

Nrf2 and HO-1 in Atherosclerosis

Subjects: Cardiac & Cardiovascular Systems

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Oxidative stress mediated by reactive oxygen species (ROS) plays a key role in the atherosclerotic process. ROS not only oxidize LDL, they also promote several pro-atherogenic effects, including inflammation, apoptosis, and alteration of vascular tone. The imbalance between pro- and antioxidant agents drives to the formation and progression of atherosclerotic plaque lesions. In this sense, the transcription factor Nuclear factor-erythroid 2-related factor 2 (Nrf2) is known to activate the expression of more than 250 antioxidant enzymes—such as heme oxygenase-1 (HO-1), glutathione peroxidase, or glutamate-cysteine ligase—making it a master regulator of oxidative stress. Nrf2 has been associated with different cardiovascular-related pathologies—such as obesity, diabetes mellitus, atherosclerosis, hypertension, or heart failure. Currently, Nrf2 signaling pathway is considered an important defense mechanism against cardiovascular diseases (CVDs).

Keywords: atherosclerosis ; oxidative stress ; heme oxygenase-1 ; Nrf2

1. Atherosclerosis and Oxidative Stress

Oxidative stress is highly involved in the initiation and development of atherosclerosis [1]. Apart from the active oxygen compounds, free radicals ($O_2^{\cdot-}$, $\cdot OH$) and non-free radicals (H_2O_2 , $\frac{1}{2} O_2$), reactive nitrogen, copper, iron and sulfur species are also considered as oxidative stress related molecules, since they promote or participate through different mechanisms in the synthesis of ROS [2][1]. ROS production increases in response to many risk factors associated with atherosclerotic CVDs—such as hypertension, diabetes, smoking, or dyslipidemia [1]. Similarly, oxidative stress can result from the imbalance of oxidant and antioxidant agents, promoting cell toxicity related to atherosclerosis [3]. Under physiological conditions, antioxidants help to prevent damage from free radicals and other oxidant molecules without affecting redox reactions that are fundamental for appropriate cell function [4]. However, under pathological situations, an imbalance of oxidant (enhanced) versus antioxidant enzymes (reduced) leads to oxidative stress (**Figure 1**). Then, many different processes can take place, including NO inactivation; oxidation of lipids, proteins, or even DNA; cell apoptosis; or enhanced expression of pro-inflammatory cytokines and ox-LDL receptors (CD36, LOX-1) [5][6]. Remarkably, ROS promotes the expression of SR in VSMCs, ox-LDL recognition and uptake, as well as their transformation into foam cells [1]. In the same way, ROS can activate the release of matrix metalloproteinases (MMPs) which could degrade the basement membrane and atheroma plaque structure, disrupting it. Additionally, ECs and SMCs can generate different oxidant agents through several enzymes [7].

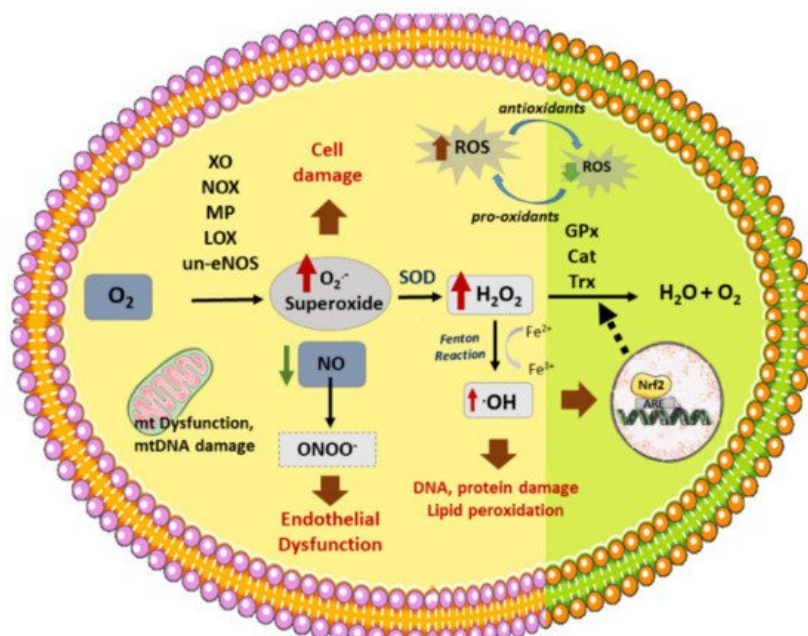


Figure 1. Vascular sources of ROS and related enzymes. Different enzymes participate in the formation of ROS: NOX (NADPH oxidase), XO (xanthine oxidase), un-eNOS (uncoupled nitric oxide synthase), LOX (lipoxygenase), MP (myeloperoxidase), generating superoxide ($O_2^{\cdot-}$) from O_2 . In addition, dysfunctional mitochondrial (mt) respiratory chain is another source of ($O_2^{\cdot-}$). On the other hand, SOD (superoxide dismutase) produces H_2O_2 (hydrogen peroxide) from superoxide, which can then be converted to H_2O by several antioxidant enzymes: GPx (glutathione peroxidase), Cat (catalase), or Trx (Thioredoxin). H_2O_2 reacts with transition metals such as Fe^{2+} (through the Fenton reaction) to produce hydroxyl radicals ($\cdot OH$). Nitric oxide (NO) reacts with $O_2^{\cdot-}$ to produce peroxynitrite ($ONOO^-$). ROS stimulates Nrf2 activation and translocation to the nucleus, activating the synthesis of antioxidant enzymes. ROS production induces cell death, DNA and lipid peroxidation, and endothelial dysfunction, among other effects, which triggers the atherosclerotic process.

2. NRF2 Antioxidant Roles in Atherosclerosis

Nrf2, encoded by NFE2L2 gene, constitutes a transcription factor that has been closely linked to atherosclerosis, although its specific role in this pathology is not clear. Nrf2 might play antagonistic roles, both preventing and enhancing atherosclerotic development [8][9][10]. For instance, several studies related to laminar and oscillatory blood flow have revealed that the former stimulates the antiatherogenic activation of Nrf2, while the latter promotes the opposite effect [11]. Studies with ApoE^{-/-} and Nrf2^{-/-} or Nrf2^{+/-} mice indicate a reduction of the atherosclerotic process in these animals, suggesting a deleterious role of the transcription factor [8][12]. Nrf2 expression in ApoE Knockout mice seems to accelerate the late stages—but not the early stages—of atherosclerosis [13], while in absence of macrophage Nrf2, both atherosclerotic stages are affected in LDLR KO mice [9]. Nrf2 might potentiate atherosclerosis through different pathways, as described [14], promoting among others the formation of foam cells [15] or the monocyte recruitment to the lesion areas by upregulation of IL-1 α in macrophages [8]. Nrf2 modulates the expression of CD36 scavenger receptor in macrophages, responsible for ox-LDL uptake and accumulation and subsequent formation of foam cells [16]. In this regard, controversial results have been assigned to the Nrf2/CD36 pathway in atherosclerosis, with studies indicating reduced ox-LDL accumulation in Nrf2 KO macrophages and decreased CD36 levels in atherosclerotic lesions in ApoE^{-/-} Nrf2^{-/-} mice [13][16]. Conversely, lack of Nrf2 in peritoneal macrophages seems to increase modified LDL uptake, suggesting that CD36 is not the only factor responsible for ox-LDL accumulation [9][16].

The majority of studies, however, have focused on the athero-protective properties of Nrf2, mainly as a key agent against oxidative stress [17]. Apart from being able to activate the expression of many antioxidant enzymes, Nrf2 has the potential to modulate NADPH oxidase activity as well. Thus, the levels of pro-atherogenic Nox2 appear to increase significantly in absence of Nrf2, whereas Nox4 is upregulated when Nrf2 is constitutively activated [18][19]. Interestingly, ROS produced by NADPH oxidase can also activate Nrf2, at least in cardiomyocytes and pulmonary epithelial cells [20]. Such activation may represent an endogenous protecting mechanism against mitochondrial damage and cell death in the heart during chronic pressure overload [21]. Studies with Nrf2-KO mouse glia-neuronal cells have shown higher rates of mitochondrial ROS production in these cells than in wild type Nrf2 cells, suggesting a modulatory role for Nrf2 over the mitochondrial respiratory chain [18]. Indeed, Nrf2 enhances mitochondrial biogenesis, as well as the activation of OxPhos, among others. Finally, mitochondrial ROS also stimulates Nrf2 activation, thus representing a continuous regulatory loop [18][22].

Nrf2 not only protects against oxidative stress in ECs through activation of different antioxidant genes, it also exerts anti-inflammatory and angiogenic effects on these cells [23]. Indeed, Nrf2 suppresses the expression of VCAM-1 and TNF α —induced expression of MCP-1 in HUVECs [24] and HAECs [25]. Likewise, endothelial Nrf2 antioxidant response can be activated in areas exposed to oscillatory shear stress, exerting an athero-protective role [26]. Similarly, Nrf2 reduces the inflammatory response in macrophages and foam cells by modulation of several inflammatory cytokines such as IL-6 or TNF α [9]. Finally, Nrf2 could protect VSMCs against oxidative stress, and may protect against atherosclerosis progression by activation of athero-protective genes, such as HO-1 or NAD(P)H dehydrogenase quinone 1 (NQO1), which suppress proliferation of SMCs and VSMCs respectively [27][28].

Modulation of Nrf2 Activity

Nrf2 belongs to “Cap’n’Collar” (CNC) family of transcription factors with a b-ZIP domain [29][30] which modulate the cellular redox status [29]. Nrf2 has seven domains that regulate the stability and functional activity of this factor [31]. Under normal homeostatic conditions, Nrf2 is trapped in the cytosol, associated with the Kelch-like ECH-associated protein-1 (KEAP-1) through the N-terminal (Neh2 domain) [31] (**Figure 2**). Keap1 acts as an endogenous inhibitor of Nrf2 [32]. In fact, Keap1 is the major regulator of Nrf2 activity [33], through the aforementioned interaction with Nrf2, which promotes the assembling

with the Cullin3 (Cul3)/Rbx1 (Ring box-1)-based E3-ubiquitin ligase complex that targets Nrf2 for constant proteasomal degradation [31]. This complex keeps Nrf2 in the cytosol, avoiding its translocation to the nucleus and the transcription of antioxidant related genes [33].

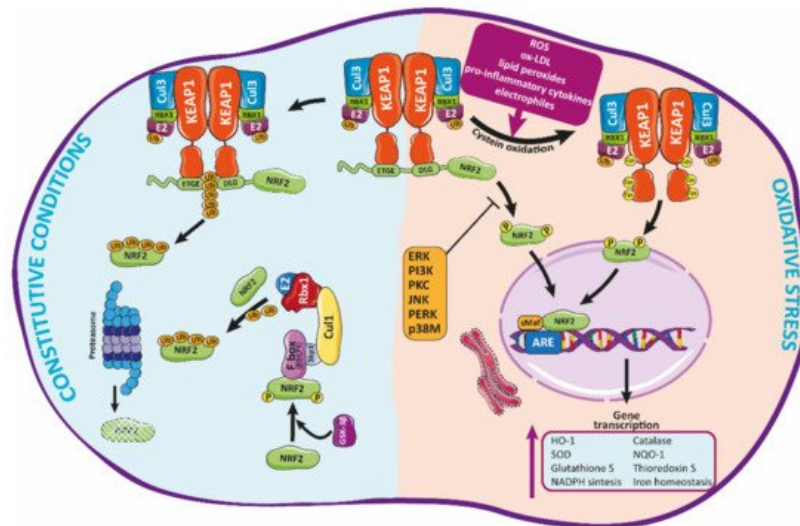


Figure 2. Regulation of Nrf2 signaling and HO-1 expression. Under basal conditions, Nrf2 is trapped into the cytosol associated with the Kelch-like ECH-associated protein-1 (KEAP-1) through the N-terminal (Neh2 domain). Herein, Nrf2 is transferred to proteasomal degradation after being ubiquitinated. In response to pathological agents such as ROS, Nrf2 is released from this complex after oxidation of Keap1 cysteine residues, moving toward the nucleus, where it binds to ARE regions, inducing the expression of antioxidant enzymes such as HO-1.

Many factors—such as ROS, ox-LDL, lipid peroxides and their metabolites, electrophiles, pro-inflammatory cytokines, and many other molecules related to oxidative stress—can induce an alteration of Keap1 conformation (by modification of its cysteine residues) [34][15], promoting the release of Nrf2 which then translocates to the nucleus [15]. Herein, Nrf2 binds through the Neh1 functional domain to small musculoaponeurotic fibrosarcoma (sMaf) proteins, forming heterodimers with them [35]. Such dimerization allows Nrf2 to interact with the DNA through the antioxidant response element (ARE) sequence in specific gene regulatory regions [31]. Through this mechanism, Nrf2 modulates the expression of many genes encoding different antioxidant proteins—e.g., HO-1, catalase, SOD, or NQO-1 [36] among others—which will contribute to reduce cellular oxidative stress [15][37]. Likewise, Nrf2 regulates the glutathione and thioredoxin systems, NADPH production and utilization, and iron homeostasis. Many of these antioxidant factors actively participate in the detoxification and elimination of pro-oxidant compounds [36].

Currently, the Nrf2/Keap1 system is considered the principal mechanism against oxidative stress, which is widely conserved, mainly in mammals [38]. This system is mainly modulated, as indicated above, by the interaction of diverse substances with several cysteine residues present in Keap1. Indeed, Keap1 contains more than 27 cysteine residues that have been described as potentially involved in the modulation of this protein and its interaction with Nrf2 [39]. Among them, Cys151 promotes Nrf2 release and activation, while Cys273, 288, and 297 facilitate the interaction of Keap1 with Nrf2, therefore inactivating this protein [40]. The current model postulates that, in response to different stimuli, modification of Keap1 thiols promotes conformational changes that negatively affect the ubiquitin ligase activity of the Keap1-Cul3 complex, as described before, releasing Nrf2 which then translocates to the nucleus and activates the synthesis of antioxidant genes [33]. Alternatively, the “hinge and latch” model suggests that, in response to stimuli, Nrf2 is synthesized *de novo*, instead of being released from Keap1, accumulating as well in the nucleus [41].

The Nrf2/Keap1 system can also be regulated by the autophagy regulator protein p62 (Sequestosome1, SQSTM1). Phosphorylation of p62 in response to many factors such as oxidative stress [42] allows the direct binding of this protein to Keap1, promoting its degradation by autophagia, and subsequent displacement and activation of Nrf2 [43]. Remarkably, p62 expression is also induced by Nrf2 in response to ROS [44]. Thus, the p62/Nrf2 route might represent an attractive candidate to promote Nrf2 activation and antioxidant response. However, given the strong correlation found between p62 upregulation and activation of Nrf2 pathway with several cancer types, major efforts have been made mainly toward the identification of potential pharmacological inhibitors of this p62/Nrf2 pathway [45].

Apart from Keap1, other molecules can regulate Nrf2 activity through additional control points at transcriptional, post-transcriptional, translational, and post-translational level [34][46] (Table 1). Several kinases, such as glycogen synthase kinase-3-beta (GSK3β), have been described as modulators of Nrf2 activity [47]. Nrf2 phosphorylation by GSK-3β activates the recognition and binding of Nrf2 to β-transducin repeat-containing protein (β-TrCP), an adaptor for the Skp1-Cul1-

Rbx1-F-box protein (SCF) E3 ubiquitin ligase complex. Similarly to the Nrf2-Keap1-Cul3 complex, this complex promotes Nrf2 ubiquitination and further proteasomal degradation [48][49]. Other kinases—such as protein kinase-C (PKC), extracellular signal-regulated kinase (ERK), PERK, p38 MAPK, JNK, and PI3K—can also influence Nrf2 activity [10][50][51]. Remarkably, while phosphorylation of certain residues of Nrf2 (Ser40, Ser558) by kinases like PKC or AMPK-mediated phosphorylation enhance nuclear translocation of Nrf2 and transcription of antioxidant enzymes [50][51], phosphorylation or other residues (Tyr576, Ser44, Ser347) by enzymes such as Fyn kinase or GSK3B, can promote Nrf2 to be exported from the nucleus to the cytosol, which activates its proteasomal degradation [48].

Table 1. Modulators of Nrf2. Protein kinases and miRNA known to regulate Nrf2 activity are shown. Acronyms: GSK-3 β : Glycogen synthase kinase-3 β ; FYN: Proto-oncogene tyrosine-protein kinase Fyn; p38: p38 MAP kinase; PI3K/Akt: Phosphoinositide 3-kinase/Protein kinase B (Akt); PKC: Protein kinase C; JNK: c-Jun NH2-terminal protein kinase; ERK: Extracellular signal-regulated kinase; PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase; AMPK: AMP-activated protein kinase; miRNA: micro RNA.

Protein Kinases	Identified in	Effect	References
GSK-3 β	HEK293T	Inhibits NRF2	[48] Rada et al., 2011 [52] Salazar et al., 2006
	HepG2	Inhibits NRF2	[53] Jain et al., 2006
FYN	HepG2	Inhibits NRF2, via GSK-3B	[47] Jain et al., 2007
P38 MAP kinase	Thymocytes and 293T; HepG2	Inhibits HO-1/Nrf2	[54] Thornton et al., 2008;
		Activates HO-1/Nrf2	[55] Shen et al., 2004 [56] Elbirt et al. 1998
PI3k /Akt	293T	Activates NRF2, by Gsk-3 β inhibition.	[52] Salazar M et al. 2006
PKC	HepG2	Activates NRF2	[50] Huang et al., 2002
JNK	HepG2	Activates NRF2	[55] Shen et al., 2004
ERK	HepG2	Activates NRF2, through GSK-3 β inhibition.	[55] Shen et al., 2004 [56] Elbirt et al., 1998
PERK	Mouse embryonic fibroblasts	Activates NRF2	[57] Cullinan et al. 2003
AMPK	HepG2, HEK293	Activates Nrf2	[51] Joo et al. 2016
miRNA	Identified in	Effect	References
miR-28	Breast cancer cell lines	Inhibits	[58] Yang et al., 2011
miR-34a	Hepatocytes	Inhibits	[59] Huang et al., 2014
	Cardiomyocytes		[60] Wang et al., 2019
miR-132, miR-200c	Renal proximal tubular cell line	Inhibits	[61] Stachurska et al., 2012
miR-144	Neuronal cell lines; Lymphoblast cell lines (k562 cell line)	Inhibits	[62] Zhou et al., 2017
miR-153, miR27a, miR-142-5p	Neuronal cell lines	Inhibits	[63] Narashimhan et al., 2012
miR-200a	OB-6 osteoblastic cells	Increases	[64] Zhao et al., 2017
miR-140-5p	HK2 cells	Increases NRF2 expression	[65] Liao W et al., 2017
miR873-5p	Mouse renal tubular epithelial cells (mRTECs)	Increases NRF2 and HO-1 expression	[66] Wang J et al., 2019

Alternatively, several studies suggest that different microRNAs (miRNAs) (i.e., miR-27a, miRNA-28a or -34a) can also regulate, directly or indirectly, Nrf2 expression and moreover, to control the antioxidant activity of the Keap1-Nrf2 complex and Nrf2/ARE signaling pathways [46][67]. miRNAs are small noncoding molecules that bind to specific regions of mRNA (UTRs), promoting either their degradation or blocking their translation, reducing their levels. Thus, several miRNAs—such as miR-28, miR-507, mi-450a or miR-634—have been found to inhibit Nrf2 expression in studies involving cancer

cells [45]. Another study found that downregulation of miRNA-93 increases Nrf2 expression, reducing ROS and cell apoptosis and diabetic retinopathy [68]. Additionally, miRNA-153 promoted oxidative stress by inhibiting Nrf2 activity in an in vitro model of Parkinson's disease [69]. As concerning Keap1, miR-141 was the first miRNA identified to suppress Keap1 levels in ovarian cancer cell lines [70]. Similarly, miR-432-3p was found to impair Keap1 mRNA translation in esophageal squamous cell carcinoma, positively modulating Nrf2 activity [71], while increased levels of miR-200a promoted Keap1 degradation and Nrf2 stabilization in OB-6 osteoblastic cells [64]. Overall, there is a growing number of studies supporting the potential use of miRNAs as an strategy to modulate the Nrf2/Keap1 pathway, either inhibiting (as it happens in cancer) or promoting the activation of Nrf2 as an antioxidant approach. Future research could benefit from these findings.

3. HO-1 Antioxidant Role in Atherosclerosis

HO-1 has many important roles that contribute to the protection against atherosclerosis, such as antioxidant, anti-inflammatory, antiapoptotic, or immunomodulatory effects [14]. HO-1 is expressed in the main cell types detected in human and mouse atherosclerotic lesions, including EC, macrophages, or SMCs [14][72].

HO-1 is a highly conserved enzyme—also known as 32-kDa heat shock protein—encoded by the hmox1 gene [73][74]. There are currently three isoforms of HO: HO-1, HO-2, and HO-3, although the last one seems to derive from HO-2 transcripts [75]. While HO-2 is constitutively expressed, HO-1 is usually present at low levels in many tissues but it can be stimulated by many factors [14]. For instance, HO-1 expression can be enhanced in response to ROS, ischemia-reperfusion, carcinogenesis, atherosclerosis itself, or other inflammatory processes [76]. Moreover, different transcription factors such as Nrf2, activator protein-1 (AP-1), or nuclear factor-kappa B (NFkB) have been shown to activate HO-1 expression, together with several up-stream kinases, like protein kinases A and C or phosphatidylinositol 3-kinase [76][77].

In mammals, heme oxygenase enzymes are located on the surface of the endoplasmic reticulum, anchored to it by hydrophobic amino acids at its COOH-terminal ends. Its catalytic part contains a “heme binding pocket” of 24 amino acids, oriented to the cytosol [78][74], together with a histidine-imidazole residue where it binds to the heme iron [78]. HO-1 enzymatically catalyzes the oxidative cleavage of the heme group (mainly heme b) of metalloproteins in equimolar amounts of carbon monoxide (CO), Fe^{2+} and biliverdin IX α [79] (**Figure 3**). Biliverdin is then converted to bilirubin by the cytosolic biliverdin reductase (BVR). All these three products have anti-inflammatory, antioxidant, antiapoptotic, antithrombotic, and antiproliferative functions in vascular cells [14][79]. Thus, the anti-atherogenic role assigned to HO-1 appears to be associated, among others, with the bioproducts, biliverdin, bilirubin, and the vasodilator CO, providing arterial protection against oxidant-induced injury [79].

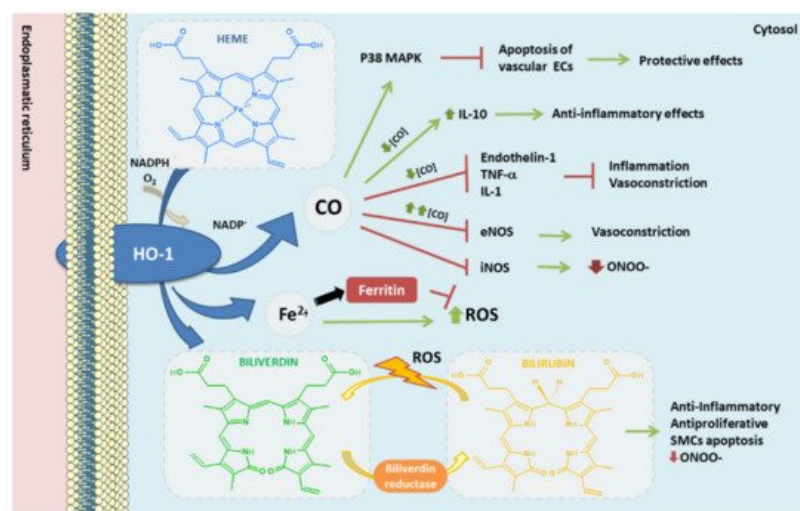


Figure 3. Heme oxygenase-1 antioxidant products derived from heme degradation. Heme oxygenase-1 (HO-1) catabolizes heme degradation into equimolar amounts of carbon monoxide (CO), Fe^{2+} and biliverdin. Biliverdin is converted to bilirubin by biliverdin reductase (BVR). All these products have shown antioxidant, anti-thrombotic and anti-inflammatory properties. In addition, HO-1 induces the production of ferritin, an iron storing protein—reducing the levels of Fe^{2+} which could derive into ROS via Fenton reaction.

Different cell-based and in vivo research studies have demonstrated that HO-1 upregulation protects vascular walls from endothelial dysfunction and pathological remodeling, significantly inhibiting the atherosclerotic process [79][80][81][82][83]. Besides, diverse genetic population studies have indicated the importance of HO-1 expression as a protecting phenomenon against atherosclerosis [14]. Under basal conditions HO-1 is under-expressed in most tissues, however when

a vascular injury occurs, the expression is higher [79]. High blood HO-1 levels have been found in different chronic diseases, such as diabetes mellitus [84] or Parkinson's disease [85][86], mainly released into the plasma by different cell types in response to inflammation or oxidative stress [87]. Remarkably, the expression of HO-1 arising from macrophages during oxidative conditions was found higher in coronary artery disease (CAD) patients when compared to healthy subjects [88]. Likewise, HO-1 plasma levels are higher in patients with carotid plaques compared to those without plaques, and these levels increase with the severity of the plaques [89]. HO-1 expression is high in atherosclerotic lesions, correlating with plaque instability and pro-inflammatory markers [73]. However, such upregulation enhances the stabilization of atherosclerotic plaques, therefore representing an athero-protective mechanism [79][90]. Similarly, increased levels of bilirubin in plasma have been associated with lower incidence of CVDs [91] while low levels of bilirubin correlate with endothelial dysfunction and increased intima-media thickness [73][92], being inversely correlated the levels of bilirubin in serum with the severity of atherosclerosis in men [93]. Such anti-atherogenic role relies mainly on the antioxidant properties of bilirubin/biliverdin. Bilirubin seems capable to scavenge oxygen radicals and inhibit oxidation of LDL and other lipids, as well as presenting anti-inflammatory and anti-proliferative roles [14]. For instance, bilirubin enhances SMCs apoptosis, preventing its accumulation in vascular walls, and it blocks the recruitment and infiltration of leukocytes into these walls [94]. Finally, bilirubin cytoprotective action might be related to its capacity to inhibit inducible NOS (iNOS), reducing the production of the free radical ONOO⁻ [95].

In the same way, CO inhibits apoptosis in vascular ECs by activation of p38 mitogen-activated protein kinase (MAPK) signaling pathway [96]. Besides, CO blocks LPS-derived upregulation of iNOS and therefore an overproduction of NO in macrophages [97], modulating the response of these cells against bacterial LPS [98]. At low concentrations, CO inhibits vasoconstrictor enzymes, such as endothelin-1, as well as the production of TNF α and IL-1 (pro-inflammatory interleukin) and increases IL-10 (anti-inflammatory interleukin) [99]; while at high concentrations it has a vasoconstriction effect, inhibiting the action of eNOS. Among other properties of CO, the antioxidant role of this molecule might be due to its capability to bind Fe²⁺ in the heme group, preventing oxidation of hemoproteins, and avoiding the release of free heme [14][100].

The heme molecule itself is fundamental for many biological functions, due to its role as prosthetic group in hemoproteins like Hemoglobin (Hb), a major transporter of O₂. However, free heme can be toxic for several reasons [101]. For instance, heme can affect vascular ECs integrity by oxidation of LDL [73][102], and it can enhance cellular cytotoxicity through different sources of ROS [103]. Moreover, free heme increases the chances of releasing Fe²⁺ from its porphyrin pocket [101], which can be oxidized to Fe³⁺ through the Fenton reaction, an important source of ROS. Fe³⁺ can promote LDL oxidation in presence of superoxide anion (O₂⁻) [73]. Moreover, excessive amounts of iron can also modulate the activity of several enzymes directly involved in regulating cholesterol and triglyceride levels, which could have a negative effect over lipid metabolism [104]. For instance, different studies have demonstrated the correlation between iron overload and endothelial dysfunction, suggesting a critical role of redox active iron in the proinflammatory response of ECs [105]. Notably, elevated concentrations of iron can be found in atherosclerotic lesions compared to non-atherosclerotic vessels [78]. Therefore, the presence of redox active iron in atherosclerotic plaques may enhance the progression of atherosclerotic lesions by promoting an increase of free radicals, lipid peroxidation, and endothelial dysfunction.

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