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Reactive Oxygen Species: Physiological Roles in the Regulation of Vascular Cells

D. Vara and G. Pula*

Department of Pharmacy and Pharmacology, Centre for Regenerative Medicine, Claverton Campus, University of Bath, Bath, BA2 7AY, UK

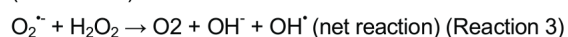
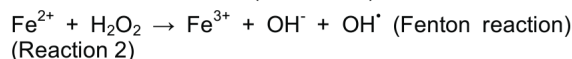
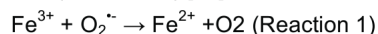
Abstract: Reactive oxygen species (ROS) are now appreciated to play several important roles in a number of biological processes and regulate cell physiology and function. ROS are a heterogeneous chemical class that includes radicals, such as superoxide ion ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}) and nitric oxide (NO^{\cdot}), and non-radicals, such as hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), hypochlorous acid (HOCl), and peroxynitrite (NO_3^-). In the cardiovascular system, besides playing a critical role in the development and progression of vasculopathies and other important pathologies such as congestive heart failure, atherosclerosis and thrombosis, ROS also regulate physiological processes. Evidence from a wealth of cardiovascular research studies suggests that ROS act as second messengers and play an essential role in vascular homeostasis by influencing discrete signal transduction pathways in various systems and cell types. They are produced throughout the vascular system, regulate differentiation and contractility of vascular smooth muscle cells, control vascular endothelial cell proliferation and migration, mediate platelet activation and haemostasis, and significantly contribute to the immune response. Our understanding of ROS chemistry and cell biology has evolved to the point of realizing that different ROS have distinct and important roles in cardiovascular physiology. This review will outline sources, functions and molecular mechanisms of action of different ROS in the cardiovascular system and will describe their emerging role in healthy cardiovascular physiology and homeostasis.

Keywords: Cardiovascular system, endothelial, hydrogen peroxide, platelet, reactive oxygen species, redox, smooth muscle, superoxide anion.

BIOCHEMISTRY AND REGULATION OF ROS

ROS are a heterogeneous class of molecules characterized by one or more highly reactive oxygen atoms characterized by a partially reduced state, short half-life and strong oxidant activity (Table 1). They are a product of aerobic metabolism and are generated by the respiratory chain [1] or by other cellular enzymes, including nicotinamide adenine dinucleotide phosphate-oxidase (NOX [2]), xanthine oxidase (XO [3]), lipoxygenase (LOX [4]), cyclooxygenase (COX [5]) and nitric oxide synthase (NOS [6]). The superoxide anion ($O_2^{\cdot-}$) is generated by one-electron reduction of molecular oxygen and represents the precursor of most ROS [7]. The dismutation of $O_2^{\cdot-}$ leads to the generation hydrogen peroxide (H_2O_2) via spontaneous reactions in aqueous solution or via reactions catalyzed by superoxide dismutases (SODs [8]). H_2O_2 can be converted into highly reactive hydroxyl radicals (OH^{\cdot}) via the Haber-Weiss reaction [9], in which H_2O_2 can be partially reduced to OH^{\cdot} in a reaction requiring $O_2^{\cdot-}$. This process is slow but greatly enhanced in the presence of the redox-cycling metal $Fe^{2+/3+}$, which functions as electron acceptor to generate O_2 from $O_2^{\cdot-}$ (Reaction 1) and as electron donor in the Fenton reaction leading to H_2O_2 degradation (Reaction 2). The

net reaction is the consumption of one H_2O_2 and one $O_2^{\cdot-}$ molecule to generate one O_2 , one OH^{\cdot} and one OH^{\cdot} (Reaction 3) [10]:



Alternatively, the enzyme myeloperoxidase (MPO) can convert H_2O_2 into the highly reactive ROS hypochlorous acid (HOCl) [11]. Another important ROS in cell biology is singlet oxygen (1O_2), which is a transiently excited state of molecular oxygen generated by natural photochemical and photobiological processes [12]. 1O_2 plays an important role in degenerative phenomena such as photodegradation, aging and photocarcinogenesis [13]. Finally, nitric oxide (NO^{\cdot}) is also an important ROS and is generated by the oxidation of L-arginine to L-citrulline catalyzed by nitric oxide synthases (NOS). Three types of NOS have been described so far: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [14]. NO^{\cdot} has been shown to exert important physiological functions via activation of soluble guanylyl cyclase (sGC) and generation of cyclic guanylate phosphate (cGMP), which include vasodilation, platelet inhibition and immune response [15-19]. NO^{\cdot} reacts rapidly with $O_2^{\cdot-}$ to generate the physiological oxidant peroxynitrite (NO_3^- or $NO_3^{\cdot-}$) [20, 21]. NO_3^- reacts with different biomolecules including CO_2 (that generates

*Address correspondence to this author at the Department of Pharmacy and Pharmacology, Centre for Regenerative Medicine, Claverton Campus, University of Bath, Bath, BA2 7AY, UK; Tel: +44(0)1225385755; Fax: 01225 386114; E-mail: g.pula@bath.ac.uk

Table 1. Relevant reactive oxygen species (ROS). This table presents chemical structure (first column), name and abbreviation (second column) and most characterized metabolic origin of the most important ROS for human health.

	Superoxide Anion ($\cdot\text{O}_2^-$)	<ul style="list-style-type: none"> • Mitochondrial respiration • NADPH oxidase (NOX) • Uncoupled nitric oxide synthase (NOS) • Xanthine oxidase (XO)
	Hydrogen Peroxide (H_2O_2)	<ul style="list-style-type: none"> • Superoxide dismutase (SOD) • NOX4, DUOX1 and DUOX2
	Hydroxyl Radical ($\cdot\text{OH}$)	Reaction of H_2O_2 with Fe^{2+} or Cu^{2+}
	Hypochlorous acid (HOCl)	Myeloperoxidase (MPO)
	Singlet Oxygen ($^1\text{O}_2$)	Natural photochemical and photobiological processes
	Nitric Oxide (NO \cdot)	Nitric oxide synthase (NOS)
	Peroxynitrite (NO $_2^-$)	Reaction of NO with O $_2^-$
	Carbonate Radical ($\cdot\text{CO}_3^-$)	NO $_3^-$ reaction with CO $_2$
	Nitrogen dioxide ($\cdot\text{NO}_2$)	NO $_3^-$ reaction with CO $_2$

other active oxidants such as carbonate radical ($\text{CO}_3^{\cdot-}$) and nitrogen dioxide (NO_2), peroxiredoxins and glutathione peroxidase (that generates nitrite (NO_2^-)), and different heme proteins [22, 23]. Collectively, oxidants derived from NO^\cdot are indicated as reactive nitrogen species (RNS). These include NO_2 and N_2O_3 and play an important role in the regulation of cell death and apoptosis [24].

Redox homeostasis is critical in maintaining healthy biological systems; therefore ROS can be eliminated by transformation into less reactive molecules. The most important example is catalase [25], which catalyzes the decomposition of H_2O_2 to water and oxygen. In view of the activity of catalases, above-mentioned SODs can be considered crucial for ROS detoxification because

they provide the substrate of catalases by transforming O_2^- into H_2O_2 [26]. Enzymes of the glutathione redox cycle are also important for controlling intracellular ROS concentrations by coupling the reduction of peroxides with the oxidation of glutathione (GSH) to glutathione disulfide (GSSG), which in turn is reduced back to GSH by glutathione reductase [27], thioredoxin reductase [28] and glutaredoxins [29]. In order to maintain the redox homeostasis of the cells following oxidative stress events, the reduction of GSSG and oxidized thioredoxins is guaranteed by an increase in the production of NADPH, which is induced by the inhibition of the glycolytic enzyme pyruvate kinase by direct oxidation of Cys358 and the consequent upregulation of the pentose phosphate pathway [30].

Finally, another possible mechanism of ROS detoxification is their non-enzymatic reaction with ascorbate and α -ketoacids, such as pyruvate, α -ketoglutarate and oxaloacetate [31, 32].

Besides enzymatic degradation or transformation of ROS, antioxidants can directly react with ROS to eliminate them or transform them into less reactive molecules [33]. A common feature of antioxidant molecules is an aromatic ring that can accommodate unpaired electrons of ROS. This is the case of vitamin E (lipophilic) and vitamin C (hydrophilic), which can donate electrons to hydroxyl or peroxy radicals to form water or stable hydroxyl molecules, respectively (Fig. 1). Interestingly, vitamin C can also regenerate vitamin E and other antioxidants after their oxidation by transferring an unpaired electron to them, while the regeneration of active vitamin C depends on the activity of NADH/NADPH-dependent reductases [34]. The antioxidant potential of vitamin E and vitamin C has suggested their use to protect the cardiovascular system from degenerative disease such as atherosclerosis [35]. Another physiological molecule with antioxidant properties initially proposed to protect against cardiovascular diseases is uric acid [36]. Despite its ability to detoxify ROS [37], the role of uric acid in the cardiovascular system has been re-evaluated and this molecule is now considered a cause of cardiovascular diseases rather than a mechanism of defense [38]. The tripeptide GSH has been proposed as an important physiological antioxidant involved in the redox regulation of cell physiology and health [39]. Because of the potent reducing activity of the thiol group of its cysteine residue and the millimolar concentrations that this molecule reaches within cells, GSH can be considered the most important line of defense against ROS accumulation. Glutathione S-transferases (GST) and glutathione peroxidases (GPx) catalyze the reduction of ROS, which is associated with the oxidation of GSH and its disulfide bridge mediated dimerization to GSSG. The reduction of GSSG to GSH by glutathione reductases maintains the balance between GSH and GSSG that is critical for cell homeostasis and survival. Recent evidence in non-mammalian eukaryotic cells suggests that the GSH:GSSG is different in different cell compartments, which can have an important function in redox stress detection and response [29]. In addition to physiological antioxidants, several synthetic and plant antioxidants are known. The former are mainly utilized for food conservation and not for human health because of their potential cell toxicity, where the latter are heavily investigated for biomedical purposes. Phenolics, flavonoids, carotenoids, steroids and thiol compounds from a variety of plants, such as grape, green tea, garlic, ginger, beetroot, ginseng, curcuma, ginkgo, green tea and rosemary have been proposed as natural antioxidants for human consumption in order to fight the negative effects of free radicals in aging and disease [40-44].

ROS-DEPENDENT POST-TRANSLATIONAL MODIFICATION OF PROTEINS

Direct reaction with amino acid residues and protein post-translation modification is critical for the physiological function of ROS. ROS can directly react with amino acid residues (Fig. 2) and affect protein folding, trafficking, degradation and activity (Table 2) [45], which have very important physio-pathological consequences for human health. The most important and investigated protein modification by ROS is cysteine oxidation by H_2O_2 or HOCl [23]. The products of cysteine oxidation by H_2O_2 or HOCl are H_2O or Cl^- , respectively, and most importantly cysteine sulfenic acid that can undergo further oxidation to generate cysteine sulfinic acid and cysteine sulfonic acid or can form intramolecular or intermolecular disulfide bridges [45]. Recently, the generation of cysteine sulfenic acid by ROS has been described as necessary for endothelial cell migration and pro-angiogenic responses [46]. The post-translational modification of proteins by OH^\cdot can also occur *via* reversible reaction with methionine residues to form methionine sulfoxide [47], which can be reduced back to methionine by the enzyme methionine sulfoxide reductase [48], or irreversible oxidation of lysine, arginine, proline and histidine to form protein carbonyls [49]. Besides its role in cysteine oxidation described above [50], H_2O_2 can also post-translationally modify proteins by oxidizing histidines to 2-oxo-histidines [51]. Thiol groups of cysteines can react with NO^\cdot , which leads to generation of S-nitrosylated cysteines [52]. Although, the activity of certain enzymes including S-nitroso-glutathione (GSNO) reductase facilitates cysteine S-nitrosylation, this reaction does not appear to be catalysed by a specific group of enzymes [53]. Cysteine S-nitrosylation affects protein-protein interactions in multiprotein complexes and regulates the activity of a variety of proteins including metabolic enzymes, oxidoreductases, proteases, protein kinases and phosphatases, membrane receptors, ion channels and transcription factors [53, 54]. In addition, NO_3^\cdot has been shown to efficiently react with proteins by tyrosine nitration, which adds in a covalent manner a nitro group (NO_2) to the aromatic of this amino acid and forms 3-nitrotyrosine [55, 56]. Tyrosine nitration significantly affects the activity and the proteolytic degradation of proteins [57], which underlies the key role of this post-translational modification of proteins in aging and degenerative diseases such as atherosclerosis and neurodegeneration [58, 59]. Finally, HOCl can also modify proteins by oxidation of cysteines to sulfenic acid or tyrosines to chlorotyrosine [11].

The oxidation of cysteine by ROS has important functional consequences for protein phosphatases and protein kinases [60]. Several protein phosphatases are inhibited by ROS as a consequence of the oxidation of key cysteine residues in their catalytic site [61-63]. This results in a net increase in the phosphorylation of their targets and is often mistaken as a positive regulation of a protein kinase with similar specificity. Examples of

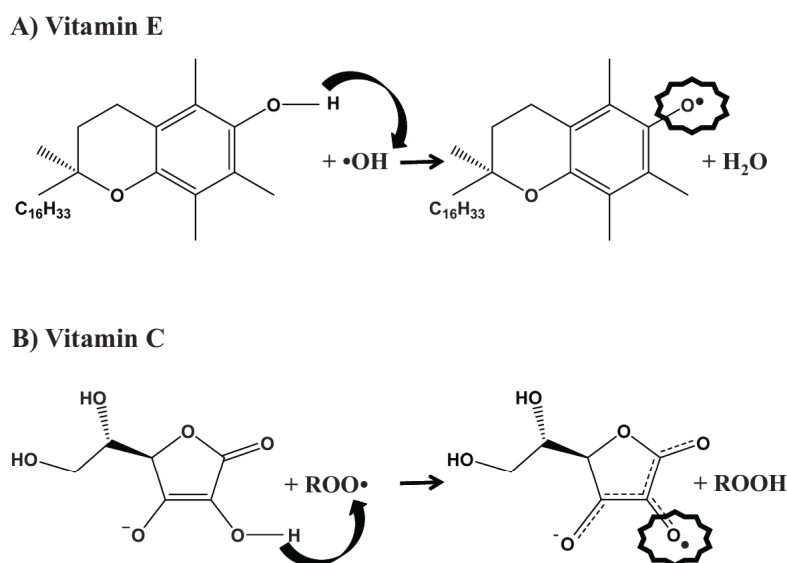


Fig. (1). Natural antioxidants. This figure represents the structure and chemical mechanism of action for vitamins E as detoxifier of hydroxyl radicals (A) and vitamin C as detoxifier of peroxy radicals (B).

protein phosphatases negatively regulated by ROS are protein tyrosine phosphatase 1B (PTP1B) [64], tyrosine phosphatase 2a and 5 (PTP2A and PTP5) [65], mitogen-activated protein kinase phosphatase (MKP) [66] and focal adhesion kinase (FAK) tyrosine phosphatase [67]. ROS-dependent modification of protein phosphatase has been suggested to play a dual role of temporary inhibition of the catalytic activity while protecting the phosphatase from permanent inactivation and degradation, such as in the case of PTP1B [68]. On the other hand, several protein kinases are activated by ROS, which plays an important role in vascular homeostasis [69]. For example, apoptosis signal-regulated kinase 1 (ASK1) of the MAPK family is directly activated by cysteine oxidation [70] and inhibited by interaction with and consequent reduction by thioredoxin [25, 71]; ROS-dependent oxidation of two specific cysteines within the redox centre of thioredoxin abolishes the interaction with ASK1 and facilitates the activation of the kinase in a redox-dependent manner [72]. Similarly dependent on cysteine oxidation by ROS and disulfide bridge formation is the activation of Src family kinases, with the activation in response to NO and H₂O₂ depending on the oxidation of a yet unidentified cysteine residue [73] and two cysteines residues in the kinase domain responsible for the activation in response to LOX-derived ROS [74]. The regulation of Src family kinases by ROS is nevertheless still controversial because of studies reporting an inhibition of these enzymes by H₂O₂ in fibroblast and endothelial cell cultures [75]. Members of protein kinase C (PKC) family also display ROS-dependent regulation, although their regulation appears to be more complex. Several reports describe ROS-dependent oxidation of cysteines within regulatory and catalytic domains of PKC leading to kinase

activation [76-78]. In contrast, redox-dependent oxidation and formation of intra- and inter-molecular disulfide bridges have been shown to inactivate different PKC isozymes [79, 80]. The different types and concentrations of ROS seem therefore critical to determine how protein kinase activity is affected. Interestingly, PKC has been shown to be activated in a H₂O₂-dependent manner in pulmonary smooth muscle and vascular cells [81], which is likely to play a key role in the increase in endothelial cell contractility and the resulting oedema induced by H₂O₂ [82]. Direct oxidative modification of amino acids has been shown to activate other important kinases, such as: 1) calmodulin-dependent kinase II (CaMKII), which is activated in a calcium-independent manner by redox-dependent methionine oxidation [83]; 2) protein kinase G1 α (PKG1 α), which is also activated by ROS with the oxidation of Cys-42 by H₂O₂ responsible for forming an intermolecular disulfide bond and generating a highly active homodimer of this protein kinase [84]; 3) protein kinase B (PKB/Akt), which is oxidized at a cysteine residue (Cys124) and redox-dependently activated [85]. Although in contrast with existing literature [65], the activation of protein phosphatase 2A in a ROS-dependent manner has been suggested to dephosphorylate and inactivate PKB [86], which therefore display complex or perhaps tissue-dependent redox regulation.

Amongst the effect of redox regulation of protein kinases, there is the modulation of certain transcription factors. Forkhead box O (FOXO) transcription factors have been shown to be finely regulated by oxidative stress, with ROS-dependent inhibition of phosphatase 2A and PTEN responsible for the increase in PI3K/PKB activity leading to FOXO inactivation counterbalanced by ROS-dependent activation of ASK1 and JNK

Table 2. Examples of ROS-dependent protein regulation. Protein name, function and redox-dependent type of regulation and post-translational modification are reported. Abbreviations: AP-1 (activator protein 1); ASK1 (apoptosis signal-regulated kinase 1); ATM (Ataxia Telangiectasia Mutated Protein); CaMKII (calmodulin-dependent kinase II); FAK (focal adhesion kinase); FOXO (Forkhead box O); HDAC (histone deacetylase); HIF1 (hypoxia-induced factor 1); MKP (mitogen-activated protein kinase phosphatase); NFkB (nuclear factor kappa beta); PHD (prolyl hydroxylase); Nrf-2 (nuclear factor erythroid 2-related factor 2); PKB/Akt (protein kinase B); PKC (protein kinase C); PKG1 α (protein kinase G1 α); PTEN (phosphatase and tensin homolog); PTP1B (tyrosine phosphatase 1B); PTP2A (tyrosine phosphatase 2a); PTP5 (tyrosine phosphatase 5); SFKs (Src family kinases); sGC (soluble guanylyl cyclase); Sox (Sry-related HMG box); TRPA1 (Transient Receptor Potential Ankyrin 1).

Name	Category	Oxidation-Dependent Protein Regulation (In Brackets We Indicate the Associated Molecular Modification, If Known)
SFKs	Kinase	Activation (S-nitrosylation/disulfide bond formation) Inactivation (homodimerization)
PKCs	Kinase	Activation Inactivation (disulfide bond formation)
ATM	Kinase	Activation (phosphorylation)
CaMKII	Kinase	Activation
PKG1 α	Kinase	Activation (homodimerization)
PKB/Akt	Kinase	Activation
ASK1	Kinase	Activation (multimerization)
PTP1B	Phosphatase	Inactivation (phosphorylation)
PTP2A	Phosphatase	Inactivation (phosphorylation)
PTP5	Phosphatase	Inactivation (phosphorylation)
MKP	Phosphatase	Inactivation (phosphorylation)
FAK Tyr phosphatase	Phosphatase	Inactivation (phosphorylation)
PTEN	Phosphatase	Inactivation
FOXOs	Transcription factor	Activation (ASK1-dep. phosphorylation) Inactivation (acetylation and PKB-dep. phosphorylation)
AP-1 (Fos/Jun)	Transcription factor	Inactivation
NFkB	Transcription factor	Activation (phosphorylation and degradation of inhibitor I κ B) Inactivation (disulfide bond formation)
Nrf-2	Transcription factor	Activation
HIF-1	Transcription factor	Activation (reduced degradation by inhibition of PHD)
SoxR	Transcription factor	Activation (prosthetic group oxidation)
HDACs	Transcriptional regulators	Inactivation (acetylation)
Integrins	Adhesion receptors	Activation (disulfide bond formation)
Caspase 9	Protease	Activation (disulfide bond formation)
TRPA1	Ion channel	Activation
sGC	Enzyme	Activation (heme nitrosylation)

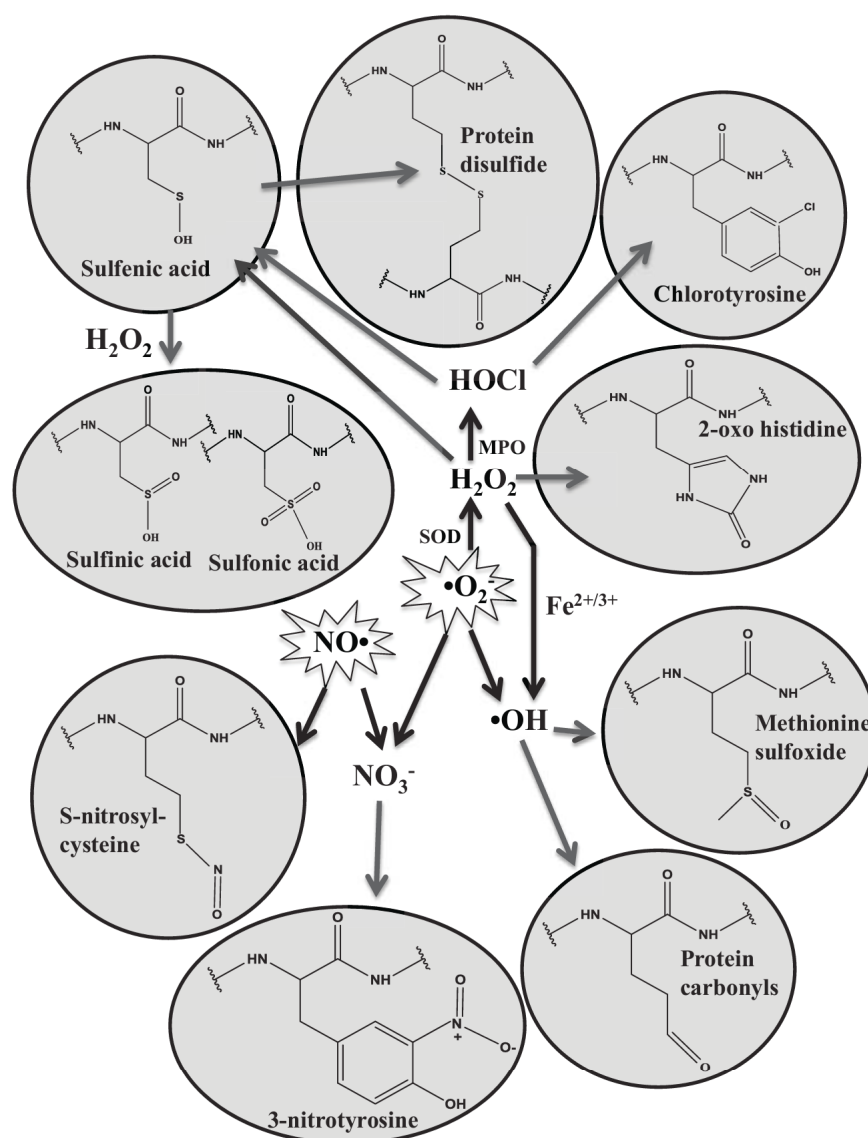


Fig. (2). Major protein modifications by ROS. Direct oxidation of histidines and cysteines by H_2O_2 leads to 2-oxo-histidines and sulfenic acid, respectively. Sulfenic acid can be oxidized further to sulfinic and sulfonic acids or can promote disulfide bond formation. Oxidation of cysteine to sulfenic acid can also be promoted by HOCl, whereas the reaction of this ROS with tyrosine leads to formation of chlorotyrosine. $\text{OH}\bullet$ generates methionine sulfoxide or protein carbonyls. $\text{NO}\bullet$ can directly S-nitrosylate cysteine residues or can interact with $\text{O}_2^{\bullet-}$ to form NO_3^- , which in turn determines the formation of 3-nitrosotyrosine. Partial reduction of H_2O_2 to $\text{OH}\bullet$ is promoted by transition metals iron and copper (Fe^{2+} and Cu^{2+}). Formation of HOCl from H_2O_2 is catalysed by myeloperoxidases (MPO), while superoxide dismutase (SOD) is responsible for the formation of H_2O_2 from $\text{O}_2^{\bullet-}$.

responsible for FOXO-dependent activation of antioxidant genes [87]. Direct acetylation of FOXO in response to ROS treatment has also been shown and is responsible for the inhibition of the DNA-binding activity of this transcription factor [87]. In the case of NF- κB , ROS-induced serine and/or tyrosine phosphorylation, ubiquitination and consequent proteolytic degradation of the inhibitor of NF- κB (I κB)

are responsible for nuclear transportation and gene activation [88]. The protein kinase phosphorylating and regulating I κB in a RPS-dependent manner are several, including I κB kinases (IKKs), phosphoinositide 3 kinase (PI3K), Src and CaMKII [89].

Another important mechanism for ROS-dependent transcription factor regulation is the ROS-dependent

modulation of their proteolytic degradation. The most suggestive example is the hypoxia-induced factor 1 (HIF1), which is proposed to be regulated by ROS-dependent inhibition of its proteolytic degradation [90]. In this case, the activity of the enzyme prolyl hydroxylase appears inhibited by H_2O_2 [91]. This in turn decreases the proteasome-mediated proteolysis of HIF1 α , which heterodimerizes with HIF1 β , translocates to the nucleus and stimulates the expression of key anti-hypoxic genes, such as vascular endothelial growth factor (VEGF), angiopoietin, stromal-derived factor 1 (SDF1) and erythropoietin [92]. Finally, transcription factors and other genes can be down-regulated in a ROS-dependent fashion at the transcription level by hypermethylation of their promoter regions [93-95]. This ROS-dependent epigenetic regulation of gene expression appears to be particularly important in tumor development and growth.

Besides direct interaction with amino acids, ROS can react with the prosthetic groups of enzymes and growth factors, thus significantly affecting their physiological activity. As mentioned above, the most important example is the activation of sGC by NO^* , which occurs by binding of the heme moiety within the enzyme and leads to a 200-fold increase in the enzyme activity [96]. Transcription factors are also important targets of ROS-dependent regulation and their regulation often involves the modification of their prosthetic groups rather than the modification of their amino acids. An important example of transcription factor regulated by redox-dependent modification of a prosthetic group is bacterial Sox, which regulate the expression of antioxidant enzymes [97]. In the case of SoxR, an iron-sulphur cluster in the RNA polymerase-binding domain is responsible for responding to redox regulation by showing increased ability to activate gene transcription in its O_2^{2-} -dependent oxidized state [98].

ROS-DEPENDENT MODIFICATION OF NUCLEIC ACIDS

Besides proteins, ROS can also directly modify nitrogen bases within nucleic acids, which is an important and well-characterized response to ROS generation (Fig. 3). Amongst nitrogen bases, guanine is the most susceptible to ROS-dependent modification and the product 8-hydroxyguanine (8-OHG) is a key product of DNA oxidation [99, 100]. 8-oxoguanine (8-oxoG) [101] and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FapyGua) [102] are other oxidative guanine modifications found in cellular DNA. The most important ROS for DNA modification is 1O_2 , which appears to target guanine specifically, and OH^* , which generates a multiplicity of products from all four DNA bases [103]. OH^* has been shown to react with C4, C5 and C8 of guanines and adenines, leading to several possible products, including 8-OHG, FapyGua, 8-hydroxyadenine (8-OHA), and 5-formamido-4,6-diamino-pyrimidine (FapyAde). The reaction of OH^* with pyrimidines gives rise to 5,6-dihydroxy-5,6-dihydrothymine (5,6-diOH-5,6-diHT), 5-hydroxy-5-

methylhydantoin (5-OH-5MeI), 5,6-dihydrothymine (5,6-diHT), 5-hydroxymethyluracil, 5-hydroxyuracil (isobarbituric acid), 5,6-dihydroxyuracil, 5-hydroxycytosine (5-OHC) and 5,6-dihydroxycytosine (5,6-diOHC). OH^* can react directly with the deoxyribose moiety of the DNA leading to the release of purines and pyrimidines and the formation of abasic sites and to strand breaks [104, 105]. Besides oxidation, guanine is also a preferential target for RNS-mediated modification. Amongst possible nitration products of guanine, 8-nitroguanine (8- NO_2G) is an important product proposed to play a role in inflammatory diseases [106]. NO_3 reacts with G to yield 8-nitroguanine (8- NO_2G) [107, 108] and 5-nitroguanidinohydantoin (NI) [107, 109]. Furthermore, ROS-dependent generation of 8-OHG and 5-hydroxymethylcytosine (5-OHMeC) has been shown to have important consequences on the interaction of DNA with methyl-binding proteins (MBPs) recognition sequence [110, 111], thus providing evidence that ROS-induced DNA damage interferes with DNA methylation-dependent regulation of gene expression. This is responsible for the down-regulation of different transcription factors and anti-tumor proteins [93-95]. Gene expression can also be increased by DNA oxidation. The most important example is the VEGF promoter, which is oxidized by mitochondrial ROS thus resulting in increased interaction with the transcription factor HIF1 α [112, 113]. Another important example of ROS-induced gene expression is associated with the expression of important genes such as oestrogen receptor, androgen receptor, retinoic acid receptor, thyroxin receptor and activating protein 1 [114]. Specific regulatory sites in these genes are oxidized by the H_2O_2 produced by lysine-specific histone demethylase 1A (KDM1A), which leads to generation of 8-oxoG and in turns attracts the interaction of the enzyme 8-oxoG DNA glycosylase I (OGG1). Ultimately, OGG1 causes single strand DNA breaks responsible for the recruitment of topoisomerase II β , which changes DNA conformation at promoter regions, stimulate transcription factor binding and initiates DNA transcription [115].

ROS GENERATION IN CARDIOVASCULAR TISSUES AND CELLS

NADPH Oxidases

NADPH oxidases (NOX) are a group of multimeric enzymes whose activity results in the production of O_2^{2-} as a consequence of the transfer of electrons from NADPH to molecular oxygen. First identified in phagocytic cells, they mediate the respiratory burst associated with pathogen phagocytosis and innate immune response [116]. The deficiency in the ability of NOXs to generate O_2^{2-} is responsible for reduced microbicidal activity of leukocytes and the development of the rare condition known as chronic granulomatous disease (or CGD) [117]. There are seven NOXs, NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 [118]. They represent the catalytic core of a multimeric complex which generates primarily O_2^{2-} ,

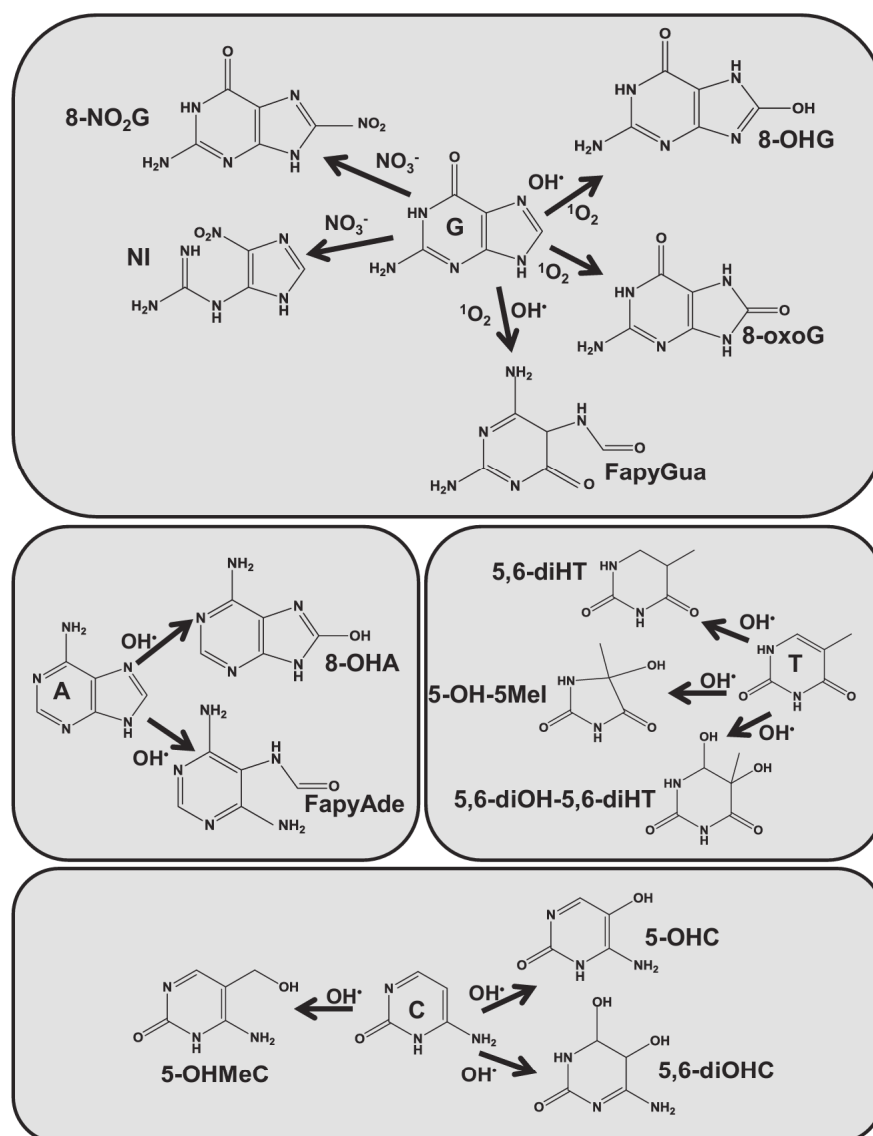


Fig. (3). Major nucleic acid modifications by ROS. 1O_2 , selectively oxidizes guanine leading to formation of 8-hydroxyguanine (8-OHG) 8-oxoguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FapyGua), while OH^\cdot generates a multiplicity of products from all four DNA bases, including 8-OHG, 8-oxoG, FapyGua, 8-hydroxyadenine (8-OHA), 5-formamido-4,6-diamino-pyrimidine (FapyAde), 5,6-dihydroxy-5,6-dihydrothymine (5,6-diOH-5,6-diHT), 5-hydroxy-5-methylhydantoin (5-OH-5MeI), 5,6-dihydrothymine (5,6-diHT), 5-hydroxycytosine (5-OHC), 5,6-dihydroxycytosine (5,6-diOHC) and 5-hydroxymethylcytosine (5-OHMeC). Moreover, NO_3^- reacts with G to yield 8-nitroguanine (8-NO₂G) and 5-nitroguanidinohydantoin (NI).

although H_2O_2 appears to be generated by DUOX1, DUOX2 and NOX4 due to delayed dissociation of $O_2^{\cdot-}$ and its dismutation before release [119, 120]. Di-/tri-tyrosine and protein cross-linking can also be generated by the peroxidase-like domain of DUOX1 and DUOX2 [121]. NOX complexes also include several regulatory proteins, which participate in the maturation and localization of the NOX complexes in

biological membranes (p22^{phox}, DUOX activator 1 or DUOXA1 and DUOX activator 2 or DUOXA 2), regulate their enzymatic activity (p67^{phox} and NOX activator 1 or NOXA1) and determine their spatial organization (p47^{phox}, p40^{phox} and NOX organizer 1 or NOXO1) [122, 123]. The small cytoplasmic GTPase Rac1 and Rac2 are also involved in the activation of certain NOX1, NOX2 and NOX3 [124].

NOXs are important sources of ROS in the vascular wall, with endothelial cells expressing NOX1, NOX2, NOX4 and NOX5 [125], vascular smooth muscle cells expressing NOX1, NOX4 and NOX5 [126], and adventitial fibroblasts expressing NOX2 and NOX4 [127]. The hyperactivity or upregulation of NOXs in the vascular wall and the consequent overproduction of $O_2^{\cdot-}$ are critical for the development of endothelial injury associated with vascular diseases. The link with vascular diseases has been demonstrated for NOX2 [128-130] and NOX 1 [131-134], whereas the role of NOX4 remains controversial possibly as a consequence of the different catalytic activity of this enzyme compared to NOX1 and NOX2 (i.e. co-production of H_2O_2 rather than $O_2^{\cdot-}$) [119, 120]. Amongst circulating cells in the cardiovascular system, as mentioned above, NOX2 has long been known to be expressed and functionally relevant in cells involved in the innate immune response, such as neutrophils, monocytes and macrophages [135]. Red blood cells have also been shown to express NOXs [136] and the enzymatic generation of has been linked with the cell fragility of sickle cell disease [137]. NOX2 (or Gp91^{phox}) and different regulatory subunits of the NOX complex (p22^{phox}, p47^{phox}, and p67^{phox}) have been known to be expressed in human platelets [138-141], while only recently we proved also the expression of NOX1 [142].

Uncoupled Endothelial Nitric Oxide Synthase

All nitric oxide synthase (NOS), including the endothelial form (eNOS), require tetrahydrobiopterin (BH4) and L-arginine to dimerize and generate NO^{\cdot} , respectively. The availability of BH4 is determined by regulation of its synthesis and degradation to BH2 [143]. The reduction of BH4 levels results in the "uncoupling" of NOS activity from NO^{\cdot} generation, which results in oxygen reduction and generation of $O_2^{\cdot-}$. Oxidative stress, angiotensin II, homocysteine, folate and vitamin C have been shown to reduce BH4 bioavailability and induce eNOS uncoupling, which is implicated in the vascular complications of diabetes, coronary artery disease, cardiac failure and ischemia-reperfusion injury [143]. The validity of this hypothesis has been suggested by studies showing that the administration of exogenous BH4 helps reducing the vascular dysfunction associated with different cardiovascular diseases [144].

Xanthine Oxidase

Xanthine oxidase (XO) is generated by oxidation of sulfhydryl groups and proteolysis of the precursor enzyme xanthine oxidoreductase (XOR) during inflammatory events, such as ischemia and age-related tissue damage [145, 146]. XO contains six electrons that can be transferred to molecular oxygen to generate two $O_2^{\cdot-}$ and two H_2O_2 per XO molecules and catalyzes the two-step conversion of hypoxanthine to uric acid [145, 147]. Alternatively, in healthy conditions, XOR is mostly converted to xanthine dehydrogenase (XDH), which preferentially transfers electrons to NAD^+ to generate NADH. The transition from XDH to XO is

regulated by disulfide bond formation and proteolysis [148]. The increase in XO in inflammatory vascular conditions is at least partially responsible for the elevation of ROS generation in myocardial failure, coronary artery disease and vascular aging [145, 146, 149, 150]. The reaction of XO-derived $O_2^{\cdot-}$ with NO^{\cdot} has also been shown to generate NO_3^- , which is responsible for endothelial dysfunction in cigarette smokers [151]. For these reasons, inhibitors of XO have been proposed for the treatment of vascular aging and cardiovascular diseases [152-154].

Mitochondrial Respiratory Metabolism

$O_2^{\cdot-}$ can be generated non-enzymatically by transfer of a single electron to molecular oxygen from prosthetic groups such as flavins, ubisemiquinone, or iron-sulfur clusters. The mitochondrial electron transport chain is characterized by several redox centers, which transfer electrons to oxygen and may release $O_2^{\cdot-}$ as a consequence of the incomplete reduction of molecular oxygen [155]. Although there are tissue-specific and physiological state-specific variations in the relative contribution of different mitochondrial redox complexes to the generation of $O_2^{\cdot-}$, complex III has an important role in ROS generation within heart, lungs and the vascular system [90, 156]. Complex III transfers electrons from ubiquinol to cytochrome c and couples this process to the translocation of protons across the mitochondrial inner membrane. The electron transfer occurs *via* a series of intermediate acceptors including cytochrome b_{566} , cytochrome b_{562} , Rieske Fe-S protein and cytochrome c1 [157]. Experimental evidence obtained pharmacologically suggests that $O_2^{\cdot-}$ is generated downstream of the Rieske Fe-S proteins as a consequence of the autoxidation of the ubisemiquinone intermediate within the mitochondrial inner membrane and sequestration of one electron by membrane-permeable molecular oxygen [90, 158, 159]. Overall, the generation of $O_2^{\cdot-}$ is balanced by the enzymatic activity of manganese superoxide dismutase (MnSOD) within the mitochondrial matrix or copper-zinc superoxide dismutase (CuZnSOD) in the intermembrane space [160, 161], which tightly regulate the accumulation of ROS in mitochondria. The production of ROS by the mitochondria is an important response to hypoxia, which activates a series of adaptive changes in gene expression and cell physiology. Complex III also appears critically involved in the hypoxic mitochondrial generation of ROS [90].

Cyclooxygenases and Lipoxygenases

Cyclooxygenases (COXs) are responsible for the conversion of arachidonic acid into prostaglandin H2 (PGH2) *via* a two-step process leading to formation of prostaglandin G2 (PGG2) and its subsequent reduction to PGH2 by the peroxidase site of the enzyme. PGH2 serves as precursor for the production of different members of the prostaglandins family and for the generations of thromboxanes [162]. Although cyclooxygenases function *via* formation of a tyrosine hydroxyl radical able to catalyze the final reduction of

PGG2 to PGH2, their ability to generate ROS in the cellular environment has been questioned in several experimental models [163, 164]. Interestingly, recent studies in Chang Liver (CHL) cells suggested that COXs upregulate the expression of NOXs through generation of prostaglandins and stimulation of prostaglandin receptors, thus indirectly increasing the generation of $O_2^{\cdot-}$ [165]. On the other hand, in the cardiovascular system, the activity of endothelial COXs has been suggested to be necessary for endothelial-dependent vascular constriction and COXs have been shown to directly generate ROS [166]. In *ex-vivo* experiments on explanted blood vessels, the contracting effect of COXs was dependent on the generation of ROS, as proved by the inhibitory effect of ROS scavengers [167-169]. More recently, treatment of hypertensive animals with COX inhibitors reduced the expression of NOXs and the generation of ROS, which ultimately abolished the hypertensive phenotype both *in vivo* and *ex vivo* [170]. Unfortunately, the results from this study do not clarify whether the activation of COX is directly responsible for ROS generation *in vivo* or whether it acts *via* upregulation of the ROS-generating enzymes NOXs, which was previously suggested [165].

Lipoxygenases (LOXs) are another class of enzyme able to generate ROS. LOX catalyze the oxygenation of fatty acids (mostly arachidonic acid) to fatty acid-hydroperoxide and they are classified as 5-, 8-, 12- or 15-LOX depending on which carbon atom they target. The oxidized products that they generate can induce oxidative changes in the redox balance of the cell [171]. Similarly to COXs, the generation of ROS associated to LOX activation seems to be mediated by expression, membrane translocation and activity stimulation of NOXs [172, 173]. The functional coupling of LOXs and NOXs has also been observed in the

physio-pathology of the cardiovascular system, where 12/15-LOX contributes to vascular hyperpermeability *via* a NADPH oxidase-dependent mechanism [174]. Unfortunately, it is impossible to determine whether previous studies reporting LOX-dependent ROS generation in vascular tissues and cells also described a NOX-dependent phenomenon [175, 176].

Hemoglobin Autoxidation

The spontaneous oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) leads to formation of methemoglobin and $O_2^{\cdot-}$ at low oxygen concentration or oxide radical ($\cdot OOH$) in conditions of high oxygen [177]. Moreover, $O_2^{\cdot-}$ can be dismutated to H_2O_2 , which oxidises ferrous and ferric ion further to ferryl ion (Fe^{4+}) or ferryl protein radicals, respectively [136]. Hemoglobin autoxidation leads to loss of oxygen transporting function and heme group/iron ions liberation, which has been suggested to participate in inflammatory response and vascular diseases [178, 179].

FUNCTIONS OF ROS IN THE HEALTHY CARDIOVASCULAR SYSTEM

The role of ROS in oxidative stress, cell death and disease is a highly investigated area of biomedical research that requires in depth discussion that lies outside the scope of this review. Some examples of redox-dependent mechanisms of disease development are shown in Table 3. This aspect of ROS biology has been efficiently summarized in other literature reviews [180, 181]. Within the cardiovascular system, the toxic effect of ROS on vascular cells has been associated with $O_2^{\cdot-}$ - and H_2O_2 -dependent stimulation of mitogen activated protein kinases (MAPKs), tyrosine kinases, Rho kinase and several transcription factors including NF- κ B, and HIF-1 [182, 183]. Here, we will focus on the

Table 3. Examples of ROS-dependent disease mechanisms. This table presents a list of diseases characterised by redox-dependent pathogenesis (left column), the accepted mechanism of involvement of redox unbalance in disease etiology (central column) and a recent study/review explaining the link between ROS generation and the disease (right column).

Disease	Redox Mechanism	Reference
Alzheimer	Lipid peroxidation in cortical neurons	[321]
	Protein nitration/oxidation in cortical neurons	[322, 323]
Neurodegeneration	Oxidative DNA damage in neurons	[324, 325]
Multiple sclerosis	Protein/DNA oxidation in oligodendrocytes and astrocyte	[326, 327]
Type 1 diabetes	ROS-dependent release of inflammatory cytokines and β cell destruction	[328]
Type 2 diabetes	ROS-dependent destruction of β cells	[329]
Vascular complications of diabetes	ROS-dependent dysfunction of endothelial cells	[330]
Chronic kidney disease	Oxidative stress/oxidative nucleic acid damage in renal cells	[331]
Atherosclerosis	Inactivation of nitric oxide by ROS and redox-dependent endothelial cell dysfunction	[332]
Hypertension	ROS-dependent DNA modification and gene expression changes in vascular cells	[332, 333]
Pulmonary hypertension	Hydrogen peroxide-dependent smooth muscle cell proliferation and vascular medial thickening	[334]
Atrial fibrillation	ROS-dependent alteration of atrial conductances	[335]

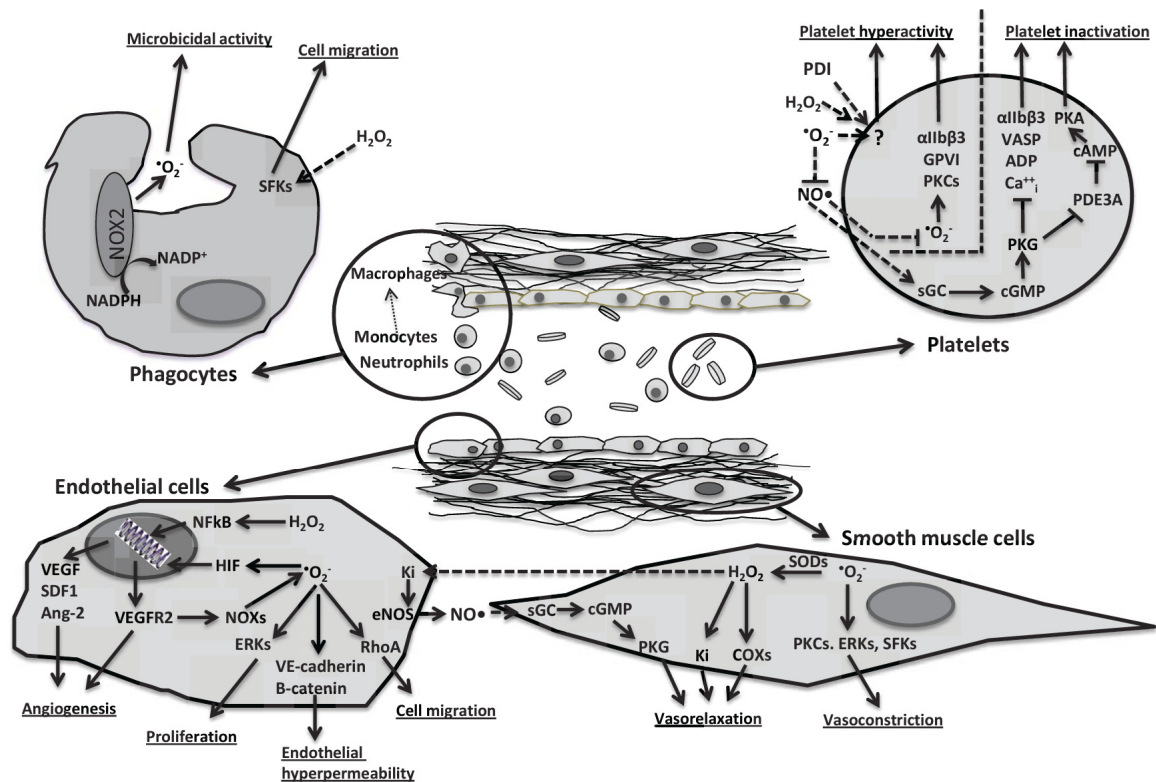


Fig. (4). Roles of ROS in the vascular system. ROS play several roles in the cardiovascular system, including promoting microbicidal activity and cell migration in phagocytes, stimulating endothelial cell motility and proliferation, endothelial permeability and angiogenesis. Vascular tone is dually regulated by ROS, with H_2O_2 and NO acting as vasorelaxing agents and $O_2^{\bullet -}$ acting as a vasoconstricting agent. Similarly, platelets and the haemostatic response display dual regulation by ROS, with $O_2^{\bullet -}$ and H_2O_2 promoting platelet activation while NO inhibits platelet function. Abbreviation: $\alpha IIb\beta 3$ (integrin $\alpha IIb\beta 3$); ADP (adenosine diphosphate); cAMP (cyclic adenosine monophosphate); cGMP (cyclic guanosine monophosphate); COX (cyclooxygenase); eNOS (endothelial nitric oxide synthase); ERK (extracellular-regulated kinase); HIF1 (hypoxia-induced factor 1); Ki (potassium channel); NADH (nicotinamide adenine dinucleotide); GPVI (glycoprotein VI); NADPH (nicotinamide adenine dinucleotide phosphate hydrogen); NFkB (nuclear factor kappa beta); NO (nitric oxide); NOX (NADPH oxidase); Nrf-2 (nuclear factor erythroid 2-related factor 2); PDE3A (phosphodiesterase 3A); PDI (protein disulfide isomerase); PKA (protein kinase A); PKC (protein kinase C); PKG (protein kinase G); SDF-1 (stromal-derived factor 1); SFKs (Src family kinases); sGC (soluble guanylyl cyclase); SOD (superoxide dismutase); VASP (vasodilator activated phosphoprotein); VEGF (vascular endothelial growth factor); VEGFR2 (vascular endothelial growth factor receptor 2).

physiological roles of ROS in the cardiovascular cell physiology and signaling [184] rather than their involvement in the aetiology and progression of cardiovascular diseases [185-187]. The roles of ROS in the healthy cardiovascular system are schematically represented in Fig. (4), which include regulation of: 1) phagocytes and innate immunity; 2) smooth muscle cells and vascular contractility; 3) platelets and haemostasis; 4) endothelial cells and angiogenesis.

Phagocytes and Innate Immunity

The evidence that ROS are important for the innate immune response and the activity of phagocytes (i.e. monocytes, macrophages, neutrophils, mast cells and dendritic cells) is amongst the first observations of a physiological role for oxidant molecules [188], which

was recently confirmed by the identification of NOX2 mutations as the cause for impaired immune response in chronic granulomatous disease (CGD) patients [189]. The ability of ROS to oxidize proteins, DNA, lipids, carbohydrates and metal-containing prosthetic group of the respiratory chain is responsible for the microbicidal activity of these class of physiological molecules [190]. In this context, NOXs seem to play a particularly important role as a source of antimicrobial ROS in the innate immune response [191]. In this respect, it might be physiologically relevant that NOX2 is expressed in phagocytic cells and other NOXs are expressed by barrier cells, such as intestinal, skin and lung epithelium [192]. Other indications of a role in the immune response for NOX-derived ROS are the induction of NOX1 expression by the inflammatory

mediator interferon γ [193] and its activation by lipopolysaccharide-dependent activation of Toll-like receptor 4 (TLR4) [194]. Similarly, DUOX enzymes expressed in mucosal cells of bronchi, trachea and salivary glands have been shown to actively participate in the immune defense of the respiratory tract by releasing antimicrobial H_2O_2 in saliva and bronchial fluids [195].

Besides a fundamental role in the antimicrobial activity of phagocytes that has been amply documented in the last 4 decades [196], ROS have been shown to play a key role in stimulating the migration of immune cells to the sites of infection/inflammation [197]. In fact, the activity of endothelial dual oxidases (DUOXs) and the production of H_2O_2 have been shown to direct the migration of leukocytes [198]. Leukocyte migration is stimulated Src family kinase activation (especially LYN) [199] and phosphatidylinositol-3,4,5-triphosphate (PIP3) accumulation due to phosphatase PTEN inhibition [200]. Interestingly, besides inducing immune cell migration, high ROS levels at the sites of inflammation might stimulate leukocytes retention by inhibiting cytoskeletal rearrangement in an actin glutathionylation-dependent manner [201].

Smooth Muscle Cells and Vascular Contractility

A critical observation suggesting a role for ROS in vascular constriction is that the enzymatic activity of SODs relaxes arterial constriction [202, 203], which suggests a role for $O_2^{\cdot-}$ in smooth muscle contraction. Although it remains to prove a role for direct protein kinase activation in several experimental systems, ROS-dependent vasoconstriction depends on protein kinase activation as suggested by its abolishment in the presence of non-specific protein kinase inhibitors [204] or inhibitors specific for protein kinase C [205], Src kinases [206-208] and extracellular signal regulated kinases (ERKs) [206]. ROS have also been shown to regulate the enzymes responsible for the opposite biochemical function: the protein phosphatases [209, 210]. Similarly to regulation of other protein function, the regulation of protein kinase and protein phosphatases in smooth muscle cells has been suggested to be associated with ROS-dependent oxidation or nitrosylation of reactive cysteine residues [211] or tyrosine nitrosylation [212].

Interestingly, the effect on vascular tone appears to depend on the chemical nature of ROS, with different effects of $O_2^{\cdot-}$ and H_2O_2 . Besides a vasoconstricting effect due to the scavenging of endothelial NO^{\cdot} [213, 214], $O_2^{\cdot-}$ has also been proposed to directly contract smooth muscle cells *via* activation of different kinases, including Src kinases [207], Rho kinases [207] and ERKs [215]. On the other hand, the role of H_2O_2 is more complex with reports of a vasoconstricting effect mediated by Src kinases [216] or protein kinase C [205, 217] and contrasting reports of a vasorelaxing role on arteries precontracted with phenylephrine or prostaglandin $F_{2\alpha}$ *via* either potassium channel regulation and cell hyperpolarization or protein kinase

modulation [218-220]. Some clarity on this matter has been made by more recent studies describing a range of different effects elicited by H_2O_2 in explanted aorta, consisting of: 1) direct cyclooxygenase activation and consequent contracting effect on blood vessel; 2) activation of endothelial potassium ion channels and increase in NO^{\cdot} generation in endothelium (which leads to vessel relaxation); 3) activation of smooth muscle cell potassium ion channels and endothelium-independent relaxation; 4) permanent damage of smooth muscle cells and reduction of their contractility [221]. Initial indications that OH^{\cdot} might be responsible for the contraction induced by the activity of xanthine oxidase have been dismissed by a study demonstrating that the OH^{\cdot} -dependent non-physiological permanent damage of vascular tissue in the experimental settings used was at the base of these claims [222-224].

NO^{\cdot} deserves a separate description in the context of vascular tone regulation. In fact, NO^{\cdot} is one of the most characterized vasodilating physiological signals [15]. Shear stress and paracrine mediators such as acetylcholine and bradykinin stimulate the release of NO^{\cdot} from endothelial cells [225], where it is synthesized by eNOS in a Ca^{++} - and calmodulin-dependent manner [226]. Conversely, the inducible form of NOS (iNOS) is released by many cell types as a consequence of cytokine exposure and participates in inflammatory vasodilation [227]. Endothelial NO^{\cdot} diffuses to the deeper layers of the vascular wall and activates guanylate cyclase (GC) in smooth muscle cells, which in turn leads to increased intracellular levels of cGMP, activation of protein kinases and phosphorylation of several downstream effector including sarcolemmal potassium channels [228] and plasma membrane calcium-activated potassium channels [229]. This leads to membrane hyperpolarization and inactivation of calcium channels, which in turn causes reduction in the intracellular calcium increase necessary for calmodulin activation, myosin light chain (MLC) phosphorylation and ultimately smooth muscle contraction. Amongst different physiological sources of NO^{\cdot} , deoxygenated hemoglobin prevalent in hypoxic conditions has been shown to possess nitrite reductase activity, generate NO^{\cdot} from nitrite and nitrate ions [230], and stimulate hypoxia-dependent vasodilation [231].

Notably, other ROS of endothelial origin have been shown to increase smooth muscle cell contraction. In particular, endothelial cyclooxygenases appear to be responsible for the generation of ROS [166] and their transfer to smooth muscle cells *via* myo-endothelial gap junctions [232]. Endothelial ROS seem to promote vascular contraction by amplifying the response of thromboxane receptors in smooth muscle [167, 233].

Platelets and Haemostasis

Original observations of a potentiatory effect of $O_2^{\cdot-}$ on platelet activation appeared in the 1970s [234]. More recently, $O_2^{\cdot-}$ has been suggested to stimulate platelet hyperactivity in anoxia/reoxygenation conditions [235] and hypercholesterolemia [236]. Besides classical agonist-induced protein phosphorylation pathways

[237, 238], platelet activation is therefore also regulated by ROS. The dependence of platelet activation on ROS generation explains the inhibition of platelets and the anti-thrombotic effect of antioxidants [239].

Besides responding to exogenous ROS [240], platelets also generate different ROS upon activation [241]. Although the molecular mechanism of platelet regulation by $O_2^{\cdot-}$ is still largely undefined, the pharmacological inhibition of $O_2^{\cdot-}$ generation has been shown to attenuate platelet aggregation in response to different agonists [242-244] and the genetic ablation of NOX2 resulted in the reduction of platelet recruitment to the vascular wall in hypercholesterolemic mice [236]. Several potential mechanisms of action have been proposed, including increasing the bioavailability of platelet-derived ADP [243], scavenging of endothelial cell- and platelet-derived nitric oxide (NO^{\cdot}) [245], directly potentiating integrin $\alpha IIb\beta 3$ activation [246, 247] or collagen receptor GPVI signalling [248, 249] or protein kinase C [78]. A credible mechanism of action for the dependence of collagen-induced platelet activation is that GPVI signaling depends on disulphide-dependent dimerization [249] and consequent dimerization of GPVI receptors [248]. This is also supported by our results [142]. Notably, although previous studies reported the dependence of the other major platelet collagen receptor integrin $\alpha 2\beta 1$ on ROS [250], the use of the GPVI-specific agonist convulxin and collagen related peptide (CRP) confirmed that NOX-dependent generation of $O_2^{\cdot-}$ directly potentiates GPVI signaling. Besides $O_2^{\cdot-}$, also H_2O_2 has been shown to activate platelets when added exogenously [251, 252], to be generated by the platelets in response to collagen stimulation [253] and to potentiate the response to this agonist [254]. Despite this wealth of information, it is currently difficult to reach a definitive explanation of the ROS-dependence of platelet activation. This is probably at least partially due to the fact that different ROS have different effect on platelet activation. For example, in our laboratory we observed the ablation of collagen signaling and consequent functional responses following NOX inhibition with 2-acetylphenothiazine [142], whereas thrombin responses are abolished by the non-specific inhibition of ROS accumulation by apocynin [142, 255]. Besides NOXs, other ROS-generating enzymes have been suggested to participate in the cascade of molecular events leading to platelet activation, such as LOXs [176, 256] and mitochondrial respiratory complexes [257]. Another aspect to consider is the effect of extracellular redox regulation of platelets. So far in this paragraph, we discussed intracellular generation and regulatory activity of ROS, but extracellular ROS accumulation in response to platelet release has also been observed. Once again, there are discrepancies in literature regarding the regulation and mechanisms of extracellular ROS generation. Several studies report that thrombin stimulates intracellular but not extracellular ROS generation [242, 243], whereas other investigators present data in favor of thrombin-dependent stimulation of extracellular ROS generation [138, 142]. Our experiments with luminol and platelet

supernatants are in agreement with the latter position. Similarly, collagen has been shown to induce extracellular ROS formation in certain reports [138, 243], whereas only intracellular ROS generation was reported in other studies [242, 258]. The extracellular redox state affects platelet responsiveness by affecting surface receptor redox state, conformation and function [259]. Because of their redox-dependent modulation [260, 261], thiol isomerases have been proposed as important redox-dependent regulators of platelet activity [259, 262]. Their release and re-association with platelet surface and their ability to modulate disulfide bridge formation within or amongst extracellular domains of platelet receptors such as integrin $\alpha IIb\beta 3$ has important potentiatory effects on platelet activity [263, 264]. Because of the important activatory/potentiatory role of ROS in platelets and the key role of these cells in the development of thrombotic conditions and vascular affections, a significant amount of research has been dedicated to the identification and characterization of natural antioxidants to be utilized as antiplatelet drugs or food supplement in our continuing fight against cardiovascular disease [265, 266], although there is still little evidence of their efficacy [267].

In this section dedicated to platelets, the well-documented and characterized role of NO^{\cdot} as physiological antithrombotic signal cannot be omitted. In contrast to other ROS, NO^{\cdot} is a key physiological inhibitor of platelet activation and is constantly released by intact endothelium to maintain platelet resting state and guarantee blood flow [18, 268]. Because of its lipophilic nature, NO^{\cdot} crosses the plasma membrane and activates soluble guanylate cyclase (sGC) of platelets [269]. The binding of NO^{\cdot} to the heme moiety of sGC leads to a 200-fold increase in enzyme activity and the accumulation of intracellular cGMP, which in turns activates protein kinase G (PKG) and reduces platelet responsiveness [270]. Moreover, the phosphorylation and inactivation of phosphodiesterase 3A by PKG leads to cAMP accumulation and protein kinase A (PKA) activation [271]. The stimulation of PKG by NO^{\cdot} has several important consequences in platelet signaling, including: 1) ablation of intracellular calcium increase by phosphorylation and inactivation of the inositol triphosphate receptor (IP3R) [272] and the IP3R-associated cGMP kinase substrate (IRAG) [273] on the endoplasmic reticulum; 2) inhibition of the cytoskeletal rearrangements necessary for platelet adhesion and aggregation *via* phosphorylation of vasodilator activated phosphoprotein (VASP) and consequent suppression of actin polymerization [274]; 3) abolishment of adhesion and spreading through inhibition of dense granule release and integrin signalling [275].

Endothelial Cells, Angiogenesis and Vascular Permeability

As mentioned in section B on protein transcription factor regulation, ROS has been proposed to play a key in role in initiating the angiogenic response to

hypoxia [90, 91]. The hypoxia-dependent increase in ROS generation is responsible for the inhibition of prolyl hydroxylation and degradation of HIF1 α , which leads transcriptional activation of HIF-dependent genes that include VEGF and other pro-angiogenic factors such as angiopoietin 2 and stromal derived factor 1 (SDF1) [92]. Hypoxia-dependent ROS generation in the mitochondria has been proposed as the trigger for the activation of the HIF-dependent response [91, 276-278]. Besides mitochondrial ROS, NOX enzymatic activity is critical in the regulation of angiogenesis, as proved in models of tumor angiogenesis experiments using NOX1 null mice [279]. This observation confirms previous studies showing that NOX1-derived ROS trigger angiogenesis [280], that NOX4 activity is necessary to sustain high HIF1 α and VEGF expression levels in cancer cells [281] and that NOX5 is required for the formation of endothelial capillary networks [282]. Besides VEGF, the expression of other relevant effectors of the angiogenic response is stimulated in a ROS-dependent manner. For example, the expression of VEGF receptor 2 (VEGFR2) in bovine aortic endothelial cells is upregulated by H₂O₂ in an NF κ B-dependent manner, which plays a relevant role in the response of these cells to oxidative aggression [283].

As well as an upstream effect on the expression of angiogenic factors and receptors, ROS play a key role in the signalling of the angiogenic response. The angiogenic responses stimulated by VEGF [284-287], angiopoietin [288], leptin [289] or seeding on the reconstituted extracellular matrix support Matrigel™ [290] are all characterised and at least partially dependent on an increase in intracellular ROS. Early signalling events of VEGF stimulation have been shown to depend on ROS generation that includes receptor autophosphorylation [291] and peroxynitrite-dependent Src kinase activation [292]. Although there is some pharmacological evidence for the involvement of lipoxygenase in the generation of ROS downstream of VEGF [291], amongst the possible source of endothelial ROS in response to the angiogenic stimulus, NOX2 appears to be an important component of the signalling pathway of VEGF *in vitro* [286, 293]. Experiments *in vivo* confirmed the role of NOX2 in the signalling pathways and functional response induced by VEGF. NOX2^{-/-} mice are in fact characterised by impaired neovascularisation in response to VEGF [286] and reduced blood-flow recovery after experimental ischemia [294]. An interesting aspect of the NOX-dependent regulation of endothelial cell physiology and angiogenesis is the participation of the small GTPase Rac and in particular the role of Rac-dependent ROS in the regulation of endothelial cell-cell interactions [295] and angiogenesis [291]. Rac has been shown to play an important role in the formation of the NOX complex and its activation [124], so Rac-dependent ROS are likely to rely on the enzymatic activity of NOXs, as shown for the angiogenic response induced by VEGF [293]. Interestingly, α -catenin phosphorylation in response to Rac-dependent ROS generation has been shown to induce cell-cell interaction reduction and promotion of endothelial cell motility [295]. The

interaction of NOXs with Rac is critical for the localization of NOX at the leading edge of migrating endothelial cells and for the local accumulation of ROS [296-298]. PI3K [296] and WAVE1 [299] are other important effectors in the compartmentalization of NOXs and ROS generation downstream of VEGF-dependent stimulation of endothelial cell motility and angiogenesis. IQGAP1 is another partner of VEGFR2 and is implicated in the generation of intracellular ROS in response to VEGF stimulation, which is necessary for Akt phosphorylation, endothelial migration, and proliferation [287]. From the molecular point of view IQGAP1 is a scaffolding protein fundamental for the formation of the macromolecular signaling complex including VEGFR2 and NOX2 at the leading edge of migrating endothelial cells [300]. The localization of NOX at the leading edge is critical for the local formation of ROS and the formation of sulfenic acid by oxidation of cysteine residues of key signaling proteins, including ERK, protein phosphatase 1B (PTP1B) and IQGAP1 itself [46]. This post-translational modification of signaling proteins by VEGF plays an important role in the promotion of endothelial cell motility and angiogenesis [46]. Besides regulating cell motility the function of IQGAP1 within the VEGFR2 signalosome is to modulate ROS-dependent tyrosine phosphorylation of VE-cadherin and loss of cell-cell contacts necessary to initiate angiogenesis [301].

Besides their role in the migration and proliferation of mature endothelial cells, ROS have also been shown to play a role in the regulation of endothelial progenitor cells, hence affecting vasculogenesis besides angiogenesis [302]. Similarly to mature endothelial cells, NOX2 appear to play a major role in the participation of endothelial progenitor cells in the process of neovascularization [303]. Interestingly though, ROS levels in endothelial progenitor cells tend to be lower than in mature endothelial cells because of higher levels of physiological antioxidants [304]. Low levels of ROS appear to be important to maintain the proliferative and undifferentiated phenotype of endothelial progenitor cells [304, 305]. Because of the importance of ROS in the regulation of angiogenic and vasculogenic responses, it is not surprisingly that natural antioxidants have often been shown to interfere with the revascularization/neovascularization response [306-310]. Nonetheless, the complexity of the ROS-dependent regulation of angiogenesis is suggested by studies in which excessive ROS generation had an inhibitory effect, hence suggesting that optimal ROS concentrations are necessary for an efficient angiogenic response [311, 312].

ROS also modulate endothelial cell permeability by regulating adherens junction formation. Oxidative stress has in fact been shown to increase microvascular permeability [313, 314] and to play a role in the vascular dysfunctions linked diabetic microangiopathy. Although cell damage by ROS has been considered the cause for increase vascular permeability in the presence of ROS, more subtle molecular mechanisms are becoming apparent. Interestingly, besides initiating angiogenesis [295],

VEGF-induced Rac-mediated disruption of adherens junctions associated with tyrosine phosphorylation of adhesion proteins (VE-cadherin and β -catenin) also leads to increased endothelial permeability [315]. ROS have been shown to compromise endothelial barrier function in different experimental systems by stimulation of p38 mitogen-activated protein kinase (MAPK) and cytoskeletal rearrangements in bovine lung microvascular endothelial cells [316], or inhibition of GSK-3 β , activation of Akt and cytoskeletal rearrangements in culture human microvascular cells [317], or activation of RhoA and PI3 kinase leading to protein kinase B (PKB/Akt) activation and redistribution of tight junction proteins claudin-5 and occluding in cultured in cultured brain endothelial cell [318], or stimulation of synthesis and release of interleukin-6 (IL-6) in human umbilical vein endothelial cells [319]. ROS appear to play an important role in the endothelial hyperpermeability associated to inflammatory conditions. In this context it is important to mention the proposed use of ROS scavengers to counteract the vascular hyperpermeability associated with inflammation [320].

CONCLUSION AND REMARKS

Great advances have been made in the understanding of the sources, physiological roles and mechanisms of action of ROS in the cardiovascular system in the last decade. Although key roles in the regulation of immune response, vascular tone, haemostasis, vascular permeability and angiogenesis have been elucidated, there is a lot more to understand regarding the redox-dependent regulation of vascular health. An improvement in the experimental tools and a standardization of investigation methods and experimental models appear necessary in order to clarify key aspects of this area of cardiovascular research and exploit the interventional opportunities that it offers.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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