

Review

Oxidative Stress-Induced Endothelial Dysfunction in Cardiovascular Diseases

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Abstract

Cardiovascular disease (CVD) is a major cause of mortality worldwide. A better understanding of the mechanisms underlying CVD is key for better management or prevention. Oxidative stress has been strongly implicated in the pathogenesis of CVD. Indeed, several studies demonstrated that reactive oxygen species (ROS), via different mechanisms, can lead to endothelial cell (EC) dysfunction, a major player in the etiology of several CVDs. ROS appears to modulate a plethora of EC biological processes that are critical for the integrity of the endothelial function. This review seeks to dissect the role of oxidative stress-induced endothelial dysfunction in CVD development, with emphasis on the underlying mechanisms and pathways. Special attention is given to ROS-induced reduction of NO bioavailability, ROS-induced inflammation, and ROS-induced mitochondrial dysfunction. A better understanding and appraisal of these pathways may be essential to attenuate oxidative stress or reverse EC dysfunction, and hence, reduce CVD burden.

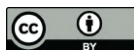
Keywords: endothelial cell dysfunction; oxidative stress; reactive oxygen species (ROS); cardiovascular diseases (CVDs); nitric oxide; eNOS uncoupling; inflammation; mitochondrial dysfunction

1. Introduction

Cardiovascular disease (CVD) remains the number one killer disease where it claims 17.9 million lives each year accounting for 31% of the total deaths in the world. Several risk factors such as sedentary lifestyle, tobacco smoking, air pollution, an unhealthy diet, physical inactivity, and obesity contribute to the increasing incidence of CVDs [1,2]. Importantly, most of these risk factors are modifiable [3], providing a means of preventive and therapeutic interventions. CVD risk factors could manifest as elevated blood pressure (hypertension), increased blood glucose levels (diabetes mellitus), in addition to overweight, abnormal blood lipids (dyslipidemia), and obesity [3–5]. Of note, inflammation has been recently recognized as a critical CVD risk factor [6–9]. Relatedly, oxidative stress has been implicated, at least in part, in the incidence and pathogenesis of several CVDs [10] and as such, this review will highlight the involvement of oxidative stress in the pathogenesis of CVDs.

Despite extensive efforts to curb their incidence and progression, CVDs continue to cause increasing economic and health burden of across the globe [1,10,11]. Consequently, there is an immediate need to find new therapeutic routes of CVDs in order to reduce their burden. Hence, understanding the role of oxidative stress in relation to the progression of different CVDs is a step in this direction.

Oxidative stress can result from the over-production or accumulation of free radical reactive species such as the oxygen reactive species (ROS), nitrogen reactive species (RNS), and reactive sulfur species (RSS) [12]. Inside the cell, oxidative stress is under a tight control [13]. Very low levels of the free radical reactive species are normally produced as by-products of cellular metabolic processes [14] where several of these species, such as hydrogen peroxide (H₂O₂), participate in the physiological signal transduction taking place inside the cell [13]. In addition, they are implicated in balancing the redox state of the cell and can participate in redox signaling, such as redox reactions of cys-



teine residues of proteins leading to activation/inactivation of proteins [15]. On the other side, excessive production of free radical reactive species is associated with several pathologies, including CVDs [14,16–19].

Among the reactive species, ROS gets the lion's share of investigations, apparently because it contributes to physiological signaling and the maintenance of cellular redox state. ROS, which include molecules such as H₂O₂, superoxide anion (O₂^{•-}), and hydroxyl free radical (•OH), are generated as byproducts of mitochondrial metabolism and by the activity of several enzymes such as heme oxygenase 1, xanthine oxidase, and an extensive family of NADPH oxidases (NOXs) [20]. Importantly, ROS levels are kept under balance by an elaborate antioxidant system [21]. For example, the Nrf/Keap1 signaling pathway can activate several antioxidant enzymes when ROS accumulates at high levels [22]. Antioxidant enzymes include catalase, SOD (superoxide dismutase), and GSH/GSS (glutathione/glutathione synthase). Overproduction of ROS can occur if the balance is shifted towards ROS generation and when the antioxidant enzyme system cannot neutralize the excessively generated ROS. As such, any upregulation of the ROS generation systems or defect in the ROS antioxidant systems can lead to unbalanced ROS generation, and consequently oxidative stress, leading to adverse pathological outcomes [13]. In this respect, the use of antioxidants has proven to be effective in certain pathologies, while it failed to show any therapeutic advantages in several other settings [23,24]. In this regard, antioxidants, like vitamin C or selenium, have shown therapeutic potential *in vitro* and in animal models of CVD but could not improve outcomes in human patients [25]. This discrepancy was linked to the poor bioavailability of antioxidants in the human body. It is thought that higher concentrations of antioxidants need to be used in human patients than anticipated from *in vitro* studies. Currently, antioxidants with better bioavailability and pharmacokinetics are undergoing clinical trials in human patients of CVD and other diseases [9,23–25]. For example, clinical trial NCT05024526 (clinicaltrials.gov) employs edarvone [26] as a targeted ROS scavenger in human patients of stroke. Another example is mitoquinone [26], a mitochondrial-targeted antioxidant, which can reduce the formation of free radicals without affecting mitochondrial oxidative phosphorylation is undergoing a clinical trial (NCT03960073) in patients of heart failure.

Accumulating evidence strongly implicates oxidative stress in the pathogenesis of CVDs, including hypertension, atherosclerosis, aortic aneurysms and vascular restenosis [27–29]. In fact, oxidative stress-induced alterations of endothelial cells (ECs) or vascular smooth muscle cells (VSMCs) are among the critical factors that regulate blood pressure [30]. Activation of NOXs, which are expressed by ECs and VSMCs [27], leads to excessive generation of ROS [31] and, in extension, oxidative stress. This status is permissive for the onset of some CVDs by inducing EC dys-

function and inflammation, depressing the levels of nitric oxide (NO), promoting VSMC proliferation, migration and deposition of extracellular matrix (ECM) proteins, as well as altering vascular response and vasotone (Fig. 1) [27,32–34]. It is not surprising, then, that inhibiting ROS generation, via antioxidants, reduces blood pressure in rodent animal models [29].

Obviously, endothelial dysfunction by itself may not be sufficient to elicit all the pathological aspects of CVD. That is mainly because VSMCs and perivascular adipose tissue contribute to vascular homeostasis by virtue of their ability to produce vasoactive compounds such as adipokines, ROS, and NO [35]. Given the variety of cells and processes involved, this review will focus on oxidative stress-elicited vascular alterations, specifically those that can induce endothelial dysfunction, contributing to the pathogenesis of CVD. EC dysfunction is reversible, making approaches that can reverse it attractive avenues in the management of CVDs [36]. Heightened oxidative stress can cause EC dysfunction in several ways. ROS can compromise endothelium-dependent vasorelaxation, induce apoptosis of ECs, increase ECs adhesion to monocytes, or modify ECs angiogenesis potential (Fig. 1) [37]. Below, we discuss the major mechanism of oxidative stress-induced EC dysfunction, namely ROS-induced reduction of NO bioavailability, ROS-induced inflammation, and ROS-induced mitochondrial dysfunction.

2. Oxidative Stress Promotes Cardiovascular Pathology: the Endothelial Cell Connection

Excessive ROS production through NOX activation or other oxidative stress generating systems inside the cell, like monoamine oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, or mitochondrial metabolism, can result in a state of oxidative stress [31]. This stress can lead to vascular damage by targeting a repertoire of vascular processes. Indeed, augmented ROS depresses levels of NO, induces monocyte invasion, elevates lipid peroxidation, promotes phenotype switching of VSMCs, induces EC dysfunction, precipitates inflammation as well as alters vascular responses and vasotone (Fig. 1) [27,32,33]. Collectively, evidence greatly implicates oxidative stress in the development of CVD including hypertension, atherosclerosis, heart failure, atrial fibrillation, aortic aneurysms and vascular restenosis [10,27–29].

2.1 Oxidative Stress in CVD

2.1.1 Oxidative Stress in Hypertension

In the case of hypertension, the evidence includes the fact that oxidative stress of ECs or VSMCs is among the factors that regulate blood pressure [30]. Consistently, the use of antioxidants to inhibit ROS generation has been shown to reduce blood pressure in rodent animal models [29]. Further evidence includes the fact that ROS production is enhanced in animal models of experimental as well as genetic

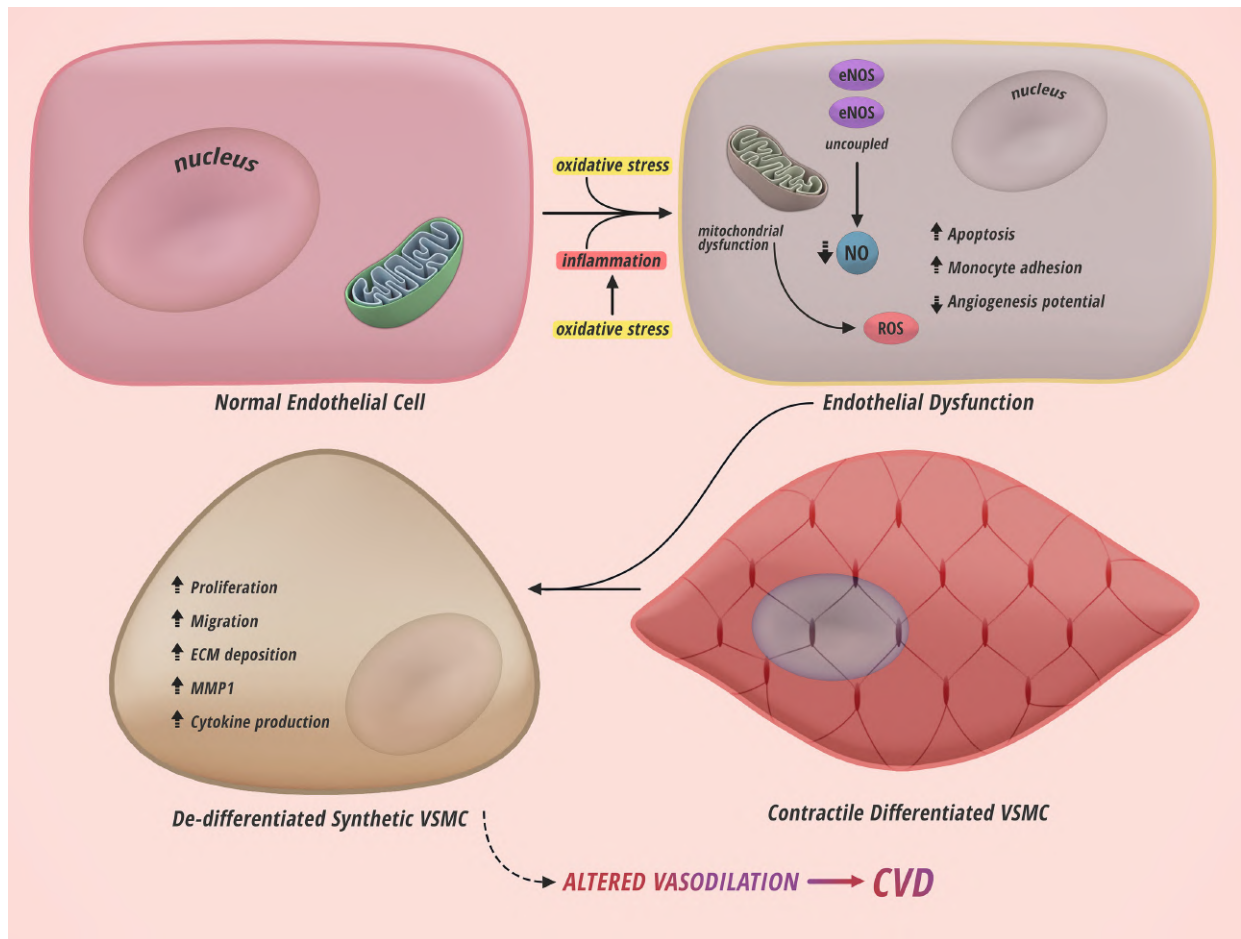


Fig. 1. Mechanism of oxidative stress-induced endothelial dysfunction leading to CVD. Oxidative stress, high levels of ROS for example, can induce inflammation, mitochondrial dysfunction, and eNOS uncoupling and decreased NO bioavailability. These in turn contribute to endothelial dysfunction by increasing ECs adhesion to monocytes, elevating the rates of apoptotic cell death, impairing ECs angiogenic potential, among others. Dysfunctional ECs can contribute to CVD directly by increased monocyte adhesion in atherosclerosis for example, or indirectly by enhancing the phenotypic switching of VSMCs. VSMCs switch into the synthetic de-differentiated phenotype that contributes to dysregulation of vasotone and development of CVD. In this scenario, ROS-induced mitochondrial dysfunction leads to the production of additional ROS, by altered mitochondrial metabolism or NOXs for example, causing the exacerbation of the endothelial dysfunction. ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ECs, endothelial cells; CVD, Cardiovascular disease; VSMCs, vascular smooth muscle cells; NOXs, NADPH oxidases.

hypertension [29,38–41]. Also, when ROS generation is dampened by NOX inhibitors, xanthine oxidase inhibitors, free radical-scavenging antioxidants, or SOD mimetics, then blood pressure will drop and hypertension will not develop in rodent models of hypertension [23,24,38,42–53]. In human patients, there is a clear increase of ROS generation during essential hypertension, renovascular hypertension, and other kinds of hypertension such as malignant hypertension and salt-sensitive hypertension [30,44,48,53–56]. Also, in human population-based observational studies, there is an inverse relation between blood pressure and the levels of blood antioxidants. Similarly, clinical studies in hypertensive patients have revealed a negative correlation between diastolic blood pressure and the levels of NO and antioxidant enzymes [53,57]. On the other hand, there

is a positive correlation between high blood pressure and an elevation of tissue concentrations of $O_2^{\bullet-}$ and H_2O_2 , an increase of the activation of NOXs and xanthine oxidase, and an increase in the levels of biomarkers of oxidative stress, such as plasma and urinary levels of thiobarbituric acid reactive substances (TBARS) which is a measure malondialdehyde (MDA) generated from lipid peroxidation [48,53,58,59]. In addition, the plasma levels of asymmetric dimethylarginine, an endothelial-derived NOS inhibitor, and 13-hydroxyoctadecadienoic acid, a lipid peroxidation product of linoleic acid and a marker of increased ROS production, were inversely related with the incidence of microvascular endothelial dysfunction and elevated blood pressure in patients of hypertension [53,56,60]. It should be noted that despite the multitude of data which associate

oxidative stress in the etiology of hypertension, oxidative stress is still not a confirmed primary cause of hypertension in humans. This is due to intricate reasons that are largely related to (1) absence of methods of accurate measurement of redox states in clinical studies, (2) studies are rarely carried out on the appropriate disease-related tissues in the human body, and (3) shortage of pharmacological agents that can directly and specifically inhibit NOX isoforms, or other ROS generation systems, in human patients [53,61].

2.1.2 Oxidative Stress in Atherosclerosis

The role of EC dysfunction in the pathogenesis of atherosclerosis has been well-established. Indeed, EC dysfunction facilitates the leakage of low-density lipoprotein (LDL) molecules into the sub-endothelial cell layer (intima) where they accumulate and then become oxidized in a mechanism that largely depend on ROS. Oxidized LDL (ox-LDL) then induces dysfunctional ECs to produce cell adhesion molecules, such as VCAM-1 and ICAM-1, which attract inflammatory leukocytes into the sub-endothelial space [62,63]. These inflammatory cells release interleukins and other pro-inflammatory cytokines which eventually potentiate proliferation and migration of VSMCs, as well as increase matrix and lipid deposition, leading to the formation of an atherosclerotic plaque [32,63–66]. Importantly, atherosclerotic plaques usually advance more readily in the presence of dysfunctional ECs that have lost their NO production ability. This is not surprising as atherosclerosis is an inflammatory disease and NO has potent anti-inflammatory properties [32,66].

One of the key players in oxidative stress-precipitated atherosclerotic disease is Nox2. Indeed, expression of Nox2 has been found to be upregulated in macrophages and aortic ECs of apolipoprotein E (ApoE) knockout mice just before the incidence of atherosclerotic lesions [67]. Importantly, a direct evidence linking the incidence of these early lesions to reduced NO bioavailability and increased $O_2\cdot$ production has been established [67].

2.1.3 Oxidative Stress in Other CVDs

Evidence that implicates oxidative stress in the development of heart failure encompasses different experimental as well as clinical studies that correlated an increase in ROS generation with the incidence of heart failure [10,68–70]. Similarly, there is evidence for the involvement of ROS in other CVDs [10,71]. Noteworthy, it is suggested that the common risk factors for CVDs like diabetes mellitus, smoking, aging, and others progress, at least in part, through further generation of ROS, thus leading to the exacerbation of oxidative stress and the pathogenesis of CVDs [71,72].

2.2 Endothelial Cell Function

Endothelial cells line the interior surface of blood and lymphatic vessels. They serve as the mechanical barrier between the circulating blood and VSMCs. As of recent,

ECs are no longer thought of as an inert entity. Indeed, ECs have been shown instead to have both sensory and effector regulatory abilities as well as both metabolic and synthetic functions [73]. Indeed, ECs have been found to be involved in key homeostatic, immune, and inflammatory processes along the cardiovascular network [71,73]. Endothelial cells are now acknowledged as major players in diverse physiological and metabolic functions including the control of thrombosis and thrombolysis, blood clotting system, platelet and leukocyte interaction with the wall of the vessel, formation and growth of blood vessels (angiogenesis), redox balance, the orchestration of acute and chronic inflammation and regulation of vascular tone [74,75]. For example, ECs can control the tone of the underlying VSMCs by secreting various relaxing and contracting factors. Indeed, ECs secrete numerous vasodilator molecules including NO, prostacyclin, H₂S, and EDHFs (Endothelium-Derived Hyperpolarizing Factor) or vasoconstrictive molecules such as thromboxane A₂, endothelin-1, and PDGF, effectively contributing to the regulation of vascular tone [11,35,73,76]. As such, endothelium functions are regulated and maintained by multiple cell surface receptors where the activation of a set of which can induce ECs to release vasoactive agents that modulate VSMCs proliferation and vascular tone [11,77]. Local as well as circulating signals can stimulate the vascular endothelium to release either vasodilators or vasoconstrictors; depending on the receptor activated. Imbalance of the released vasoactive agents can cause an increase in ROS generation which can then lead to endothelial dysfunction and eventually CVDs, including hypertension and atherosclerosis [11,44,78,79]. Given the versatility of functions carried out by ECs, the vascular endothelium can be viewed as an extended and distributed organ with a dynamic and adaptable interface. Furthermore, at the single cell level, ECs can act as integrators of the local pathophysiological microenvironment; hence any dysfunction of the integrators, even broadly, would encompass implications for CVDs incidence and progression [75]. Overall, it is not surprising that endothelial dysfunction can predict the progression of anatomically overt vascular diseases and prominently correlate with the progression of CVDs including hypertension and atherosclerosis [11,80].

2.3 Endothelial Dysfunction: a Hall Mark of CVDs

Consistent with the above discussion, endothelial dysfunction has been acknowledged not only as a pathological state of the endothelium, but also as a predictor of various CVDs or even mortality [35,71,81,82]. Notably, endothelial dysfunction is considered as the hallmark of hypertension [79]. As well, endothelial dysfunction is the earliest observable change in the chronology of an atherosclerotic lesion [75]. Indeed, the ability to measure endothelial dysfunction in patients, through measurement of acetylcholine-dependent dilation or flow-mediated dila-

tion (FMD), makes EC dysfunction a measurable as well as early predictor of CVDs [35]. In accordance, impaired endothelial function has been demonstrated in patients with CVDs including peripheral arterial occlusive disease, coronary artery disease, or heart failure [83].

Endothelial dysfunction is mainly caused by an imbalance of the production and the bioavailability of vasodilating versus vasoconstricting agents [75]. It culminates in impaired endothelium-dependent relaxation of vessels mostly due to decreased vascular bioavailability of NO [84]. In this context, the definition of endothelial dysfunction can be expanded to include all of the maladaptive alterations in the functional phenotype of ECs that can be correlated with a CVD [75]. The imbalance in the bioavailability of vasodilators versus vasoconstrictors can be due to impaired production of different vasoactive agents released by ECs, VSMCs, or perivascular adipose tissue. This imbalance can result in an altered endothelial cell phenotype characterized by being vasoconstrictor, pro-inflammatory, pro-atherothrombotic, and pro-proliferative; and this EC phenotype leads to impaired regulation of blood flow and/or vascular tone. Collectively, this EC state may be referred to as endothelial dysfunction (Fig. 1) [35]. Overall, endothelial dysfunction can be considered a hallmark of vascular injury in most CVDs.

2.4 ROS-Induced Endothelial Dysfunction

The pathophysiological events that can drive endothelial dysfunction are diverse and include hypercholesterolemia (e.g., oxidatively modified lipoproteins), metabolic syndrome (e.g., advanced glycation end products, ROS, adipokines), hypertension (e.g., angiotensin-II, ROS), aging (e.g., advanced glycation end products, cell senescence), proinflammatory cytokines (e.g., Interleukin-1 (IL-1), Tumor Necrosis Factor- α (TNF- α), hemodynamic forces (e.g., disturbed blood flow), and oxidative stress (multi-factorial), among others [75]. Here it is interesting to note that several of the stimuli that can elicit endothelial dysfunction are themselves risk factors for CVD and that several of them involve an increase in oxidative stress, as noted earlier. These stimuli are considered as multi-factors that contribute to endothelial dysfunction and more importantly these factors can synergize to exacerbate the development of endothelial dysfunction; and hence CVDs [35].

Of particular interest to this review, endothelial dysfunction is associated with increased vascular ROS production, oxidative stress, and vascular inflammation in patients with hypertension [53]. In this regard, ROS levels increase in isolated arteries exposed to high pressure *in vitro* [85] leading to endothelial dysfunction [86]. Furthermore, short-term increases in blood pressure can also disrupt endothelial function and enhance oxidative stress *in vivo* [83,87–89]. Also, ROS contribute to EC dysfunction in experimental and clinical atherosclerosis [37,71]. To add, excessive ROS production damages endothelial cells,

especially at the terminal arteries, and causes the modification of intracellular endothelial redox homeostasis. In fact, many studies have demonstrated the notion that elevated production of ROS contributes to the oxidative alterations in the arterial wall, leading to alteration of the intracellular redox homeostasis and cellular damage [90,91]. Finally, increased levels of oxidative stress can cause EC dysfunction in several ways as has been discussed (Fig. 1) [37].

3. Mechanisms of ROS-Induced Endothelial Dysfunction

3.1 ROS-Induced Reduction of NO Levels and eNOS Uncoupling

Nitric oxide signaling is critical for metabolic and vascular health including normal EC function. It is a key vasodilator produced by ECs and it regulates vascular tone and has anti-inflammatory, antioxidant, and antithrombotic effects [92]. It can prevent aggregation of platelets and multiplication of VSMCs [29]. It is also known to regulate metabolic homeostasis. Of note, endothelial dysfunction is evident in endothelial cells that do not produce sufficient amounts of NO and, therefore, are not capable of inducing suitable vasodilation of the vasculature. Also, NO release by ECs in the vasculature can be measured in patients as an alteration of FMD, and this is highly correlated with the extent of endothelial damage in patients. In agreement, NO release by endothelial cells is the chief cause of FMD [93]. Lastly, dysregulation of NO production or its signaling responses can be correlated with cardio-metabolic disorders [92].

Four distinct isoforms of nitric oxide synthase (NOS) are responsible for NO production namely: NOS 1 or neuronal NOS (nNOS), NOS 2 or inducible NOS (iNOS), NOS 3 or endothelial NOS (eNOS), and NOS 4 or mitochondrial NOS (mtNOS). NOS converts its substrate, L-arginine, into L-citrulline in the presence of O₂, NADPH, and the cofactor BH₄ (tetrahydrobiopterin) to produce NO (Fig. 2) [92]. eNOS is the main producer of NO in the vasculature. eNOS-generated NO then diffuses into nearby VSMCs and activates the soluble guanylate cyclase (sGC) which cyclizes GTP into cGMP. cGMP as a second messenger acts on several downstream VSMC effectors such as cGMP-regulated phosphodiesterases and cGMP-dependent protein kinases (cGKs), also called protein kinases G (PKGs). The main effect of this signaling on VSMCs is a modulation of their Ca²⁺ channels and, by extension, relaxation of VSMCs (Fig. 2) [92,93]. In fact, blockade of NO signaling or reduction of NO bioavailability is a conduit for CVDs [93].

One manifestation of ROS-induced endothelial dysfunction is a decrease in NO bioavailability which leads to a vasoconstrictive, proinflammatory, proliferative and thrombotic status; that is EC dysfunction [44,93]. Reduction of NO bioavailability, and hence induction of endothelial dysfunction, can take place in the following ways: (1)

inactivation of NO via its reaction with $O_2^{\bullet-}$ to produce $ONOO^-$, which causes a decrease in the effective concentration of bioavailable NO (2) a reduction in NO production due to a decrease in eNOS expression levels, (3) a reduction in the levels of eNOS substrate or cofactor; for example a decrease in the levels of eNOS substrate, L-arginine, through its degradation by arginase, (4) a reduction in NO production due to an alteration in eNOS activity such as eNOS uncoupling, or (5) a buildup in the levels of asymmetric dimethylarginine, the endogenous eNOS inhibitor (Fig. 3) [93].

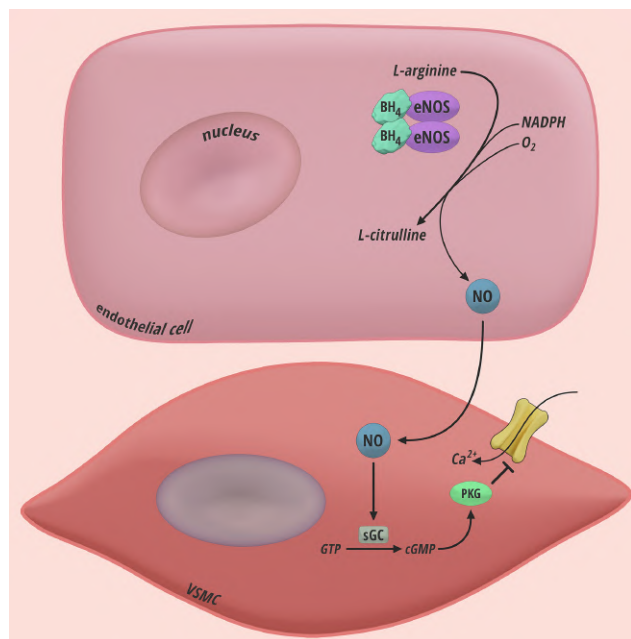


Fig. 2. Physiological Nitric Oxide (NO) signaling pathway leading to vascular relaxation. NO is generated in ECs mainly due to the catalytic activity of eNOS. eNOS converts L-Arginine into L-Citrulline and releasing NO, in the presence of O_2 , NADPH, and BH₄, cofactor of eNOS. NO then diffuses and acts on the nearby VSMCs initiating a series of events that will lead to the relaxation of VSMCs. In VSMCs, NO activates sGC which cyclizes GTP into cGMP. cGMP is a second messenger that activates PKG. PKG can inhibit Ca^{2+} entry into VSMCs leading to vascular relaxation. NO, nitric oxide; ECs, endothelial cells; eNOS, endothelial NO synthase; BH₄, tetrahydrobiopterin; sGC, soluble guanylate cyclase; PKG, protein kinase G; VSMCs, vascular smooth muscle cells; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate.

ROS can directly interact with NO, effectively reducing the bioavailable NO levels, to produce $ONOO^-$ which can modulate cellular processes through protein nitration and modulation of mitochondrial function leading to apoptosis. This can cause an exacerbation of EC dysfunction. eNOS uncoupling significantly reduces the lev-

els of bioavailable NO. eNOS uncoupling usually involves the dissociation of the active, NO producing, dimer form of eNOS into eNOS monomers, that can mainly produce $O_2^{\bullet-}$. During eNOS uncoupling, eNOS transfers an electron from NADPH to molecular oxygen, instead of NO, causing an increase in $O_2^{\bullet-}$ levels and a decrease in NO generation [94] (Fig. 4). Furthermore, production of $O_2^{\bullet-}$ by uncoupled eNOS contributes to a vicious cycle of $ONOO^-$ production and NO consumption where eNOS-produced $O_2^{\bullet-}$ can further reduce the levels of available NO by reacting with it, converting it to $ONOO^-$ (Fig. 4). Notably, $ONOO^-$ by itself can cause eNOS uncoupling as well [95,96]. Also, decrements in the levels of BH₄ or L-Arginine can cause eNOS uncoupling partly through eNOS monomerization [94,96]. Consistently, BH₄ treatment, of a rat model of adjuvant-induced arthritis, can alleviate EC dysfunction [95], and the antioxidant resveratrol can improve vascular function in patients with hypertension partly through enhancing production of BH₄ enhancing eNOS activity [97]. In addition, targeted overproduction of BH₄ by endothelial cells can ameliorate endothelial dysfunction and atherosclerosis in ApoE-knockout mice [98]. Importantly, oxidative stress has been shown to deplete the levels of BH₄ leading to eNOS uncoupling. This can take place partly through BH₄ oxidation into BH₃[•] radical (Fig. 4) [99–102]. BH₃[•] can be reduced by eNOS or the presence of antioxidants, leading to the recoupling of eNOS [101,102]. This can be, in part, the reason of increased NO levels following treatment with antioxidants [97,103] and can be the basis of eNOS recoupling therapy of CVDs.

It should be noted that there is crosstalk between the different ROS generating pathways, leading to amplified generation of ROS in a manner that leads to a feed-forward vicious cycle of ROS generation [35,104–106]. For example, it is well established that mitochondria and NOX, mitochondria and eNOS, NOS and xanthine oxidase, or eNOS and NOX can crosstalk to enhance ROS generation in what has become to be recognized as “ROS-induced ROS release”. This crosstalk is required to sustain oxidative stress and the consequent pathogenesis of CVDs [25,35,104–107]. Using bovine aortic ECs, Doughan *et al.* [108], demonstrated that angiotensin II can activate PKC which induces NOX to produce $O_2^{\bullet-}$. $O_2^{\bullet-}$ subsequently interacts with eNOS-produced NO to form $ONOO^-$ which can then induce mitochondrial dysfunction due to the increased production of mitochondrial H_2O_2 . Mitochondria-produced H_2O_2 feeds back to further activate NOX and the cycle continues to eventually culminate in EC dysfunction (Fig. 5).

Overall, ROS, mainly $O_2^{\bullet-}$, scavenging of NO into $ONOO^-$ and the consequent induction of monomerization of eNOS by $ONOO^-$ to produce more $O_2^{\bullet-}$ and subsequently more $ONOO^-$, is one of the mechanisms by which ROS uncouples eNOS to decrease NO levels [96]. Furthermore, eNOS-produced $O_2^{\bullet-}$ in addition to ROS, including

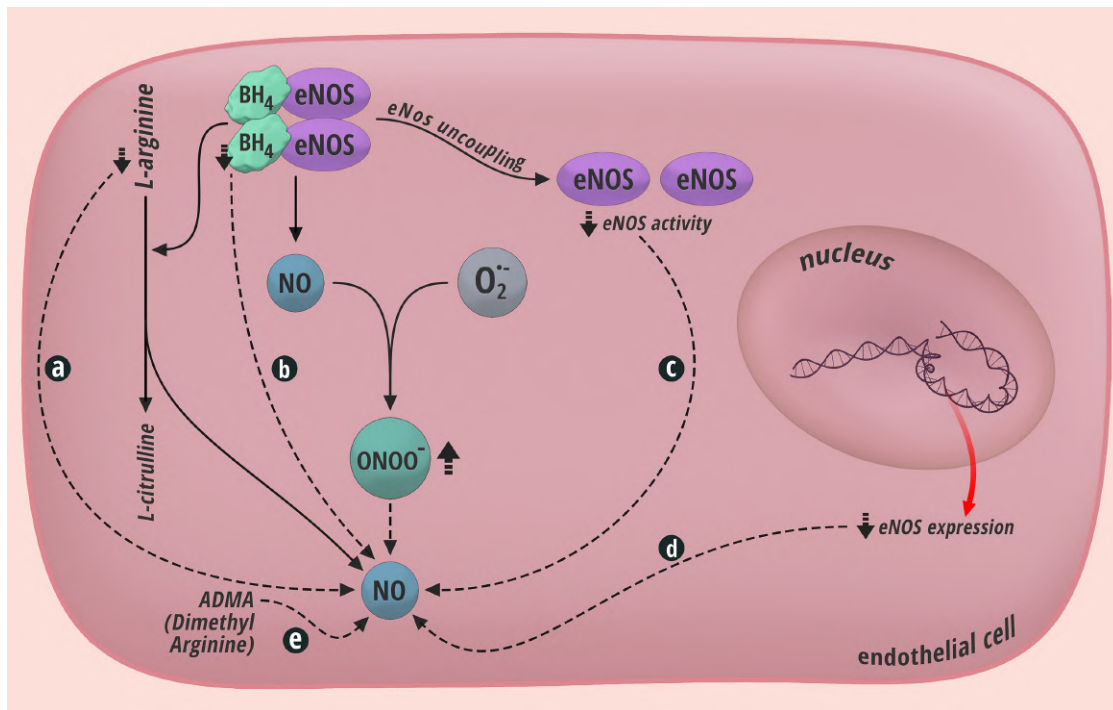


Fig. 3. Major mechanisms leading to reduction of NO bioavailability contributing to induction of endothelial dysfunction. The dashed arrows indicate pathways that lead to a decrease in NO levels. (a) and (b) A reduction in the levels of eNOS substrate (L-Arginine) or cofactor (BH4). (c) Inactivation of NO through reaction with $O_2^{\bullet-}$ to produce $ONOO^-$, effectively decreasing the concentration of bioavailable NO. (c) A reduction in NO production due to an alteration in eNOS activity for example in the case of eNOS uncoupling. (d) A decrease in NO production due to a decrease in eNOS expression levels. (e) A buildup in the levels of asymmetric dimethylarginine, the endogenous eNOS inhibitor. NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite; eNOS, endothelial nitric oxide synthase; BH4, tetrahydrobiopterin; ADMA, dimethylarginine.

$O_2^{\bullet-}$, produced from other sources, such as NOX, xanthine oxidase, or mitochondrial metabolism, can sequester NO and decrease its bioavailability, thus contributing to tissue damage and hypertension [109,110]. In fact, eNOS uncoupling is a hallmark of most CVDs [14]. Altogether, initial formation of ROS by several pathways like NOX, mitochondria, or xanthine oxidase leads to further oxidative stress in a self-amplifying loop that depletes NO and contributes to endothelial dysfunction and the development of several CVDs.

3.2 ROS-Induced Inflammation Contributes to Endothelial Dysfunction

Inflammation is a major contributor to vascular health and the pathogenesis of CVDs. Indeed, inflammation is a risk factor of several CVDs [6]. Elevated levels of several cytokines like IL-1, IL-6, IL-17A, and TNF- α have been positively correlated with CVDs. ROS-evoked inflammation provokes ECs to upregulate the expression of numerous proinflammatory agents like IL-1 β , IL-6 and TNF- α and cell adhesion molecules like VCAM-1 and ICAM-1. TNF- α , IL-6 and IL-1 β , can promote insulin resistance of the vasculature and monocyte infiltration of the endothelium [111], and this can lead to chronic inflammation and EC

damage. Numerous inflammatory, chemokines, cytokines and other agents alter the activities of ECs. This alteration culminates in a proinflammatory, proliferative and prothrombotic state characteristic of dysfunctional endothelial cells [93,112]. In addition, several genome wide association studies (GWAS) correlated inflammatory processes or loci of inflammatory genes (e.g., SNPs in the genes of PECAM1 or PROCR) with the progression of CVDs [25]. Recently, results of the CANTOS study have revealed that anti-IL-1 β therapy can reduce the rates of cardiovascular events [6]. During inflammation ECs undergo Type I and then Type II activation. Activation of ECs involves increased permeability of plasma proteins into the vasculature, enhanced expression and display of adhesion molecules, and higher expression of proinflammatory cytokines and chemokines. Most of these events, like the expression of the chemokines MCP-1 and the cytokine IL-8 among other proinflammatory cytokines, are mediated by activation of the nuclear factor (NF)- κ B signaling pathway [112].

It is well recognized that ROS can activate NF- κ B. ROS can induce the oxidation of the I κ B kinase (IKK) complex causing NF- κ B activation and translocation to the nucleus [113]. Also, H_2O_2 can induce the translocation of NF-

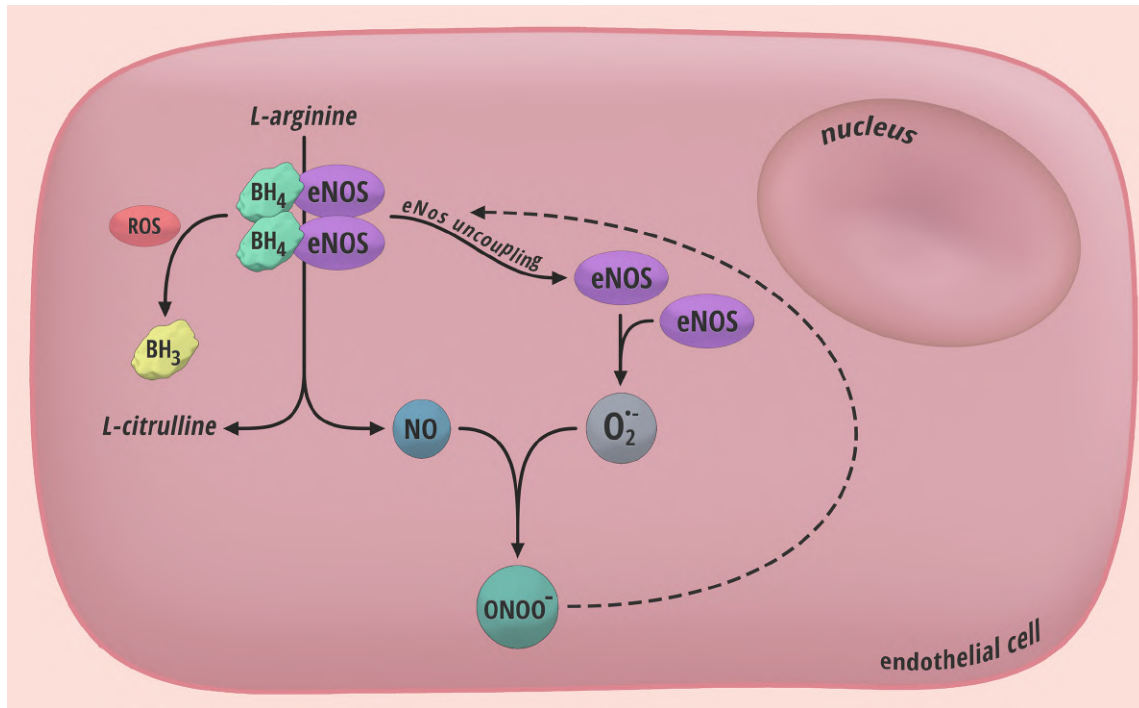


Fig. 4. Oxidative stress-induced uncoupling of eNOS leading to endothelial cell dysfunction. Oxidative stress, mainly ROS, can reduce the levels of the eNOS cofactor BH4 by converting it into BH3• radical leading to eNOS uncoupling. Uncoupled eNOS transfers electrons to O₂ instead of NO, generating O₂•⁻. O₂•⁻ can react with the available cellular NO reducing its levels by converting it to ONOO⁻. Furthermore, ONOO⁻ can directly promote eNOS uncoupling. eNOS, endothelial nitric oxide; ROS, reactive oxygen species; BH4, tetrahydrobiopterin; NO, nitric oxide; O₂•⁻, superoxide anion; ONOO⁻, peroxynitrite.

κ B to the nucleus [114] in a process that involves p65 phosphorylation on Ser 529 [115]. Consistently oxidative stress-mediated activation of NF- κ B can be prevented by overexpression of the antioxidant enzyme SOD-2 [116]. In the nucleus, NF- κ B mediates the activation of its target genes including numerous players of EC dysfunction like cell adhesion molecules VCAM-1 and ICAM-1, and inflammatory mediators like IL-6 and TNF- α (Fig. 6) [116].

Oxidative stress and high levels of ROS result in oxidation of numerous proteins [117]. The oxidation of proteins can result in the secretion of inflammatory mediators, such as the inflammatory signaling molecule, peroxiredoxin-2. In fact, peroxiredoxin-2 is thought of as a link between oxidative stress and inflammation (Fig. 6) [118].

In another mechanism, oxidative stress contributes to the activation of NLRP3 (NOD-like receptor protein 3) inflammasome complex through several means [119,120]. ROS produced by injured mitochondria can induce the activation of the NLRP3 inflammasome to produce IL-1 β (Fig. 6) [119,121]. Moreover, ROS can cause the dissociation of thioredoxin-interacting protein, which blocks the action of thioredoxin, from thioredoxin. Thioredoxin-interacting protein then binds to the NLRP3 inflammasome complex causing its activation and production of inflammatory IL-1 β [122].

A major evidence of ROS-induced inflammation is that knock-out of ROS producing enzymes, like Nox, attenuates ROS-induced inflammation [25]. In addition, NOX activation is known to trigger inflammation [123]. Noteworthy, there is an interplay between oxidative stress and inflammation during EC dysfunction. For example, inflammation, once initiated by ROS, can in turn affect the activity of NOXs [124,125].

3.3 ROS-induced Mitochondrial Dysfunction of Endothelial Cells

Endothelial cells obtain much of their energy (around 75–99%) from glycolysis instead of oxidative mitochondrial metabolism [126]. Nevertheless, EC mitochondria remain physiologically relevant partly due to their ability to produce ROS. Mitochondrial ROS production can take place at complex I or complex III [127], and mitochondria-produced ROS are major contributors of oxidative stress and, hence, EC dysfunction [109]. In addition, mitochondrial ROS generation can be increased by other sources of ROS such as NOX-derived ROS, in a crosstalk scheme as discussed earlier [128]. In fact, this crosstalk is required for NOX-derived ROS to act on the endothelium [128].

ROS is not only produced by the mitochondria, but also the mitochondria is affected by ROS where excessive ROS has critical effects on mitochondria and may even lead

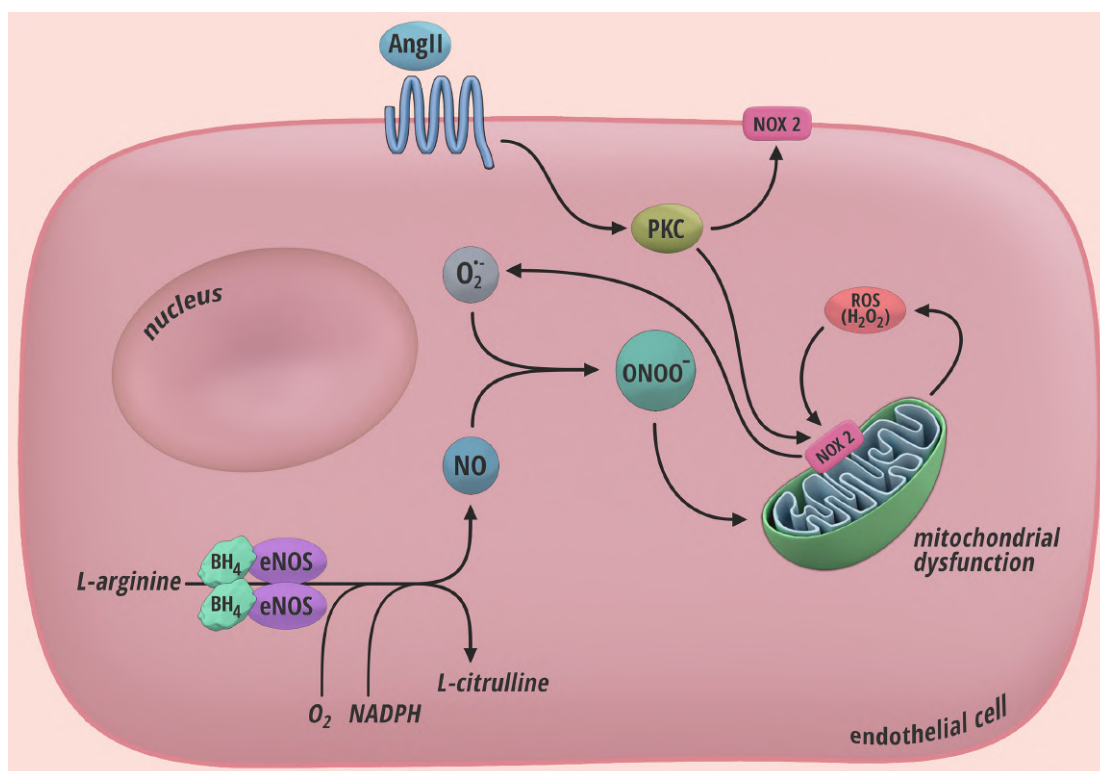


Fig. 5. Cross-talk between vascular endothelial dysfunction and mitochondrial damage during angiotensin II signaling. Signaling of Ang II through its receptor activates PKC as one of its downstream targets. Ang II signaling leads to mitochondrial dysfunction due to the increased PKC-dependent activation of NOXs which produce $O_2^{\cdot-}$. $O_2^{\cdot-}$ subsequently interacts with eNOS-released NO to form $ONOO^-$, depleting NO levels and leading to endothelial dysfunction. At the same time, $ONOO^-$ can further cause mitochondrial dysfunction through additional production of mitochondrial H_2O_2 . Excessive H_2O_2 levels feedback to further activate NOX, exacerbating both mitochondrial damage and endothelial dysfunction. Ang II, angiotensin II; PKC, protein kinase C; NOX, NADPH oxidase; $O_2^{\cdot-}$, superoxide anion; $ONOO^-$, peroxynitrite; eNOS, nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species.

to mitochondrial dysfunction. Importantly, mitochondrial damage can accelerate ROS-induced endothelial dysfunction. It is important to note that endothelial cells are not only exposed to endogenous oxidative species, but also to plenty of exogenous sources of reactive species that compound the ROS-induced endothelial dysfunction. For example, during atherosclerosis, activated neutrophils produce large amounts of ROS at areas of damaged endothelium [126].

Maintenance of Ca^{2+} homeostasis is crucial for normal cardiovascular physiology. ROS can disrupt the normal mitochondria-mediated Ca^{2+} homeostasis. For example, it has been reported that H_2O_2 treatment of permeabilized endothelial cells in culture increases the concentration of mitochondrial Ca^{2+} , likely through inhibition of mitochondrial Na^+/Ca^{2+} exchanger [129].

Mitochondrial-generated ROS can also target and damage the mitochondrial electron transport chain (ETC). Mitochondrial $O_2^{\cdot-}$ can react with NO to produce $ONOO^-$, and $ONOO^-$ can directly damage ETC proteins [130]. Mitochondrial ROS-induced damage of mitochondrial DNA (mtDNA) is another way of ROS-induced mitochondrial dysfunction, and consequently endothelial dysfunction.

mtDNA damage can lead to disruption of ETC and ATP production and this may result in excessive ROS generation by complexes I and III [131]. Also, excessive ROS and mtDNA damage can cause the depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) resulting in the decrease of ETC efficiency and these alterations are implicated in elevated outer mitochondrial membrane permeability [132,133]. Notably, $\Delta\Psi_m$ disruption can stimulate additional ROS formation and thus accelerate the pathogenesis of atherosclerosis [134].

Relatedly, increased outer mitochondrial membrane permeability can enhance the release of mitochondrial constituents like ROS and mtDNA into the cytoplasm. This event can induce inflammation or apoptosis in several ways [135].

It is evident that ROS-induced mitochondrial damage contributes to EC dysfunction through several mechanisms. Further, exploration of these mechanisms can give insight into novel therapies of CVDs.

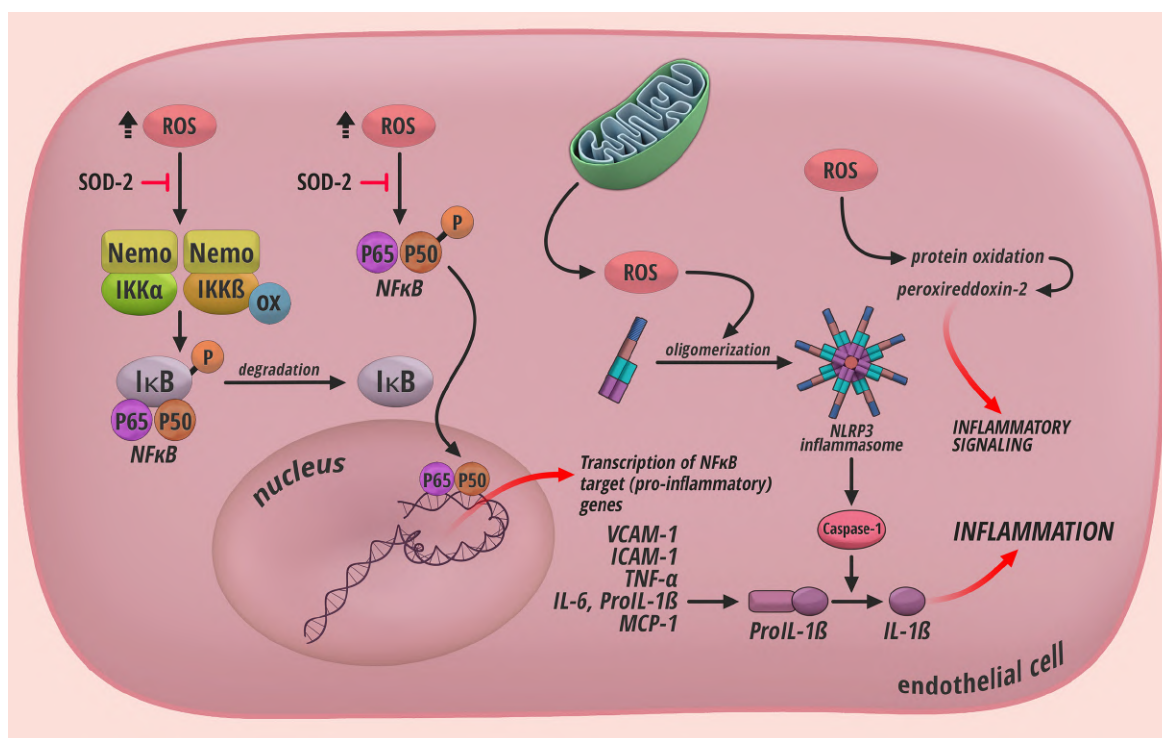


Fig. 6. Mechanisms of oxidative stress-induced inflammation leading to endothelial dysfunction. ROS can cause the activation of the IKK complex through oxidation of IKK α . Active IKK complex can activate the NF- κ B transcription factor by inducing the degradation of its inhibitor protein I κ B. NF- κ B then translocates to the nucleus leading to the transcription of several inflammatory mediators including MCP-1, IL-1 β , IL-6, IL-8, and TNF- α and adhesion molecules like VCAM-1 and ICAM-1. Also, ROS (H₂O₂) can lead to the phosphorylation of the p65 subunit of NF- κ B leading to the activation of NF- κ B and transcription of its target genes. Both of these mechanisms can be inhibited by SOD-2. In another mechanism, mitochondria-released ROS can cause the oligomerization and activation of NLRP3 inflammasome complex which activates caspase-1 to cleave pro-IL-1 β into mature active IL-1 β . Finally, oxidative stress-induced protein oxidation can cause the release of several inflammatory mediators like peroxiredoxin-2. Peroxiredoxin-2 has both oxidative and inflammatory functions and can serve as a linking point between inflammation and oxidative state.

4. Conclusions

Imbalance of the oxidative state of the vasculature contributes to initiation and progression of CVDs. Moreover, normalization of this oxidative state has been shown to benefit vascular health by acting on VSMC, ECs, and other cells of the vasculature. In particular, the ensuing ROS-induced endothelial dysfunction has a multitude of clinical manifestations, in CVD and other diseases. Importantly, blockade of ROS generation or the use of antioxidants have been shown to be able to normalize the disrupted EC functions [136], and may thus prove vital for reversing EC dysfunction and ameliorating symptoms of CVD. Further exploration of the involved mechanisms may provide insight into newer therapies that can prevent or treat CVD.

Author Contributions

Conceptualization—AHE; methodology—AS, KA, RA, AP, AO, AEY, GP, AHE; formal analysis—AS, KA, RA, AP, AO, AEY, GP, AHE; writing - original draft preparation—AS, KA, RA; writing - review and editing—AS, KA, RA, AP, AO, AEY, GP, AHE;

supervision—AHE; project administration—GP, AHE; funding acquisition—GP. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest. GP (Gianfranco Pintus) is serving as one of the Editorial Board members of this journal. We declare that GP (Gianfranco

Pintus) had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GP (Giordano Pula).

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