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Role of endothelial cell stress in the pathogenesis of chronic heart failure

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1. ABSTRACT

Endothelial cells are key modulators of diverse physiological processes, and their impaired function is a cause of numerous cardiovascular diseases. Under physiologic condition, the reactive oxygen and nitrogen mediators in endothelia lead to the signal propagation of the initial stimulus, by forming molecules with a longer half-life like hydrogen peroxide. Hydrogen peroxide is the focus of growing attention in endothelial biology, and consequently the enzymes involved in its generation and clearance are viewed as novel mediators of great importance. In particular, among peroxidases, myeloperoxidase is recognized as a key enzyme, capable of impairing intracellular NO reservoirs as well as producing oxidized amino acids such as 3-chlorotyrosine or 3-nitrotyrosine. This process switches the functional pathways from normal signalling to a condition characterized by oxidative and/or nitrosative stress. Understanding the molecular mechanisms involved in these stress responses in endothelium will lead to better therapeutic strategies for oxidative stress-driven cardiovascular diseases.

2. ENDOTHELIAL PHENOTYPE AND FUNCTION

Endothelial cells (ECs) are derived from the mesoderm germ layer, that together with haematopoietic tissues, develop early in the embryo. The first blood islands appear in the yolk sac, as a result of the proliferation of angioblasts, i.e. endothelial cell precursors (1). Subsequently, isolated foci of endothelial cells are observed in the embryo proper, as clusters of angioblasts which proliferate in the primitive heart tube. This leads to the formation of the vitelline vessels, that give rise to the circulatory system in the embryo (2,3). In these processes, a series of molecular switches between "migratory" and "non migratory" phenotypes ensures the proper distribution of angioblasts and the formation of vascular networks (4). Interestingly, angioblasts are also found in adult tissues, and endothelial progenitor cells are characterized as circulating adult precursors (5). Later in development, endothelia in other vascular territories differentiate directly from mesenchymal cells, under the induction of endoderm or ectoderm derived tissues. Initiation of vessel development requires a complex molecular signalling, with different stimuli including but not limited to basic

fibroblast growth factor (βFGF) and vascular endothelial growth factors (VEGFs). The essential role of VEGF in development was demonstrated by gene knockout experiments which resulted in embryonic lethality (6,7). Interestingly, the action of VEGFR-3 appears crucial in the fate of the vessel, to be an artery or a vein. VEGFR-3 is expressed later in development only on ECs that will form the *intima* of veins or lymphatic vessels (8). The ability of ECs to form capillary-like tubes is also regulated by the extracellular matrix, such as collagen type I and matrigel that promote the assembly of vessel-like structures *in vitro*.

2.1. *In vitro* models of endothelial biology and heterogeneity

The study of endothelial function gained a boost in the 1970s with the development of techniques for isolation and culture of endothelial cells *in vitro* (9,10,11). In particular, HUVECs (human umbilical vein endothelial cells) have been widely used as a model for understanding endothelial biology under physiologic and pathologic conditions (12,13). The need to obtain organ-specific endothelial cells arose from observations that vascular beds show a great heterogeneity in different organs and tissues (14,15,16). For this reason, the phenotypic characterization of an endothelial cell should be based on more than one molecule (e.g. on both CD31 and vWF, rather than just one) since endothelia in different vessels do not uniformly express these two markers (17,18,19). This phenomenon is particularly striking when dealing with stable endothelial cell lines, used in *in vitro* experiments. Most of the lines, in fact, lack one or more cellular markers, adhesion molecules or receptors, therefore justifying research efforts to use primary cell lines as HUVEC or organ-specific microvascular cells as primary models for cardiovascular biology and diseases (20).

2.2. Endothelial functions in vascular biology

Among the number of functions which characterize endothelial cells *in vivo* is their ability to provide a structural barrier between the circulation and the surrounding tissues. By the process of transcytosis, ECs transport molecules from the luminal to the basal side (21,22,23). In addition, ECs contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as nitric oxide (NO) and vasoconstrictors. NO is receiving great attention in view of the multiple roles that it plays in vascular biology and cellular signalling processes. ECs elaborate NO by the activity of one of the NO synthases (NOS), eNOS, which is constitutively expressed in ECs (24). All isoforms of NOS require the regulated formation of an active dimer to produce NO. EC-derived NO has several important effects on the vasculature. NO maintains basal vessel tone and provides endothelium-dependent vasorelaxation by relaxing vascular smooth muscle cells through the binding of NO to the heme prosthetic group of guanylyl cyclase. Moreover, endothelial-derived NO also inhibits platelet and leukocyte adhesion. In addition to these effects on the vasculature, endothelial-derived NO inhibits smooth muscle cell migration and proliferation, and limits neointimal proliferation after vascular injury (25,26,27,28). In addition to its stimulatory effect on EC migration and proliferation, NO sustains vascular reparative mechanisms (29).

3. ROLE OF ROS IN ENDOTHELIUM

The ROS (reactive oxygen species) family includes many molecules that have multiple effects on cellular functions, such as regulating cell growth and differentiation, limiting bioavailability of NO, and stimulating many proinflammatory genes (30). Many of these processes are directly linked to endothelial and cardiovascular physiology, and their impairment secondary to alterations of the ROS equilibrium in cells may lead to the pathological features observed in many cardiovascular diseases. Reactive oxygen species, also known as "oxygen-derived species" are intermediate products in redox reactions of the cellular metabolism. Among ROS, two main groups of compounds can be described. The first group includes the free radicals (e.g. superoxide [$\cdot O_2^-$], hydroxyl radical [OH·], nitric oxide [NO·]), which are chemical species containing one or more impaired electrons (31). The presence of an unpaired electron confers a high chemical reactivity to these ROS, while rendering the radical itself a quite unstable molecule. A second group comprises the nonradical derivatives of oxygen (e.g. hydrogen peroxide [H_2O_2], peroxynitrite [$ONOO^-$]). These compounds are less reactive than free radical ones, and more stable, with a longer half-life (32). Moreover, the chemical features of the different compounds may also have profound effects in terms of diffusibility within the cellular environment and between adjacent cellular populations. In fact, in living cells, O_2^- has a short half-life, since it is rapidly dismutated to hydrogen peroxide by superoxide dismutase (SOD) enzymes. Moreover, the charged ion crosses the cell membrane and cellular compartments slowly, while H_2O_2 has a longer biological lifespan than superoxide, being more stable, and is known to diffuse more easily between different cells and cellular compartments. Dysfunction of the endothelial cell layer is a process characteristic of several cardiovascular pathologies. Among the key factors that promote endothelial dysfunction is disequilibrium in the cellular concentrations of reactive oxygen species (ROS), which may be accompanied by, or lead to, a reduced bioavailability of NO, therefore linking together the two aspects of cellular stress events: oxidative stress and nitrosative stress (33). A number of ROS may contribute to cardiovascular pathologies by direct chemical interactions, e.g. by oxidation of the cellular constituents, by influencing the bioavailability of NO, or by influencing the signal transduction pathways (34). Endothelium constitutes a particular example of tissue in which the basal levels of some ROS, e.g. hydrogen peroxide, are more elevated than in other cell types. Moreover, several enzymes in these cells may constitute potential sources of ROS. One example is NADPH oxidase, together with uncoupled eNOS or xanthine oxidase (XO) (35,36). On the other hand, the balance between ROS-producing and ROS-scavenging mechanisms is of key relevance for the maintenance of a proper endothelial function. Therefore, the reduction of ROS-scavenging processes, characteristic of chronic heart failure, may contribute to the development of oxidative stress conditions (37). In addition, redox signalling has been shown to be fundamental in the development of the autonomous proliferative response following hypoxia,

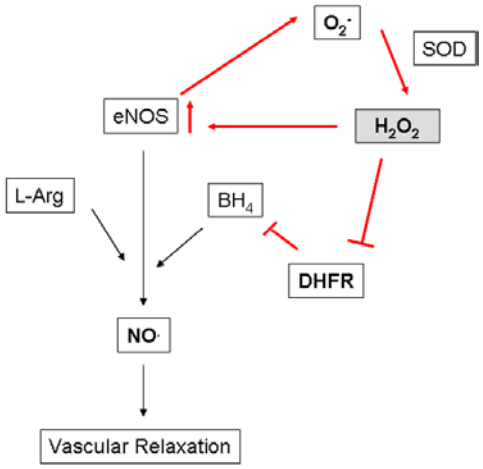

Figure 1. Physiological pathway of eNOS activity and uncoupling in response to hydrogen peroxide. In physiological conditions (black arrows), eNOS provides endothelium-mediated vasorelaxation by producing NO using L-arginine and tetrahydrobiopterin (BH4) as a substrate. In pathological conditions, an imbalance in intracellular concentration pf ROS develops (red arrows) and eNOS becomes uncoupled acting as a source of superoxide ion. Superoxide may then be dismutated to hydrogen peroxide, and prevents recovery of oxidized BH4 (via DHFR inhibition). This circuit of oxidative stress leads to the reduction of bioavailable NO.

together with the MEK/ERK pathway (38). More importantly, in heart failure additional processes involving the delicate equilibrium between ROS and RNS species have been observed. In fact, xanthine oxidase, a superoxide-producing enzyme, is upregulated following heart failure (39,40). Xanthine oxidase is a variant of XDH (xanthine dehydrogenase) resulting from modification of the native enzyme by two different mechanisms: in fact, XDH may undergo either an irreversible proteolytic cleavage or an oxidation of sulfhydryl residues. Moreover, the cofactor used by XO is molecular oxygen (which is then reduced to O2–). The production of O2•– by XO has an obvious potential pathophysiologic relevance (reviewed in 33,40). In fact, upregulation of xanthine oxidase in the failing heart may lead directly to the development of oxidative stress (41). Interestingly, inhibition of XO ameliorates the remodeling process in post-infarct heart. The beneficial effects of XO inhibition are dependent on NOS activity, therefore not only should XO activity be reduced, but also NO supply must be maintained active via the NO-producing enzymes (42). Therefore, the imbalance between enzymes (NOS versus XO) in the failing heart reflects the impairment in NO and superoxide levels. While, in normal physiologic conditions, the equilibrium is shifted towards an abundance of NO with respect to superoxide, favoring S-nitrosylation reactions, in heart failure superoxide is prevalent, and this favours the development of oxidative stress as a result of the oxidation reactions taking place (33).

3.1 Hydrogen Peroxide in endothelium: sources and functions

Hydrogen peroxide is a reactive oxygen species which is capable of exerting several physiological effects on endothelial cells. Within the cell a minimum level of hydrogen peroxide needs to be maintained for it to act on cellular proliferation (43). Importantly, H_2O_2 has emerged as a real signalling intermediate in endothelium, since its action leads to an increased transcription of VEGF, as well as of other growth factors (44,45). However, excess levels of hydrogen peroxide induce cell death. This has been demonstrated in several model systems *in vitro*, using HUVEC cells or organ-derived microvascular endothelial cells. Several reports have indicated that doses of hydrogen peroxide over 50μM in the culture medium lead to an increase in apoptosis in HUVEC cells (46,47), which increase at higher doses (48). Interestingly, even clonogenic endothelial progenitor cells showed a high sensitivity to oxidative stress (49). Moreover, similar doses of hydrogen peroxide may have different effects on cellular viability depending on the type of endothelial cells used for the experiment (e.g. artery-derived vs. vein-derived). In a very recent paper, La Rocca *et al.* showed that exposure of HUVEC to 60μM hydrogen peroxide for 3 and 6 hours resulted in a slight and insignificant decrease of viability (0.53 to 2.86 % less). When the same dose was used on endocardial endothelial cells derived from the endocardium of CHF patients, these cells responded with a lower decrease in viability (0.8 to 1.1%) thus suggesting that endothelial cells isolated from an organ "primed" for oxidative stress should be even more resistant to exogenous stressor molecules (50). In addition, nitric oxide has been shown to possess a dual contrasting effect on H2O2 mediated toxicity, limiting or enhancing its toxicity with respect to the concentrations of both species (51). Endothelial hydrogen peroxide has been shown to cause endothelium-dependent vasorelaxation, a process largely due to the action of eNOS. In fact, it has been demonstrated that "in vitro" hydrogen peroxide should upregulate eNOS transcription (52). This should lead to an increase in NO bioavailability, thereby causing vascular relaxation. It has recently been postulated that hydrogen peroxide can serve as a coupler of myocardial oxygen consumption to coronary blood flow increase. In fact, since superoxide ion is produced in proportion to the rate of cardiac metabolism, its dismutation leads to an increase in H_2O_2 concentration, which then exerts its vasoactive effects (53). More recently, Kang and co-workers suggested a novel role for the eNOS pathway, and in particular for eNOS derived hydrogen peroxide, in maintaining vasorelaxation in a hypertension animal model (54). On the contrary, in cardiovascular pathologies a number of factors should lead to the uncoupling of NOS. This process causes the arrest of NO production and formation of the superoxide ion (O_2^-). This process should have different causative factors: one could be the reduction of bioavailability of L-arginine, the enzyme substrate, due to its chlorination by hypochlorous acid (as stated below). On the other hand, the deficiency of BH_4 (tetrahydrobiopterine, an essential cofactor in NO synthesis) could be due to the down regulation of DHFR (dihydrofolate reductase) by hydrogen peroxide (see Figure 1). In fact, DHFR is involved in recycling of oxidized BH_4, a molecule which has been linked to eNOS uncoupling in several reports (55,56,57,58).

4. EMERGING ROLES FOR MYELOPEROXIDASE IN ENDOTHELIAL BIOLOGY

Myeloperoxidase is a heme protein characteristically present in different subsets of azurophilic granules of neutrophils (59). It has been recently reported that MPO expression is not restricted to the myeloid lineage. In a recent work, Green and co-workers suggested its expression in granule-containing and pyramidal neurons of the hippocampus, and the expression was demonstrated also in neuronal cell lines (60). During the differentiation of monocytes into macrophages, MPO expression is lost, but the gene can be reactivated in certain subsets of macrophages (61,62). In addition, macrophages are able to uptake the molecule from the extracellular space via receptor-mediated endocytosis, and this mechanism has also been proposed as a way to provide enzyme clearance after its activity (63,64). The historical function of this enzyme is to provide microbicidal activity against pathogens. In the typical pathway of phagocyte activation, MPO secretion is accompanied by an oxidative burst, in which NADPH oxidase provides superoxide and its dismutation product, hydrogen peroxide (65). In fact, neutrophils employ MPO together with hydrogen peroxide and halide ions (e.g. Cl-, Br-) as a powerful antimicrobial tool, by using hydrogen peroxide to form more powerful oxidants such as hypochlorous acid (HOCl) (66,67,68,69). The combined presence of MPO and peroxide, rather than peroxide alone, is able to guarantee an effective antimicrobial activity of phagocytes (70). In particular, it has been shown that while in neutrophil-mediated antibacterial defence both bacterial protein nitration and chlorination may take place during phagocytosis. The latter process is mediated by MPO, while nitration follows an alternative pathway, thereby reinforcing the idea of MPO as a key enzyme in chlorinative pathways (71).

4.1. MPO: interactions with endothelial cells

While the role of MPO following neutrophil activation in the oxidative burst is well established, the effects of this enzyme on endothelial biology have recently come to light.

One of the better characterised processes of MPO intervention in endothelial homeostasis is the depletion of intracellular NO, to produce nitrogen dioxide, which can in turn be converted to nitrogen radical ($NO_2^{\cdot}$). This mechanism requires a two-step redox reaction with the formation of intermediate compounds (compound I and compound II) by interaction with hydrogen peroxide (72,73). Nitrogen radical action results in the formation of oxidized amino acid residues, such as 3-nitrotyrosine. This covalent modification should affect both free and protein-incorporated amino acids. In the latter case, the introduction of this group would perturb protein conformation, so leading to loss of activity. This process, called “nitrosative stress” refers to the accumulation of tyrosine-nitrated proteins, leading to impairment of protein and cellular functions (74,75). Moreover, MPO is the only mammalian enzyme capable, at the physiological level of the halides, of producing HOCl, a more powerful oxidant than hydrogen peroxide. Hypochlorous acid action results in a number of modifications of biological molecules. Indeed, one of the hallmarks of HOCl action is the accumulation of chlorinated proteins, due to the formation of 3-chlorotyrosine residues. The action of such a strong chlorinating oxidant as HOCl is directed towards a number of biological molecules, for example cellular amines and lipids can undergo chlorination (76,77). However, even if there is a biochemical capability for such product formation, most of the chlorinated species have a short half-life in the cellular environment. Therefore, even if it appears as the tip of an iceberg, .3-chlorotyrosine is perhaps the best marker of HOCl activity in living cells, due to the production of low, but detectable, levels of this non-physiologic product (69). Hence, accumulation of chlorinated end-products of MPO-derived HOCl constitutes a molecular fingerprint of the activity of this enzyme.

Oxidized lipids are another important pathological end-product of HOCl action; consequently, a role of this enzyme in atherosclerosis development has been postulated. LDL appeared as a target for MPO-dependent oxidation, and recent advances suggested that HDL may be oxidized too, therefore reverting their protective effect on vasculature (78,79). Interestingly, human MPO-overexpression, in a mouse model of atherosclerosis, promoted the development of pathology in experimental animals, thereby confirming the causative link between lipid oxidation and disease (80).

4.2. MPO and endothelium: *in vivo* evidence and novel *in vitro* models

A concept which has emerged in the last few years regarding the development of chronic cardiovascular pathologies like CHF (chronic heart failure) is that myeloperoxidase levels may be causatively linked to the presence of a cardiovascular disease (81,82,83). Endothelial dysfunction, as reflected by an imbalance between oxidative and nitrosative stress, has also been related to myocyte hypertrophy and to the development of chronic heart failure (84,85). In a very recent study, Mocatta and co-workers showed that myeloperoxidase levels were elevated in plasma after acute myocardial infarction. Moreover, MPO emerged as a risk factor for long-term mortality, its prognostic value being comparable to that of proBNP (86). Given its role as a potential marker of endothelial dysfunction reflecting the grade of cardiovascular disease, there is a strong interest to discover the ways MPO can interact with endothelium and what kind of pathways may be influenced by the enzyme activity. One of the current models of MPO-endothelium interaction is based on the capability of this enzyme to cross an intact endothelial layer by a process known as transcytosis. Therefore, endothelial cells can translocate the molecule from the vessel lumen to the basement membrane, where the enzyme has been reported to specifically trigger extracellular matrix protein nitration, as recently shown for fibronectin (21,87,88). Transcytosis appears to be performed via an interaction between MPO and albumin, and with albumin binding proteins in caveolae (23,89). The open question of this paper remains the way in which a neutrophilic enzyme such as MPO may

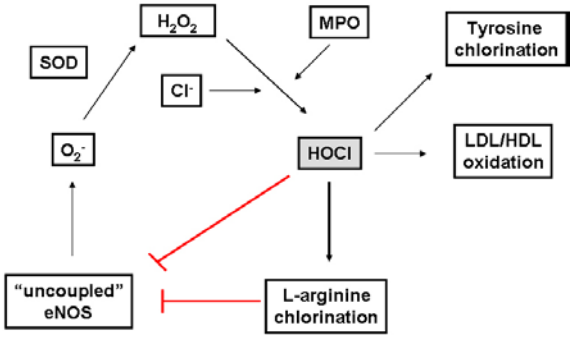

Figure 2. Pathway for production of hypochlorous acid, generation of oxidative stress and chlorination of protein and lipid targets in endothelial cells. MPO is known as the sole mammalian enzyme capable of forming HOCl *in vivo* in the presence of hydrogen peroxide and chloride ions. HOCl is a more powerful oxidant than hydrogen peroxide. One of the targets of HOCl is L-arginine, which becomes chlorinated, hence subtracting the substrate of eNOS for the formation of NO. This process leads to the uncoupling of eNOS, and to switching the enzymatic activity towards superoxide ion production. Superoxide may be dismutated to hydrogen peroxide, providing the molecules necessary for MPO activity. In addition, HOCl may disrupt the activity of eNOS dimers, reinforcing the uncoupling of the enzyme and increase in oxidative stress.

interfere with intracellular pathways in endothelial cells. Since MPO interaction with endothelium has been demonstrated *in vitro* and *in vivo*, it can be argued that in the development of the inflammatory process, the combined presence of MPO and hydrogen peroxide produced by neutrophils should have an effect on endothelial cells .In a very recent work, La Rocca and co-workers demonstrated for the first time the capability of endothelial cells, subjected to oxidative stress by hydrogen peroxide exposure, to express endogenous myeloperoxidase. This expression took place after exposure to a non-lethal dose of hydrogen peroxide for 3 and 6 hours, and led to the accumulation of 3-chlorotyrosine in HUVEC and in human endothelial cells derived from human endocardium (50). The innovative findings of this paper should add a new interpretation to the relevant role of MPO in endothelial biology. As stated previously, an enzyme like MPO enters the cellular pathways in endothelium in several ways. First, MPO should use hydrogen peroxide to produce, via the two step reaction of compounds I and II, nitric dioxide radical, an extremely reactive nitrogen species that should bind covalently to tyrosine residues, both free and incorporated in proteins, thereby forming 3-nitrotyrosine and leading to nitrosative stress (90). Moreover, MPO is known to be the sole mammalian enzyme capable of forming HOCl *in vivo*, and therefore its action should be indirectly demonstrated by the analysis of the accumulation of chlorinated end products in both endothelial cells and body fluids (90,91). HOCl is a more powerful oxidant with respect to hydrogen peroxide, and it should have several intracellular targets. One of its targets is L-arginine, which becomes chlorinated, therefore subtracting the substrate of eNOS for the formation of NO (81,92). This process leads to the uncoupling of eNOS, thereby switching the enzymatic activity towards superoxide ion production. Superoxide may in turn deplete the intracellular reservoir of NO, by a fast reaction which leads to peroxynitrite formation, or may be dismutated to hydrogen peroxide, thereby providing the molecule necessary for MPO activity (Figure 2). In a recent paper, Xu and co-workers demonstrated the capability of HOCl to disrupt the *in vitro* activity of purified eNOS. This effect was confirmed in HUVEC cells, where HOCl abolished eNOS activity by disrupting the enzyme dimers at concentrations even 100 fold lower than those needed with the recombinant protein (93).

5. CONCLUSIONS AND PERSPECTIVES

The importance of hydrogen peroxide in endothelial biology is the subject of a growing interest, and the enzymes involved in both its generation and clearance are now viewed as novel mediators of great importance. The generation of oxidative stress, represented by an imbalance between ROS production and scavenging, may lead to the accumulation of oxidized molecules and in impaired cell function. In particular, myeloperoxidase has gained much attention in recent years as a key enzyme capable of impairing intracellular NO reservoirs as well as of producing oxidized amino acids such as 3-chlorotyrosine or 3-nitrotyrosine. This process switches the functional pathways from signalling to the development of oxidative stress. The pathophysiological link between MPO levels in body fluids and severity of the cardiovascular disease is well established, and a complex picture is emerging on intracellular and intercellular regulator processes involved in oxidative stress. In assessment of the level of stress, the measurement of expression level of MPO (e.g. by determining protein concentration in body fluids), should be coupled with evaluation of its activity, and accumulation of end-products that result from such activity. Accurate measurement of level of 3-nitrotyrosine or 3-chlorotyrosine, non-physiologic end products of MPO-driven oxidative and nitrosative stress, provide an additional level of clinical information. Moreover, the target proteins of such oxidizing processes should also be evaluated, in order to provide new potential targets for therapeutic intervention. Much work still needs to be done in terms of pharmacological tools to counteract the activity of the oxidants. In fact, several clinical trials using antioxidants have provided contrasting results. Obviously, when stable lesions in post-infarct patients are present, antioxidants should have a limited, effect. On the other hand, their administration in the initial phase of disease, during the increase of ROS activity, could perhaps be beneficial. Moreover, not all oxidants act in the same way; some (e.g. HOCl) are very powerful with respect to the molecules used in pharmacological testing. Together the presented data show that a better understanding of the fine molecular mechanisms is required in treatment of the oxidative stress-driven cardiovascular diseases.

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Abbreviations: βFGF: basic fibroblast growth factor; BH4: tetrahydrobiopterine; BNP: brain natriuretic peptide; CHF: chronic heart failure; DHFR: dihydrofolate reductase; ECs: endothelial cells; ERK: extracellular signal-regulated kinase; HDL: high density lipoprotein; HUVECs: human umbilical vein endothelial cells; LDL: low density lipoprotein; MEK: mitogen regulated kinase; MPO: myeloperoxidase; NADPH: nicotinamide adenine dinucleotide phosphate; NO: nitric oxide; NOS: nitric oxide synthase; RNS: reactive nitrogen species; ROS: reactive oxygen species; VEGF: vascular endothelial growth factor;

VEGFR: vascular endothelial growth factor receptor; vWF: von Willebrand factor; XO: xanthine oxidase

Key Words: Endothelial cells, Myeloperoxidase, Hydrogen Peroxide, Oxidative Stress, Enos, Nitric Oxide, Superoxide, ROS, RNS, 3-Chlorotyrosine, 3-Nitrotyrosine, Nitrosylaton, Review

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