Reactive oxygen species in pathogenesis of atherosclerosis

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Abstract

The volume of publications on the role of reactive oxygen species (ROS) in biological processes has been increasing exponentially over the last decades. ROS in large amounts clearly have detrimental effects on cell physiology, whereas low concentrations of ROS are permanently produced in cells and play a role as signaling molecules. An imbalance in ROS production and defense mechanisms can lead to pathological vascular remodeling, atherosclerosis being among them. The aim of this review is to examine different sources of ROS from the point of view of their participation in pathogenesis of atherosclerosis and related cardiovascular risk. Among the possible sources of ROS discussed here are mitochondria, NADPH-oxidases, xanthine oxidase, peroxidases, NO-synthases, cytochrome P450, cyclooxygenases, lipoxygenases, and hemoglobin of red blood cells. A great challenge for future research is to establish interrelations, feedback and feed-forward regulation mechanisms of various sources of ROS in development of atherosclerosis and other vascular pathologies.

Keywords: NADPH-oxidase; xanthine oxidase; peroxidase; NO-synthase; cytochrome P450; cyclooxygenase; hemoglobin.

Short 'running title': ROS & atherosclerosis

Abbreviations: AII - angiotensin II, ABCA1 - ATP-binding cassette transporter A1, BH_4 – tetrahydrobiopterin, COX – cyclooxygenase, CREB - cyclic adenosine monophosphate response element-binding protein, CYP - cytochrome P450, EC – epicatechins, EDHF - endothelial-derived hyperpolarizing factor, EPO - eosinophil peroxidase, GPx - glutathione peroxidase, GSH – glutathione, Hb – hemoglobin, HDL - high-density lipoprotein, HETE - hydroxyeicosatetraenoic acid, IL - interleukin, LDL - low density lipoproteins, LO – lipoxygenase, LOX-1 - lectin-like oxidized low-density lipoprotein, LPO – lactoperoxidase, LTs - leukotrienes, MAPK - mitogen-activated protein kinases, MIF - migration inhibitory factor, MPO – myeloperoxidase, NOS - NO-synthase, NOX - NADPH-oxidase, NSAIDs - nonsteroidal anti-inflammatory drugs, PGG2 - prostaglandin G2, PGH2 - prostaglandin H2, PMA - phorbol myristate acetate, PMNs - polymorphonuclear cells, PON1 - paraoxonase 1, PS - phosphatidylserine, RBC – red blood cells, ROS - reactive oxygen species, SMC - smooth muscle cells, SOD - superoxide dismutases, TAS - total antioxidant status, TCA - tricarboxylic acid, TLR4 - toll-like receptors 4, TPO - thyroid peroxidase, XDH - xanthine dehydrogenase, XO - xanthine oxidase, XOR - xanthine oxidoreductase.

Introduction

In the 1930th the phenomenon of oxidative (respiratory) stress in phagocytosis was described [1]. This publication gave rise to many studies on reactive oxygen species (ROS) in biological processes and systems. The ROS term refers to superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), peroxyl radical (ROO•), alcoxy radical (RO•), ozone (O_3), and singlet oxygen ($^1O_2$). Sometimes, reactive nitrogen species (RNS: nitric oxide or NO, hypochlorous acid, peroxinitrite) are also included [2]. The superoxide anion is a precursor for most ROS in cells, which dismutates to H_2O_2 either spontaneously or by superoxide dismutases (SOD). ROS participate in many intra- and intercellullar processes. High levels of ROS in the normal state is a characteristic function of the so called professional phagocytes - the cells of innate immunity; in the other cells, high concentrations of ROS are a sign of oxidative stress and a prerequisite of cell death. According to several studies, concentration of both endogenous (intracellular) and exogenous (extracellular) ROS can amount to as much as 500 μM [3, 4]. The consequences of oxidative stress caused by ROS include lipid peroxidation, break of DNA threads, and oxidation of proteins [5, 6], although these are not the only effects of ROS. For example, H_2O_2 can readily inhibit mitochondrial aconitase of the tricarboxylic acid (TCA) cycle, at concentrations not high enough to initiate lipid peroxidation [7]. Although ROS in large amounts clearly have detrimental effects on cell physiology, small amounts of ROS could have a beneficial effect, suggesting its therapeutic potential for reducing ischemic tissue [8]. Low concentrations of ROS are permanently produced in virtually every cell and tissue and play a role as the secondary messengers in redox-sensitive signaling pathways [9, 10]. H_2O_2 is the most stable and interesting molecule, from the point of view of signal transduction. The signaling effects of H_2O_2 are mainly directed towards post-translational modification of proteins due to oxidation of the thiol groups of cysteine. Extracellular H_2O_2 can enter the cells through diffusion, though entry is mainly via aquaporin channels which determine the localisation and specificity of H_2O_2 effects [11]. Signaling effects of ROS are a long-known phenomenon, and Peter Proctor in 1972 was the first to suggest their special functions [12]. During the past 40 years a plethora of papers and comprehensive reviews have been published, with a review by Proctor among the first [13, 14, 15].

Oxidative stress is involved in various pathological vascular remodeling. These injuries can be minimized by other cells, reducing molecules and anti-oxidative enzymes, both intra- and extracellular: cystein (Cys), glutathione (GSH), glutathione peroxidases (GPx), thioredoxins, peroxiredoxins, SOD, catalaze, serum paraoxonase 1 and even albumin [6, 16, 17, 18]. It is clear that imbalance between ROS generation and neutralization is the principal cause of vascular and other pathologies. The aim of this review is to examine different sources of ROS from the point of view of their participation in pathogenesis of atherosclerosis and related cardiovascular risk. Among the sources of ROS are intracellular organelles (e.g. mitochondria), and enzymes such as xanthine oxidase,

myeloperoxidase (MPO), lactoperoxidase (LPO), uncoupled NO-synthase (NOS), cyclooxygenase (COX), lipoxygenase (LO), hemoglobin (Hb), cytochrome P450 (CYP) and NADPH-oxidases (the number of which is now reported to be seven; NOX1-5 and DUOX1/2) [14, 15, 19, 20 , 21].

1. NADPH-oxidases

While there are many possible sources of ROS, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) play a central role. They are a source of "kindling radicals," which affect other enzymes, such as nitric oxide synthases, xanthine oxidase, etc. This is important as risk factors for atherosclerosis (hypertension, diabetes, hypercholesterolemia, and smoking) regulate the expression and activity of NOX in the vessel wall [15]. NOX are the main sources of the superoxide anion in various pathological states of the cardiovascular system. The principal difference between NOX and other sources of ROS is that for the former the generation of ROS is their major function [22]. NOX are present in neutrophils where they mostly determine the non-specific immune response [23]. In the ischemic-reperfusion condition, NOXs substantially determine tissue injury through generating ROS in endothelial and smooth muscle cells (SMC) of blood vessels.

NOX1 has been found in the plasma membrane of endothelial and SMC, located mainly in caveolae membranes next to caveolin [24]. NOX1 regulates proliferation and motility of SMC and takes part in blood pressure regulation [25]. The enzyme is involved in progression of hypertension initiated by angiotensin II [26, 27]. Elevated activity of NOX1 in SMC results not only in elevated level of superoxide anion but also to uncoupling of endothelial NOS (eNOS) as a result of oxidation of tetrahydrobiopterin (BH_4), thus making eNOS an additional source of superoxide [28]. Moreover, superoxide generated by NOX1 (and probably by other NOXs) converts xanthine oxidoreductase to xanthine oxidase [29].

NOX2 is the most studied isoform of NOX, which generates superoxide anions in professional phagocytes and many other cells. In phagosomes, superoxide interacts with chloride ions under catalytic control of myeloperoxidase, with production of hypochlorite [30]. Activation of the lipoxygenase signaling pathway is also coupled with activation of NOX2, when ligands CD154 or CD40L interact with CD40 of B lymphocytes [31, 32]. Associated factors of TNF-receptor (TRAF) -PI3 kinase and small G-protein Rac1 - are involved in this signaling pathway. Synergism in effects of ligands of CD40 and oxidative stress developed after activation of NOX in ischemic-reperfusion conditions lead to death of hepatocytes and probably endothelial cells, which also express CD40 in stress conditions [33, 34].

NOX1/2 promote the development of endothelial dysfunction, hypertension, and inflammation [15]. For activation of NOX1/2 an interaction of several regulatory subunits is necessary: Ncf1/p47^{phox} (NOX1 and NOX2);

Cyba/p22^{phox}; Noxa1, Noxo1 (a homolog of p47^{phox}, NOX1*)*; small G-protein Rac (NOX1 and NOX2) [23, 35]. As distinct from NOX1 and NOX2, functionally active NOX4 consists of only two proteins, Nox4 and p22^{phox} [36]. NOX4 is located in the endoplasmic reticulum and nucleus [24, 37]. NOX4 has been found in endothelial cells, where it is the principal source of ROS, and also in many other cells: fetal liver, SMC, osteoclasts, hemopoietic stem cells, adipocytes and cardiomyocytes [38, 39]. It is supposed that NOX4 is part of the oxygen sensor system in proximal tubules of kidneys, carotid body type I cells, SMC of lung artery, neuroepithelial bodies of pulmonary airways, heart fibroblasts, and probably in other cells [24, 40, 41]. It was first suggested that NOX4 generates superoxide, which can neutralize NO produced in the same cells [37], but later it was established that NOX4 generates H_2O_2 [42, 43]. Activators of other NOX isoforms do not affect activity of NOX4, and generation of H_2O_2 in the cells depends upon expression of NOX4; activity of the enzyme is therefore constitutive and there is no need for regulatory subunits to be activated [42].

Nonetheless, regulation of NOX4 does exist and studies of the regulatory mechanisms could improve understanding of the pathogenesis of many diseases, including atherosclerosis. For example, insulin activates NOX4 in 3T3-L1 fibroblasts and the H_2O_2 produced inhibits tyrosine phosphatases enhancing tyrosine phosphorylation [44]. Toll-like receptors 4 (TLR4) also activate NOX4 under ischemia/reperfusion; this activation plays a key role in development of inflammation and is an important prerequisite of kidney and brain injuries [45, 46]. TGF-beta induces transformation and proliferation of SMC and endothelial cells by ROS generated via NOX4 [47, 48]. 7-Ketocholesterol, which is the principal component of low density lipoproteins (LDL), stimulates expression of NOX4 and apoptosis in SMC of blood vessels [49]. Plaque instability associated with acute coronary syndromes results in part from apoptosis and senescence of cells within the atherosclerotic lesion. An age-dependent increase in ROS in aorta from atherosclerotic mice has been observed, with evidence for elevated ROS prior to lesion development [50]. Whereas macrophage infiltration was restricted to the lesion, oxidized lipids extended beyond the plaque and into the vessel wall. Consistent with these findings, dynamic changes in the expression of NADPH oxidases in atherosclerotic vessels were observed. Specifically, Nox1 protein expression was increased early and decreased with lesion progression, while induction of Nox4 was a late event. Nox2 and p22^{phox} proteins were elevated throughout lesion development. Atherosclerotic SMC demonstrated increased generation of ROS, cell cycle arrest, evidence of senescence, and increased susceptibility to apoptosis [50]. Interestingly, NOX4 may have a role in protecting the vasculature during stress; however, when its activity is increased, it may be detrimental. Also, calcium-dependent NOX5 has been implicated in oxidative damage in human atherosclerosis [15]. It is noteworthy here that NOX5 is expressed in primates but does not occur naturally in rodents.

NADPH oxidases of the DUOX group (DUOX1 and DUOX2) also generate H_2O_2 [51, 52]. DUOX1/2 are sources of H_2O_2 for LPO, playing an important role in antibacterial activity of saliva, milk, lacrimal fluid and secretion of air-conducting pathways [51]. DUOX2 (and probably DUOX1) are functionally bound with thioperoxidase (TPO) and participate in biosynthesis of thyroid hormones generating H_2O_2 through intramolecular dismutation of superoxide [53]. TPO utilize iodide ions and H_2O_2 for generation of molecular iodine, which is necessary for biosynthesis of thyroid hormones T_3 and T_4. NOX4, together with DUOX2, is a source of H_2O_2 in the thyroid gland. Insufficient quantity of H_2O_2 is a cause of hypothyroidism, whereas elevated synthesis of H_2O_2 is observed in papillary thyroid carcinoma [54]. As for blood vessel pathologies, low normal thyroid function may adversely affect lipoprotein metabolism and atherosclerosis development. Subclinical hypothyroidism is associated with endothelial dysfunction [55]. Vascular SMC apoptosis is an early trigger of hypothyroidism-associated atherosclerosis, and activation of thyroid hormone receptors TRα1 to prevent vascular SMC apoptosis could be a therapeutic strategy in this disease [56].

2. Mitochondria

In mitochondria, oxygen can be reduced with one or two electrons to produce superoxide and H_2O_2, respectively [57]. The respiratory chain is the main source of superoxide in one-electron mitochondrial reduction of oxygen. Complex I supplies electrons for matrix oxygen, though complex III – for matrix and intermembrane space oxygen. Moreover, mitochondrial sources of superoxide can be monoamine oxidase and glycerol-b_5-reductase (located on outer mitochondrial membrane), glycerol-3-phosphate dehydrogenase and cytochrome P450 (located on inner mitochondrial membrane), aconitase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase (matrix enzymes). Two-electron mitochondrial reduction of oxygen in mitochondria takes place due to interaction of cytochrome C (cytC) and the p66shc adaptor protein. Superoxide of mitochondrial matrix dismutates to produce H_2O_2 due to Mn-SOD (SOD2), whereas superoxide of the intermembrane space – due to cytosolic Cu,Zn-SOD (SOD1) [58]. Mitochondrial-derived reactive oxygen species (mtROS) is one of the major sources of cellular ROS, and excessive mtROS is associated with atherosclerosis progression in both human and mouse models. Despite numerous association and causation studies demonstrating the importance of mtROS in atherosclerosis progression, the failure of antioxidant therapy in human randomized clinical trials demands more definitive, cell-type specific investigations [59]. For example, it was demonstrated that mitochondrial ROS of endothelial cells contribute to initiation of inflammatory reactions via NFkB to recruit leukocytes into the intima [60]. The successive oxidative burst in activated neutrophils can greatly exceed the initial generation of ROS by endothelium, bringing forth irreversible injuries to the cells [61]. In addition, mitochondrial oxidative stress in lesional macrophages amplifies

atherosclerotic lesion development by promoting NF-κB-mediated entry of monocytes and other inflammatory processes [62]. ROS interacts with ionic channels, cytoskeletal and adhesive proteins, enhancing endothelial permeability due to activation of contractile proteins and degradation of occludin, the principal tight junction protein [63, 64]. It also should be noted that mitochondrial DNA (mtDNA) defects promote atherosclerosis and plaque vulnerability independently of ROS, through effects on vascular SMC and monocytes. MtDNA damage is therefore not only causative, but also indicates higher risk for atherosclerosis [65].

3. Xanthine oxidoreductase

The enzyme xanthine oxidoreductase (XOR) catalizes the two last stages of degradation of purines (hypoxanthine → xanthine → uric acid). The initial transcription product of a single gene is NAD-dependent xanthine dehydrogenase (XDH, EC 1.17.1.4). Molybdenum-containing cofactor of XDH transfers electrons from substrate through Fe/S centres to FAD-containing cofactor followed by reduction of NAD: xanthine + $NAD^+ + H_2O$ $\rightleftharpoons$ uric acid + NADH + H^+. However, a post-translational oxidative modification of cysteine residue or irreversible proteolytic modification transforms XDH to oxygen-dependent xanthine oxidase (XO, EC 1.17.3.2) [66, 67]. As a result, the affinity decreases for NAD and increases for oxygen, and electrons proceed from the substrate via Mo^{2+} cofactor to FAD cofactor, with production of H_2O_2: hypoxanthine + H_2O + O_2 $\rightleftharpoons$ xanthine + H_2O_2; xanthine + H_2O + O_2 $\rightleftharpoons$ uric acid + H_2O_2. Also, in some conditions one-electron reduction of oxygen occurs and the superoxide-anion is produced: RH + H_2O + $2O_2$ $\rightleftharpoons$ ROH + $2O_2^-$ + 2 H^+. Moreover, XDH can also generate superoxide when NAD^+ is in deficit (for example, during inflammation and concomitant hypoxic microenvironment of the enzyme, though before XDH transforms to XO) [68, 69]. According to recent data, XO is a major contributor to oxidative stress [70]. In the atherosclerotic plaque, XO-mediated ROS formation is pro-inflammatory and XO-inhibition by febuxostat is a potential therapy for atherosclerosis [71]. However, allopurinol or siRNA against XO does not exhibit a protective effect against cell death suggesting that XO-generated H_2O_2, and perhaps H_2O_2 in general, is a consequence but not a mediator of cell death [70].

There is another possible reason for low efficiency of allopurinol. It was shown earlier that during diverse pathologic processes, XO released into plasma from tissues can circulate to remote sites and bind to target tissues low in or devoid of XO activity. Cell-bound XO may then be concentrated several thousand-fold at the cell surface or interstitial matrix, where its oxidant products could more readily react with cellular target molecules and disrupt vascular functional and barrier properties [72]. High concentrations of XO associate with endothelial cell glycosaminoglycans resulting in significant resistance to inhibition by traditional pyrazolopyrimidine-based

inhibitors such as allopurinol [73]. A new nonpurine XO-specific inhibitor - febuxostat - was shown to have superior potency for inhibition of endothelium-associated XO, as compared with allopurinol, and could be an efficient potential therapy for atherosclerosis [71].

4. Cytochromes P450

Cytochromes P450 (CYP) is a big group (superfamily) of heme-containing enzymes, which for a long time were considered to be flavin-containing monooxygenases of endoplasmic reticulum exclusively in liver, the function of which is oxygen- and NADPH-dependent oxidation and/or reduction of cholesterol, vitamins, steroids, and many other compounds. It was later revealed, however, that some enzymes of this superfamily are expressed in many tissues beyond liver, including the cells of cardiovascular system [74]. The CYP enzymes metabolizing arachidonic acid (families CYP2 and CYP4A) participate in vascular tone regulation, generating vasoactive derivatives of fatty acids and ROS. Thus, 20-HETE is produced in vascular SMC due to CYP4A activity, and CYP2C8/9 of endothelial cells produce epoxyeicosatrienoic acids responsible for NO- and PGI_2-independent endothelial-dependent relaxation of blood vessels caused by a hyperpolarizing factor [74, 75]. Like other CYP enzymes, CYP2C is inhibited by NO and therefore work in environments with minimal NO concentration. It has been found that CYP enzymes of blood vessels can generate superoxide anion, H_2O_2 and hydroxyl radical; this takes place during the NADPH-dependent transfer of electrons, which were intended for reduction of heme iron in oxygen generation [74]. In endothelial cells, CYP2C participates in generation of endothelial-derived hyperpolarizing factor (EDHF) and at the same time is a source of ROS [76]. CYP2C can produce both EDHF (vasodilator) and ROS (vasoconstrictors in case of inactivation of NO and vasodilator in case of generation of H_2O_2), therefore modulation of CYP2C activity is not always predictable, especially taking into account activity of other sources of ROS. For example, superoxide anion produced by CYP2C impairs the NO-dependent vascular relaxation and enhances activity of redox-sensitive nuclear factor NFkB and expression of VCAM-1 [76]. Inhibition of CYP2C9 by sulfaphenozole normalise endothelium-dependent NO-mediated dilation in patients with coronary artery disease [77]. A trigger role of CYP-generated ROS in pro-inflammatory effects of TNF-a has been demonstrated, which elevated expression of addressin genes (MAdCAM-1) and some other adhesion molecules of high endothelial venule; inhibitors of CYP (ketoconazole and others) blocked the effects of TNF-a [78]. In addition, expression by endothelial cells of E-selectin (CD62), ICAM-1 (CD54), VCAM-1 (CD106) depend upon ROS produced by NOX, mitochondria, XO and eNOS [78, 79, 80]. Conversely, expression and activity of endothelial CYP is stimulated by circular stretch of blood vessels, hormones, and inhibitors of HMG-CoA reductase [75, 81]; all of these induce elevation of both ROS and EDHF.

Expression of CYP increases during hypertension and hypercholesterolemia. It is worth noting that oxidized LDL (oxLDL) decreases the level of expression of CYP2 in endothelium due to H_2O_2 produced by NOX-4, and decreases expression of transcriptional regulator NF-1 by interacting with the promotor region of CYP [82]. Because of the injuring effects of oxLDL upon blood vessels, a blockade of lectin-like receptors for oxLDL (LOX-1) could be an efficient therapeutic means, which would regenerate activity of CYP.

5. NO-synthases

NO-synthases are homodimeric oxidoreductases which provide electron transfer from the C-terminal reductase to the N-terminal oxidase domain. Cofactors FAD and FMN are connected with the reductase domain to provide electron transfer from NADPH. Heme prosthetic group, BH_4, oxygen and L-arginine are bound to the oxidase domain; also, there is a site for binding calmodulin. The structure of the reductase domain is similar to that of CYP-enzymes, so in the case of “uncoupling” of the NOS superoxide anion would be a product of reaction instead of NO [83, 84]. BH_4 provides the coupling of two processes, reduction of molecular oxygen and oxidation of arginine; moreover, BH_4 stabilizes the dimeric structure of NOS [85]. ROS oxidize TH_4 to produce dihydrobiopterin, which competes with TH_4 for binding with NOS and promotes uncoupling and further generation of ROS. The principal factor of uncoupling is a ratio of BH_4 to total biopterin: at concentration of the latter above 1.5 μM the uncoupled eNOS can simultaneously produce NO and O_2^-, they react to generate peroxinitrite, which is another uncoupler of NOS [85].

It has recently been reported that oxLDL binding to lectin-like oxidized low-density lipoprotein (LOX-1) determined a significant increase in the generation of ROS, suggesting the involvement of signaling pathways such as mitogen-activated protein kinases (MAPK) [86]. LOX-1 has been identified in endothelial cells as the main receptor of oxLDL. LOX-1 is upregulated in the presence of pathological conditions including atherosclerosis, hypertension and diabetes, because it acts as a mediator of "endothelial dysfunction". It promotes the generation of O_2^-, the inhibition of NO production and the increase of endothelial adhesiveness to monocytes. Also, oxLDL inhibits macrophage migration and traps foam cells, possibly through oxLDL induced iNOS expression. Therefore, reduction of peroxynitrite and possibly lipid hydroperoxide levels in plaques represents a valuable therapeutic approach to reverse migratory arrest of macrophage-derived foam cells and to impair plaque formation [86]. In addition, ceramide may be an important target for preventing and treating vascular dysfunction associated with atherosclerosis, since it has been shown that ceramide has an integral role in the transition of NO synthesis to mitochondrial-derived H_2O_2, which regulates flow-induced dilation in microvessels [87].

6. Peroxidases

Peroxidases (EC 1.11.1.x) are widely distributed in the animal and plant kingdoms, they catalize reactions of one- and two electron oxidation of various substrates according to the general scheme: ROOR' + electron donor ($2e^-$) + $2H^+ \rightarrow$ ROH + R'OH. Recently phylogenetic relations were established for the major lines of evolution of the heme-containing animal peroxidases: myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO) and thyroid peroxidase (TPO) [88]. On the basis of their prevalence and two types of enzyme activity intrinsic to these proteins, a new superfamily of peroxidases/cyclooxygenases were classified and divided into seven subfamilies, the first of which includes heme peroxidases of the vertebrata. Enzymes of this subfamily are constituents of inner immunity (MPO, EPO, LPO), and also participate in biosynthesis of hormones (TPO, see above). Peroxidases consists of one (monomeric EPO and LPO) or two (homodimeric MPO and TPO) glycosylated alpha-helical (predominantly) domains with one autocatalytically modified heme in each domain [89].

MPO is a component mainly of the azurophilic granules of neutrophils. The product of activity of this enzyme is hypochlorous acid HOCl, which is generated in phagosomes from H_2O_2 and Cl^- during the respiratory burst of neutrophils [30]. Moreover, MPO with the help of H_2O_2 oxidizes thyrosine to thyrosine-radical, which is also cytotoxic along with hypochlorite and serves to combat against bacteria and other pathogens [90]. MPO is one of the factors promoting oxidative/halogenating modification of LDL. Both monocytes (which differentiate into macrophages) and neutrophils secrete MPO in response to the presence of damaged LDL. Binding to MPO is important for LDL to become modified and acquire proatherogenic properties. MPO binds to the LDL surfaces, and the LDL-MPO complex is uncoupled in the presence of peptide EQIQDDCTGDED that corresponds to a fragment of apoB-100. The EQIQDDCTGDED peptide, ceruloplasmin, and SCN^- can play the role of anti-atherogenic factors reducing the deleterious effect of catalytically active MPO on LDL and accumulation of cholesterol in macrophages [91]. Serum MPO levels and paraoxonase 1 (PON1) activities were significantly associated with the prevalence of coronary lesions. PON1 has antioxidant properties for high-density lipoprotein (HDL). A high MPO/PON1 ratio, when cut-off values were set at 1.59, was independently correlated with restenosis and *de novo* lesions. HDL isolated from patients with low serum MPO/PON1 ratio inhibited VCAM-1 expression, which may participate in monocyte recruitment to atherosclerotic sites, significantly greater than that with high MPO/PON1 ratio. The cholesterol efflux capacity of apoB-depleted serum from patients with high MPO/PON1 ratio was significantly lower than that with low MPO/PON1 ratio [92]. As for the mechanism, MPO may contribute to the generation of dysfunctional HDL with impaired ATP-binding cassette transporter A1 (ABCA1) efflux capacity in humans with atherosclerosis. Subjects with coronary artery disease and acute coronary syndrome had higher levels of chlorinated

Tyr192 and oxidized Met148 compared with control subjects. In contrast, plasma levels of MPO did not differ between the groups. HDL from the subjects with coronary artery disease and acute coronary syndrome was less able to accept cholesterol from cells expressing ABCA1 compared with HDL from control subjects. Levels of chlorinated tyrosine and oxidized methionine were inversely associated with ABCA1 efflux capacity and positively with atherosclerotic disease status. Quantification of chlorotyrosine and oxidized methionine in circulating HDL might be useful indicators of the risk of cardiovascular disease that are independent of HDL-cholesterol [93]. It is worth noting that epicatechins (EC) are substrates for peroxidases including the neutrophil-derived MPO. Oxidation of the catechol moiety of EC to a *o*-quinone by MPO generates potent macrophage migration inhibitory factor (MIF) inhibitors. MIF is implicated in a number of inflammatory diseases including sepsis, arthritis and colitis, and in diseases with an inflammatory component, such as atherosclerosis, diabetes and cancer. MIF has an unusual N-terminal proline with catalytic activity, and targeting of this residue by small molecule inhibitors has been shown to interfere with the biological activity of MIF. Near complete inhibition of MIF by the MPO/H_2O_2/EC system was achieved at equimolar concentrations of EC and MIF even in the presence of other MPO substrates. The modification introduced by oxidized EC on MIF occurs at the N-terminal proline. MIF inhibition by oxidized EC contributes to the anti-inflammatory activity of these compounds [94].

LPO catalyzes oxidation of some substrates with H_2O_2 according to a general scheme: substrate + H_2O_2 → oxidized substrate + H_2O. The most significant physiological implication has oxidation of thiocyanate (SCN^-) and bromide (Br^-), with hypothiocyanate ($OSCN^-$) and hypobromide (BrO^-) being produced, respectively. As for the secondary or intermediate products of halide oxidation, singlet oxygen and other ROS were revealed [95, 96]. H_2O_2 is produced from glucose and oxygen via glucose oxidase (EC 1.1.3.4). Another source of H_2O_2 for LPO is DUOX1/2 [51]. In some conditions LPO can enhance oxidative stress and the toxic effects of H_2O_2, for example with excess thiocyanate in the reaction medium [97]. Another example is oxidation of estradiol in breast cancer [98]. In addition, LPO can enhance the carcinogenic potential of aromatic and heterocyclic amines [99]. There are scarce data on the specific role of LPO in pathogenesis of atherosclerosis. LPO, prostaglandin synthetase, prostacyclin synthetase, though mainly myeloperoxidase, can generate toxic methyl glyoxal through catalyzed peroxidation of acetoacetate, and this has been proposed to be a link between cardiovascular risk factors and the initiation of atherosclerosis [100].

EPO is a haloperoxidase utilizing bromide as a substrate, which is necessary for eosinophils to combat multicellular pathogens (e.g. nematode worms) and some bacteria (e.g. *Mycobaterium tuberculosis*) [101]. This is a heterodimer of 71-77 kDa with glycosylated (more heavy) and non-glycosylated (less heavy) subunits [102]. Excessive activity of EPO and NOX, and enhanced generation of ROS is observed in some diseases, such as asthma

[103]. As with LPO, there is little evidence of direct cause-effect relations between EPO and atherosclerosis development. EPO may activate histamine release by basophils and mast cells. The content and release of heparin and histamine may play a role in preventing thrombus formation and in promoting lipolysis. The cytoplasmic lipid accumulation by neutrophils, basophils and mast cells may in turn contribute to the population of foam cells in these lesions [104]. Patients with allergic disorders such as allergic rhinitis or asthma have been reported to be at increased risk for atherosclerosis, and there is a relationship between peripheral eosinophil count and degree of albumin excretion rate, which is a useful marker of cardiovascular mortality as well as diabetic nephropathy in patients with type 2 diabetes [105].

7. Cyclooxygenase and Lipoxygenases

Cyclooxygenase (COX, prostaglandin-endoperoxide synthase, prostaglandin H2 synthase, EC 1.14.99.1) has two active centers: one of these provides the cyclooxygenase activity *per se*, arachidonic acid (20:4, ω-6) being the substrate that is transformed to 15-hydroperoxy-PG endoperoxide (prostaglandin G2, PGG2); the second active center contains heme which provides peroxidase activity of the enzyme transforming PGG2 to prostaglandin H_2 (PGH_2). In addition, the "peroxidase center" of COX generates a tyrosyl radical in Tyr385, which makes possible the initiation and implementation of the cyclooxygenase reaction [106]. The action of various agonists, toxic agents and carcinogens (PMA, high concentration of glucose, angiotensin II, 2-aminobiphenyl, to name a few) enhance expression of inducible COX-2, and the level of expression depends upon ROS, which in turn is determined by activity of NADPH-oxidases [107, 108]. The question of whether there is feedback remains unresolved: although it has been demonstrated that the level of COX-2 expression has no influence on the level of ROS [109], an early paper presented indirect evidence of such an effect [110]. If COX-2 is considered as a relevant source of free oxygen radicals, inhibiting the activity of this enzyme would reduce oxidative stress. Indeed, there is evidence to indicate that selective and timely use of COX-2 inhibitors (nimesulide or celecoxib) would be useful in preventing the onset and development of atherosclerosis by enhancing antioxidant defense system (SOD, GPx, and total antioxidant status (TAS)) [111]. Aspirin is known to reduce the incidence and mortality from ischemic heart disease and is a mainstay in the prevention of vascular complications of atherosclerosis. On the other hand, during the last decade it has been suggested that nonsteroidal anti-inflammatory drugs (NSAIDs), and in particular inhibitors of COX-2, are associated with an increase in cardiovascular morbidity and mortality. It has been shown recently that COX-1 but not COX-2 drives vascular prostacyclin in the healthy cardiovascular system; COX-2 profoundly limits atherosclerosis, and this protection is independent of local prostacyclin release [112]. Moreover, selective depletion of COX-2 in vascular smooth muscle cells and endothelial cells depressed biosynthesis of PGI2 and PGE2, elevated

blood pressure, and accelerated atherogenesis in Ldlr knockout mice [113]. Deletion of COX-2 in vascular smooth muscle cells and endothelial cells coincided with an increase in COX-2 expression in lesional macrophages and increased biosynthesis of thromboxane [113]. To reconcile the differences, a special experiment was recently conducted to show that low dose aspirin indeed reduces early atherosclerosis, while inhibition of COX-2 by meloxicam (which is also known as a potential vasculitis inducer) had a null effect on the extent of atherosclerosis in ApoE knockout mice [114]. Thus, the COX-2/ROS relations are presently in the background scene; the perspective could be the targeted delivery of COX-2 inhibitors to macrophages, which may conserve their efficacy while limiting cardiovascular risk. In any case, it now seems now that COX *per se* cannot serve as a source of pathogenic ROS.

At the same time, growing evidence suggests that lipoxygenases (LO) and COX-generated arachidonic acid metabolites can induce ROS generation by stimulating NOX and that a potential signaling connection exits between the LOX/COX metabolites and NOX [115]. Lipoxygenases (EC 1.13.11.-) are a family of iron-containing enzymes that catalyse the dioxygenation of polyunsaturated fatty acids in lipids containing a *cis,cis*-1,4-pentadiene structure. In mammals, a number of lipoxygenases isozymes are involved in the metabolism of eicosanoids: arachidonate 12-lipoxygenase (12-LO, EC 1.13.11.31), arachidonate 5-lipoxygenase (5-LO, EC 1.13.11.34), erythroid cell-specific 15-lipoxygenase (EC 1.13.11.33). 5-Lipoxygenase (5-LO) is the key enzyme involved in the synthesis of pro-inflammatory leukotrienes (LTs) [116]. The pathophysiological effects of LTs can be modulated by the selective inhibition of 5-LO and, in the last decade, significant research efforts have led to the exploitation of 5-LO pathway for developing new drugs against inflammatory diseases. In particular, attention is focused on the contribution of leukotriene B4 (LTB4), a potent bioactive eicosanoid that is derived from 5-LOX, and its receptors, BLT1 and BLT2, to NOX stimulation through a signaling mechanism that leads to ROS generation [115, 117]. Some flavonoids known as antioxidants (e.g. genistein) when taken in excess may possesses a pro-oxidant potential, and surprisingly it was demonstrated that increased generation of ROS was induced with inhibited expression of NOX4 and COX1/2 as well as increased activity of the glutathione redox system [118]. The cellular expression of 5-LO, however, was up-regulated and the addition of 5-LO inhibitor (Zileuton) decreased genistein-induced intracellular ROS level, close to that obtained following the addition of the ROS scavenger N-acetylcysteine. Besides 5-LO, pro-inflammatory cytokine stimulation induces 12-lipoxygenase (12-LO) expression in human islet beta cells; 12-hydroxyeicosatetraenoic acid (12-HETE), a product of 12-LO activity, stimulated NOX1 expression in human islets and production of ROS [119]. 15-LO plays a role in atherogenesis. 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE], the major 15-LO-dependent metabolite of arachidonic acid, stimulated the production of ROS by monocytes through the xanthine oxidase-mediated activation of NOX [120]. ROS production led to the Syk-, Pyk2-, and

MAPK-dependent production of the proinflammatory cytokine IL-17A in a manner that required the transcription factor CREB (cyclic adenosine monophosphate response element-binding protein). In addition, this pathway was required for the 15(S)-HETE-dependent migration and adhesion of monocytes to endothelial cells. Moreover, peritoneal macrophages from ApoE-/- mice fed a high-fat diet, when compared to those from similarly fed ApoE-/-:12/15-LO-/- mice, exhibited increased: xanthine oxidase and NOX activities; ROS production; phosphorylation of Syk, Pyk2, MAPK and CREB; and IL-17A production. These events correlated with increased lipid deposits and numbers of monocytes and macrophages in the aortic arches of ApoE-/- mice, which resulted in atherosclerotic plaque formation [120].

8. Hemoglobin and Red Blood Cells

Hemoglobin (Hb) is the iron-containing oxygen-transport metalloprotein in red blood cells of all vertebrates (with the exception of the fish family *Channichthyidae* [121]) as well as the tissues of some invertebrates. In mammals, Hb makes up about 96% of red blood cells' dry content (by weight), and around 35% of the total content (including water) [122]. The mammalian Hb molecule is an assembly of four globular protein subunits, and each subunit is composed of a protein chain tightly associated with a non-protein heme group. Heme is the archetypical O_2 binding molecule, a member of the porphyrin family composed of four pyrrole rings bound to a central iron atom, which can be converted from ferrous (Fe^{2+}) to ferric (Fe^{3+}) by binding of molecular O_2 [123]. The ability of heme to bind O_2 reversibly has long been of interest in terms of O_2 sensing [123, 124, 125]. Heme proteins implicated in O_2 sensing include mitochondrial cytochromes, NADPH oxidase, cytochrome *P*-450, NOS, guanylate cyclase, catalase, cystathionine β-synthase, and heme oxygenase; some of these were discussed above in relation to ROS generation. Heme itself is a pro-oxidant [126].

Red blood cells (RBC) are continuously exposed to both endogenous and exogenous sources of ROS. Endogenous ROS are continuously generated by the slow autoxidation of Hb [127]. The initial oxidative process in Hb involves the spontaneous oxidation of its ferrous iron, known as autoxidation of Hb, leading to non-functional metHb and superoxide anion. Moreover, H_2O_2 arising from O_2^- dismutation could react with Hb, either ferrous or ferric, forming ferrylHb (Fe^{4+}) or ferryl protein radicals, respectively. Finally, the formation of ferrylHb could also result in the formation of heme degradation products [21]. FerrylHb, but not haemoglobin or metHb, induce the expression of proinflammatory genes in endothelial cells *in vitro* and the recruitment of polymorphonuclear cells (PMNs) *in vivo* [128]. In hypoxia and some other conditions Hb is not fully oxygenated, and there is a dramatic increase in the rate of Hb autoxidation for this partially oxygenated Hb [129]. Of particular importance is an increase in the affinity of partially oxygenated Hb for the RBC membrane [130]; this limits the efficiency of the

antioxidant system, which is primarily cytosolic, from neutralizing the ROS formed at the membrane. The pool of un-neutralized ROS in the RBC has been shown to damage the RBC membrane [131] and to be transferred to other cells coming into contact with RBC, resulting in tissue damage and inflammation [132; 133]. The ROS generated on the RBC membrane through Hb autoxidation are ideally located to react with membrane lipids and proteins producing lipid peroxidation and modified membrane proteins that can affect the membrane structure [21]. Although exogenous ROS has been shown to cause lipid peroxidation, this lipid oxidative damage does not affect RBC deformability [134]. Damage to membrane proteins is, thus, presumably responsible for the impaired cellular deformability associated with oxidative stress. The role of protein damage in producing impaired deformability is consistent with a dominant role for the membrane cytoskeleton in regulating RBC deformability [135, 136, 137].

The contribution of oxidative stress that does not necessarily involve Hb autoxidation to deformability is indicated by caspase-3, which is activated in the RBC by oxidative reactions and has been shown to partially degrade band 3. This affects interactions of band 3 with cytosolic proteins as well as the linkage to ankyrin and the cytoskeleton, which finally induces exposure of phosphatidylserine (PS) to the outer surface [138, 1339, 140]. This dramatic rearrangement of the membrane has been shown to involve a concomitant decrease in deformability [141, 142]. In addition, oxidative stress has been shown to inhibit Ca-ATPase [143], and enhanced intracellular calcium can affect deformability by activation of the Gardos channel resulting in leakage of K^+ from the RBC [144] causing shrinkage of the cells and impaired deformability [141, 145]. An increase in intracellular calcium also activates calpain, transglutaminase-2 and some other caspases that can degrade/crosslink cytoskeleton proteins [146]. It also inhibits phosphotyrosine phosphatase increasing band 3 phosphorylation [147].

RBC-derived iron-rich heme group is one of the main sources of oxidative stress, leading to progression of chronic pathological vascular remodeling [148]. Lysis of RBC within the intraluminal thrombus of abdominal aortic aneurysm or inside the plaque associated with haemorrhage due to microvessel rupture or with RBC extravasation in initial plaques, may lead to subsequent release of the pro-oxidant Hb, that when oxidized in mtHb, liberates heme and iron within tissue and transfers them to lipoproteins [149]. The presence of these oxidized forms of Hb in ruptured advanced atherosclerotic plaques has been observed [150]. The authors demonstrated that exposure of RBC to lipids from atherosclerotic lesions causes haemolysis and oxidation of Hb, and conversely, Hb promoted further lipid oxidation. In contrast, Boyle *et al*. reported that erythrocytes, Hb and heme, released by intraplaque haemorraghe, stimulated cholesterol efflux and decrease oxidative stress in macrophages [151]. Similarly, Finn *et al*. reported that haemoglobin/haptoglobin stimulated macrophages expressed more ferroportin, had less intracellular iron and ROS production and were more resistant to cholesterol loading due to upregulation of the ATP-binding cassette transporter [152]. Two peaks were identified by mass spectrometric analysis corresponding to alpha and

beta chains of Hb associated with proinflammatory high-density lipoprotein (HDL). HDL-associated Hb is predominantly in the oxygenated (oxyHb) form with distinct physical and chemical properties. Furthermore, oxyHb-containing proinflammatory HDL potently consumed NO and contracted arterial vessels *ex vivo* [153]. Apart from the oxidative toxicity of Hb, it is a potent scavenger of NO - a critical regulator of vascular tone, endothelial function and thrombosis. Hb functions as an allosterically and redox-regulated nitrite reductase whose "enzyme activity" couples hypoxia to increased NO-dependent blood flow [154]. Hb potently inactivates the NO radical forming nitrate and metHb, producing endothelial dysfunction under haemolytic conditions [155]. Free heme can threaten vascular endothelial cell integrity through oxidative modification of low-density lipoproteins (oxLDLs) [156]. Initially, most of this oxLDL is contained within foam cell lysosomes primarily in the form of the peroxidised lipid-protein complex ceroid [157]. Specifically, ceroids are insoluble sudanophilic polymers of oxidised cholesterol and proteins; they are hallmarks of atherothrombotic pathology [158]. Development of ceroids could colocalise with iron deposits within cell and tissue [159], and presence of Hb and MPO has been identified in ceroids [160]. Similarly, ceroids can be found in intraluminal thrombus of abdominal aortic aneurysm [149]. These data also provide evidence of the role played by heme-derived iron in the genesis of tissue oxidative process, modifying both lipids and proteins. Iron is an essential element that plays crucial roles in cell proliferation and metabolism by serving as a functional constituent of various enzymes. However, when present in excess, free iron generates ROS, e.g. via the Fenton reaction. In this respect, another iron species named non-transferrin bound iron seem to play an important role in the ROS-induced toxicity both at the cell membrane but also intracellularly [161]. Conversely, ROS themselves can modulate iron homeostasis as a defense mechanism against iron-induced oxidative stress, since the expression of many genes involved in iron transport and storage is regulated by ROS [162]. Human carotid atherosclerotic lesions contain 3- to 17-fold more iron than healthy control arteries [163]. Iron can mediate both lipid and protein oxidation. However, it has been suggested that elevated levels of iron contribute to the extent of protein, but not lipid, oxidation in advanced human atherosclerotic lesions [164]. The source of redox-active iron in vascular tissue could involve both RBC lysis and erythrophagocytosis. Most of the iron within vascular lesions is associated with phagocytes [165]. The storage and processing of iron from erythrophagocytosis or other sources within vascular lesions could have an important role in disease initiation and progression.

9. Conclusion: ROS, atherosclerosis and some related points of research

In the vasculature, reactive oxygen species (ROS) generated by both mitochondrial respiration and enzymatic sources serve as integral components of cellular signaling and homeostatic mechanisms. Because ROS are highly reactive biomolecules, the cellular redox milieu is carefully maintained by small-molecule antioxidants and antioxidant enzymes to prevent the deleterious consequences of ROS excess. When this redox balance is

perturbed, because of either increased ROS production or decreased antioxidant capacity, oxidant stress is increased in the vessel wall and, if not offset, vascular dysfunction ensues. A number of heritable polymorphisms of pro-oxidant enzymes, including 5-LO, COX-2, eNOS, and NOX, have been identified and found to modulate ROS production and, thereby, the risk of atherothrombotic cardiovascular disease in individuals with these genetic polymorphisms [166]. Similarly, heritable deficiency of the antioxidant enzymes catalase, GPx, glutathione-S-transferases, heme oxygenase and glucose-6-phosphate dehydrogenase favors ROS accumulation, and has been associated with an increased risk of vascular disease. The scope of this review was to examine several possible sources of ROS as risk factors for atherosclerosis. According to literature, NOX may play a central role in development of various vascular diseases, they are considered as a source of "kindling radicals" affecting other enzymes [15]. Thus, elevated activity of NOX1 in SMC results in uncoupling of eNOS and converting XOR to xanthine oxidase, thus making them additional sources of superoxide [28, 29]. In the atherosclerotic plaque, XO-mediated ROS formation is pro-inflammatory [71]. The uncoupled eNOS can simultaneously produce NO and O2-, they react to generate peroxinitrite, which is another uncoupler of NOS [85].

Calcium-dependent NOX5 has been implicated in oxidative damage in human atherosclerosis, whereas NOX4 may have a role in protecting the vasculature [15]. CYP-generated ROS can also play a dual role in development of atherosclerosis and vascular tone, inducing pro-inflammatory effects through expression of addressin genes and adhesion molecules [78], though generating EDHF in certain conditions [82].

Nox2 and p22phox proteins were elevated throughout lesion development [50]. In phagosomes, superoxide generated by NOX2 interacts with chloride ions under catalytic control of MPO, with production of hypochlorite [30]. Serum MPO levels were correlated with restenosis and atherosclerotic lesions [92]. Activation of the lipoxygenase and cyclooxygenase signaling pathways is also coupled with activation of NOX2 [31, 32, 108, 109]. COX and evidently LO per se cannot serve as a source of pathogenic ROS, though their expression and/or activity can be affected by ROS. Reciprocally, LO- and COX-generated arachidonic acid metabolites can induce ROS generation by stimulating NOX [115, 118].

Mitochondrial ROS of endothelial cells and lesional macrophages contribute to initiation of inflammatory reactions via NFkB to recruit leukocytes into the intima, and successive oxidative burst in activated neutrophils can exceed the initial generation of ROS [60, 61, 62]. Endothelial cells and especially leucocytes can damage RBC, though in hypoxic conditions RBC can independently generate ROS due to autoxidation of Hb. Oxidative damage to RBC membrane proteins is responsible for the impaired cellular deformability [134]. Elevated levels of iron contribute to the extent of protein, but not lipid, oxidation in advanced human atherosclerotic lesions [164]. The source of redox-active iron in vascular tissue could involve both RBC lysis and erythrophagocytosis [165].

The relations between different resources of ROS and atherosclerosis are summarized in Figure 1.

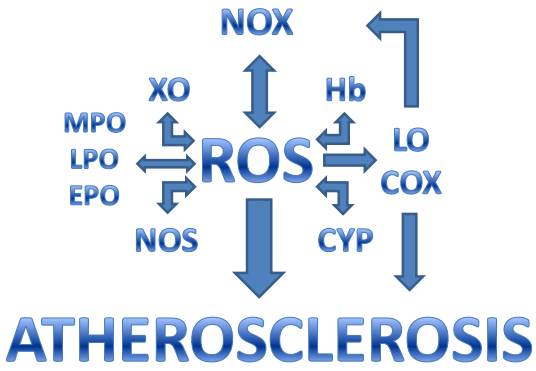

Figure 1. Contribution of various sources of ROS in development of atherosclerosis.

ROS play a critical role in vascular tone and disease. A break in integrity of endothelium can be induced by ROS effects on the key enzymes of metabolism [166, 167], as well as on different signaling and effector pathways, i.e. receptors of plasma membrane, kinases and phosphatases, transcriptional factors and their interaction with DNA, translation, processing and secretion of integrins and adhesion proteins, cytoskeleton and its interaction with plasma membrane [4, 80]. The levels of oxidative stress have been implicated in the pathogenesis associated with cancer, hypertension and atherosclerosis [168]. Among the functional effects of ROS are: metabolic control in cells [169]; regulation of HIF-1α in hypoxia [170, 171]; triggering of autophagy through a cysteine protease Atg4 [172]; formation of inflammasome NLRP3; and launch of inflammation [173, 174, 175]. ROS mediate effects of proinflammatory cytokines (TNF-alpha, IFN-gamma, IL-1) and are considered to be atherogenic factors [176, 177, 178, 179]. On the other hand, proapoptotic oxidative or endoplasmic reticulum stress inducers trigger another stress reaction in macrophages, autophagy, which becomes dysfunctional in atherosclerosis and its deficiency promotes atherosclerosis in part through inflammasome hyperactivation. Inhibition of autophagy by silencing ATG5 or other autophagy mediators enhances apoptosis and NOX-mediated oxidative stress while at the same time rendering the apoptotic cells less well recognized by efferocytes [180, 181].

Having a strategic position between blood and tissues, the endothelium controls hemostasis perfusion, inflammation, and recruitment of inflammatory cells; on the other hand, an increased level of ROS can damage endothelium and enhance expression of proinflammatory genes [182, 183]. Superoxide anion generated by monocytes/macrophages in oxidative burst is considered to be the principal source of atherosclerosis [184, 185,

186]. OxLDL induce expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin, enhancing adhesion of monocytes to endothelial cells and foam cells formation, and leading to cytotoxicity, inflammation and atherosclerotic lesions [187, 188, 189]. ROS also induce secretion of Weibel-Palade bodies (WPB) from endothelial cells [190, 191]. A similar effect coupled with elevated expression of P-selectin is induced by activated platelets [192]. P-selectin of platelets and endothelial cells, and E-selectin of endothelial cells, serve as receptors of leukocytes that recognize specific epitopes on the surface of neutrophils and monocytes [192, 193]. The level of P-selectin in blood is tightly correlated with probability of a lethal outcome for atherosclerotic patients [194]. The WPB are widely known to be reservoirs of van Willebrand factor (VWF); along with VWF and P-selectin, these unique organelles of endothelial cells secrete VWF-propolypeptide (proregion), endothelin-1, angiopoietin-2, interleukin-8 and eotaxin-3 (attractants for neutrophils and eosiniphils, respectively), CD63 antigen and some other substances [195, 196, 197, 198, 199, 200]. It should be noted here that extracellular inducers of WPB excretion are vasoactive agonists thrombin and histamine [198, 201]. It has been revealed that thrombin causes a two-phase elevation of ROS in endothelial cells (in 15 min and 3 h), accompanied by increased expression of mRNA subunit $p22^{phox}$. H_2O_2 causes a similar effect, whereas vitamin C and diphenyleneiodonium (DPI) prevents it. The effect of thrombin is mediated by p38 MAP-kinase and PI3K/Akt. The positive feedback mechanism consists of induction of a small quantity of ROS through NOX with further expression of $p22^{phox}$ and synthesis of a significant amount of ROS [201]. Also, ROS mediate the angiotensin II (AII)-induced dysfunction of endothelial cells. AII triggers translocation of Nox2 to endothelial plasma membrane and its association with adenosine receptor A2A; simultaneously, phosphorylation of $p47^{phox}$, ERK1/2, p38 MAPK and Akt is observed, with further production of ROS and reduction of endothelium-dependent relaxation in response to acetylcholine [202]. SCH58261, an antagonist of A2AR, decreases production of ROS and retains reaction to acetylcholine. Redox regulation of vascular NOXs activation provides both negative feedback and feed-forward regulation mechanisms. For example, Rac1 protein turnover is strongly dependent on the redox status of the cell, creating a negative feedback mechanism [203]. At the same time, a feed-forward mechanism exists, by which exogenous exposure of SMC or fibroblasts to H2O2-activated NOX stimulates them to produce endogenous superoxide anion, thereby amplifying the vascular injury process [204]. The self-limiting mechanism could predominate during physiological conditions, and be involved in maintaining low output of a non-phagocyte NOX, whereas the feed-forward mechanism may have a role in the NOX-dependent oxidative stress in a variety of diseases, including atherosclerosis [15].

It can be readily seen from these and many other data obtained recently that there are tight relations between ROS generation and vascular functions in the normal physiological state (signaling) and various pathologies, atherosclerosis being among them. Unfortunately, there are no embracing studies on interrelations,

feedback and feed-forward regulation mechanisms of different sources of ROS generation, described in this review, in development of atherosclerosis and other vascular pathologies. Discovery of these relations constitute a genuine challenge and would require great endeavor.

A better mechanistic understanding of mechanisms of ROS production and effects, their balance in normal state and imbalance in atherosclerosis, their control by enzymatic and non-enzymatic defense mechanisms, and their feedback and feed-forward regulation mechanisms may lead to more effective prophylactic and/or therapeutic strategies.

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