids inside the plaque.

### Oxidative stress and atherosclerosis

It seems that oxidative stress-induced endothelial dysfunction represents one of the first stages in the development of atherosclerotic lesions (13–15). It depends, at least partly, on the production of reactive oxygen species (ROS) and the subsequent decrease in vascular bioavailability of nitric oxide (NO). There is evidence that the atherosclerotic vessel wall contains increased levels of ROS, including hydroxyl radicals (HO $\cdot$ ), superoxide anions (O $_2^{\cdot-}$ ), hydrogen peroxide (H $_2$ O $_2$ ), and lipid peroxides (LOOH $\cdot$ ) (16, 17). ROS

producing enzymes involved in increased oxidative stress within the vascular tissue include NADPH oxidases, xanthine oxidase, lipoxygenase, cyclooxygenase, cytochrome p450-type enzymes, endothelial nitric oxide synthase (eNOS) uncoupling mechanisms, and mitochondrial superoxide-producing enzymes. eNOS uncoupling occurs in the absence of substrate L-arginine or co-factors BH $_4$ , in the presence of endogenous inhibitor asymmetric dimethylarginine or oxidative stress (18). Activation of vascular NADPH oxidases is common in cardiovascular diseases and has a predominant role in generating ROS in different vascular cells, including endothelial cells, SMCs and fibroblasts (19). A new family of NADPH oxidase subunits, known as non-phagocytic NADPH oxidase (NOX) proteins, have been identified. In endothelial cells, gp 91 phox (also called Nox2) has an essential role in ROS production. Mitochondrial DNA damage resulting from ROS production in vascular tissues may, in turn, also be an early event in the initiation of atherosclerotic lesions (20). However, atherogenic stimuli are associated with a prelesional increase and subsequent decrease in the expression of antioxidant enzymes such as catalase-1, superoxide dismutase, glutathione peroxidase and glutathione S-transferase (21). By affecting several redox-sensitive pathways in vascular cells, ROS induces expression of adhesion molecules and chemotactic factors by the endothelium followed by local infiltration of circulating immune cells and migration and proliferation of vascular SMCs (22, 23). ROS-produced following angiotensin II-mediated stimulation of NADPH oxidases can activate signal transducing processes leading to different events, including inflammation (19). Exposure of endothelial cells to  $H_2O_2$  increases the expression of ICAM-1 (24), while endogenously produced  $H_2O_2$  upregulates ICAM-1 and MCP1.  $H_2O_2$  is also required for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) induction of ICAM-1 and VCAM-1, upregulation of MCP-1 by TNF- $\alpha$  or hyperglycemia, as well as for the expression of platelet activating factor (PAF) and P selectin, all of which mediate neutrophil adhesion to endothelium, platelet activation and endothelium-platelet interaction (25). Selectively overproducing or removing  $H_2O_2$  significantly altered atherogenesis in animal models. Mice overexpressing p22phox (a small subunit of cytochrome b $_{558}$ ) had markedly increased atheroma formation in the carotid ligation model. This response was associated with elevated  $H_2O_2$  production in the vessel wall and was abolished by  $H_2O_2$  scavengers, implicating a critical role of  $H_2O_2$  (25). In addition, overexpressing CuZnSOD had no effect on atherosclerotic lesion formation, whilst overexpression of catalase or co-overexpression of catalase and CuZnSOD markedly retarded atherosclerosis in all aspects (26). These data suggest that  $H_2O_2$  is more atherogenic than superoxide. On the other hand, superoxide could decrease endothelial NO bioavailability. Loss of endothelial NO is associated with several cardiovascular diseases, including atherosclerosis, because NO plays a crucial role in regulating vasorelaxation, inhibition of leukocyte-endothelial adhesion, vascular

smooth muscle cell migration and proliferation, as well as platelet aggregation. Thus, defects of endothelial NO function, i.e. the dysfunctional eNOS/NO pathway is considered as an early marker for various cardiovascular disorders because it is associated with all major cardiovascular risk factors, and also has a profound predictive value for the future atherosclerotic disease progression (27).  $H_2O_2$  increases the expression of endothelial NO synthase (eNOS) through transcriptional and post-transcriptional mechanisms (28). Although eNOS was originally thought to be a constitutively expressed enzyme, currently it has become clear that its expression can be modulated by a variety of chemical, physical, and developmental stimuli (29), including oxLDL (30). In addition, superoxide produced by NADPH oxidase may react with NO released by eNOS, leading to the generation of peroxynitrite. Peroxynitrite, in turn, has been shown to uncouple eNOS, thereby switching an antiatherosclerotic NO-producing enzyme to an enzyme that may initiate or accelerate the atherosclerotic process by producing superoxide (31).

### Contribution of oxLDL to atherogenesis

Although there are data showing that low-density lipoprotein (LDL) could transmigrate across the intact endothelium into the subendothelial space, this process is more pronounced if LDL is oxidatively modified (oxLDL) (32). LDL has been demonstrated to undergo modification by endothelial cells (33), smooth muscle cells (34) and monocyte-macrophages (35).

OxLDL can activate ECs via the lipid peroxide species generated by oxLDL which may be involved in NF- $\kappa$ B activation. Lysophosphatidylcholine, one of the active molecules present in oxLDL, has been shown to activate NF- $\kappa$ B in primary cultured endothelial cells via a pKC-dependent pathway (36). Similarly, when unmodified human LDL particles are injected into a rat model, they localize in the arterial wall where they undergo oxidative modification which is accompanied by an increase in endothelial NF- $\kappa$ B activation and expression of NF- $\kappa$ B-dependent genes (37). Except in endothelial cells, activated NF- $\kappa$ B has been identified *in situ* in the smooth muscle cells and macrophages of human atherosclerotic plaques, and, in contrast, is strikingly absent in vessels devoid of atherosclerotic disease (38).

During endothelial cell activation, as much as 40% of the LDL phosphatidylcholine is degraded to lysophosphatidylcholine by a phospholipase  $A_2$ -like activity. Similar results were obtained when incubation conditions were selected to favour oxidation, for example, by increasing the  $Cu^{2+}$  concentration (33). These results suggest that endothelial cells modify LDL by mechanisms involving the generation of free radicals and action of phospholipase.

A possible mechanism for oxidative modification

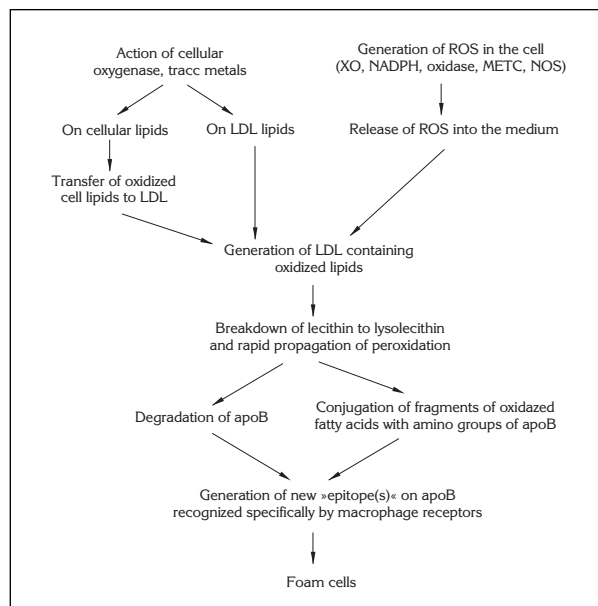


Figure 1 Mechanisms that lead to the oxidative modification of LDL by cells

of LDL has been reported to involve biologically active proteins, such as lipoxygenase, cyclooxygenase, peroxidases and heme or copper-containing proteins, which can generate free radicals and reactive oxygen species (39) (Figure 1). Lipoprotein modification may be promoted by micromolar concentrations of iron or copper in a metal ion concentration- and time- dependent manner (34, 40). Furthermore, peroxynitrite, a reactive oxidant species produced from nitric oxide and superoxide anion, is implicated in the pathogenesis of atherosclerosis through LDL oxidation, together with platelet aggregation, vascular hyporeactivity and macrophage activation (41). Stimulated expression of iNOS is documented in macrophages, foam cells and the vascular smooth muscle (42). Activated phagocytes provide one more pathway for LDL oxidation *in vivo* due to their ability to produce superoxide. Also, phagocyte-specific product, myeloperoxidase, modifies LDL by generating reactive intermediates such as tyrosyl radical, reactive aldehydes, and HOCl. Both catalytically active myeloperoxidase and dityrosine levels in LDL have been found in human atherosclerotic tissue (34). Early atherosclerosis in Watanabe rabbits (that lack LDL receptors) is associated with excessive vascular superoxide due to NADPH oxidase activity (43), i.e. Nox isoforms of different vascular cells (endothelial, smooth muscle cells). Glycation or glycooxidation can result in the formation of low molecular mass aldehydes, such as methylglyoxal, glyoxal, and glycolaldehyde, which can modify Arg, Lys and Trp residues of the apo B protein of LDL. In this way, LDL is rapidly converted to a form that is recognized by the scavenger receptors of macrophages (44). Oxidative modification of LDL is accompanied by extensive degradation of its polyunsaturated fatty acids, generating a complex ar-

ray of shorter chain-length fragments. Some of these fragments can be covalently linked to apolipoprotein B (45), through the  $\epsilon$ -amino groups of lysine residues. Thus oxidation of LDL may generate a broad spectrum of conjugates between fragments of oxidized fatty acids and apolipoprotein B. This modified form of the apolipoprotein is recognized by the acetyl-LDL receptor (46), which can also recognize malondialdehyde (MDA)-conjugated LDL. Namely, LDL is mostly taken by classical LDL-receptors present on the cell surface of parenchymal cells. These receptors cannot recognize oxidized LDL. On the other hand, macrophages have few receptors for normal LDL (47), but these cells readily bind and internalize oxidized LDL by specific »scavenger« receptors. Goldstein et al. (46) first postulated that the modification of LDL to a form recognized by the scavenger or acetyl-LDL receptor may be required for the widespread deposition of LDL-derived cholesterol esters in macrophages.

OxLDL is altered as follows: it is degraded by macrophages much faster; it cannot be uptaken through the LDL receptors; its electrophoretic mobility, density, negative charge and hydrophobic sites on apoB are increased; its magnitude, protein-phospholipid interaction and PUFA are decreased; its content of lysolecithin and oxidized cholesterol is increased; its apo B is fragmentary with decreased content of histidine, proline and lysine; it shows chemoattractant activity and inhibits macrophage motility; and, finally, it is cytotoxic. As such, oxLDL could contribute to the atherogenic process in several ways: (1) enhanced rates of macrophage uptake and degradation of the oxidatively modified LDL through the scavenger receptor (33); (2) increased recruitment of monocytes into the intima by the chemoattractant activity of oxLDL for circulating monocytes (48) due to induction of monocyte, chemotactic protein 1 (MCP-1) synthesis (49); (3) induced adhesion of monocytes to the arterial intima and stimulated intimal monocytes to differentiate into resident macrophages (50); (4) retention of macrophages in the intima through inhibition of their motility by oxLDL (48); (5) cellular injury caused by peroxidized lipid components of oxLDL.

### Inflammation and oxidative stress biomarkers in ischemic heart diseases

In order to study the biomarkers of inflammation and oxidative stress, we investigated 69 patients with ischemic heart diseases which occurred as a consequence of atherosclerosis. 30 patients with acute myocardial infarction (AMI), 23 with unstable angina pectoris (USAP), and 16 with stable angina pectoris (SAP) were included in the study. Their results were compared with those of healthy individuals (control group). At the admission to hospital (Institute of Cardiovascular Diseases »Niška Banja«), in all patients, 12-lead electrocardiogram, echocardiogram, and collection of blood samples for the evaluation of standard and specific biochemical analyses were performed. The patients' diseases were diagnosed according to objective clinical findings, functional tests, as well as according to troponin I (*Table 1*) which directly showed the degree of myocardial injury.

B-type natriuretic peptide (BNP) has been considered as a useful biomarker for the diagnosis, prognosis and therapy monitoring of patients with cardiac diseases. Plasma BNP levels are elevated in patients with left ventricular dysfunction and heart failure, and in those the last BNP predicts disease progression and increased morbidity and mortality (51). It is a useful prognostic parameter in patients with acute coronary syndrome because patients with increased BNP levels have a higher rate of cardiac complications and higher mortality after myocardial infarction (52). In this study, BNP was significantly increased in all patient groups, from  $36 \pm 11$  pmol/L in SAP patients to  $155 \pm 46$  pmol/L in AMI patients, in comparison with healthy controls ( $12 \pm 2.5$  pmol/L), which shows that it strongly depends on the degree of patient disease (*Table 1*).

Lipid status showed a slight deviation in comparison with the control group. A significant increase in LDL-C related to USAP patients and in LDL-C and TG in SAP patients was observed (*Table 1*). Biomarkers of inflammation including ESR, leukocyte count (Le), and C-reactive protein (CRP) were significantly elevated in both AMI and USAP groups, whilst in the

Table 1 Cardiac markers and lipid status in patients with ischemic heart diseases

Group	n	Troponin I ( $\mu\text{g/L}$ )	BNP (pmol/L)	Cholesterol (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	TG (mmol/L)
AMI	30	$26.98 \pm 8.78^{**}$	$155 \pm 46^{***}$	$5.9 \pm 0.3$	$3.9 \pm 0.2$	$1.02 \pm 0.04$	$2.13 \pm 0.16$
USAP	23	$0.253 \pm 0.16^*$	$120 \pm 53^*$	$5.9 \pm 0.5$	$3.9 \pm 0.4^*$	$0.91 \pm 0.08$	$2.31 \pm 0.27$
SAP	16	$0.004 \pm 0.00$	$36 \pm 11^*$	$5.7 \pm 0.3$	$3.5 \pm 0.2^*$	$1.06 \pm 0.05$	$2.92 \pm 0.39^*$
Control	16	0	$12 \pm 2.5$	$5.5 \pm 0.1$	$3.5 \pm 0.1$	$1.15 \pm 0.06$	$1.85 \pm 0.20$

The results are given as means  $\pm$  SE

\*  $p < 0.05$  vs. control group

\*\*  $p < 0.01$  vs. control group

\*\*\*  $p < 0.001$  vs. control group

Table II Biomarkers of inflammation in patients with ischemic heart diseases

Group	n	ESR	Le	CRP (mg/L)
AMI	30	30±4.4***	10.6±0.7***	34.7±8.8***
USAP	23	26±5.5*	9.1±0.8	12.4±3.7**
SAP	16	21±3.6*	7.4±0.5	3.7±0.6**
Control	16	10±1.7	6.9±0.2	1.6±0.3

The results were given as means ±SE  
 \* p<0.05 vs. control group  
 \*\* p<0.01 vs. control group  
 \*\*\* p<0.001 vs. control group

Table III Markers of oxidant stress in patients with ischemic heart diseases

Group	n	oxLDL (ng/mL)	Plasma l.p. (μmol/L)	Erythrocytes l.p. (nmol/mLEr)
AMI	30	256±84	18.6±1.3***,***	9.57±0.7 <sup>b,c</sup>
USAP	23	438±103	13.8±1.0***	8.28±0.8 <sup>a,b</sup>
SAP	16	320±113	12.3±1.3***	5.67±0.5
Control	16	280±48	5.6±0.3	5.37±0.5

The results are given as means ±SE  
 \* p<0.05 vs. USAP a-p <0.05 vs. SAP  
 \*\* p<0.001 vs. SAP b-p <0.005 vs. control group  
 \*\*\* p<0.001 vs. control group c-p <0.001 vs. SAP

SAP group a significant increase in ESR and Le was noted (Table II).

Oxidant stress was evaluated through the lipid peroxides, in both blood plasma and erythrocytes, as well as oxLDL determination. While lipid peroxides rose proportionally, correlating with the degree of the ischemic disease, there were no significant differences in oxLDL concentrations, although oxLDL was the highest in USAP patients. In this study, lipid peroxides had better predictable values than oxLDL (Table III).

Taken together, our results showed that atherosclerotic disease is associated with both inflammation and oxidative stress, and the degree of their expression gradually changes correlating with the stages of the disease.

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## INTERAKCIJA IZMEĐU OKSIDATIVNOG STRESA I INFLAMATORNIH BIOMARKERA U ATEROSKLEROZI

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*Kratak sadržaj:* Brojni rezultati pokazuju da postoji uska veza između inflamacije, oksidativnog stresa i ateroskleroze. Inicijalni događaj u aterogenezi je disfunkcija ili aktivacija endotela. Ona može biti izazvana mehaničkim, hemijskim, infektivnim ili imunološkim faktorima, što ukazuje da gotovo svi faktori rizika za aterosklerozu mogu dovesti do endotelne disfunkcije. To izaziva kaskadu inflamatornih reakcija u kojima učestvuju monociti, makrofagi, T limfociti i ćelije glatkih mišića. Ove ćelije i endotel nadalje proizvode adhezione molekule, citokine, faktore rasta i metaloproteinaze, prolongirajući aterogenezu. Aktivacija enzima koji proizvode oksidantse, kao što su NADPH oksidaza i azot-monoksid sintaza (NOS), dovodi do oksidativnog stresa i posledične oksidativne modifikacije LDL, a oxLDL potom može aktivirati endotelne ćelije. Oksidativno modifikovani LDL preuzimaju makrofagi putem »scavenger« receptora. Rezultat toga je akumulacija holesterola u makrofagima i stvaranje penastih ćelija koje predstavljaju bazu ateroskleroze. Čitav proces se ciklično ponavlja, dovodeći do formiranja uznapredovalih aterosklerotičnih promena koje karakteriše jezgro sačinjeno od lipida i nekrotičnog tkiva pokriveno fibroznom kapom.

*Cljučne reči:* ateroskleroza, inflamacija, oksidativni stres

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