REVIEW ARTICLE

Reactive Oxygen Species as Intracellular Signaling Molecules in the Cardiovascular System

Andrey V. Krylatov[1], Leonid N. Maslov[1,*], Nikita S. Voronkov[1], Alla A. Boshchenko[1], Sergey V. Popov[1], Ludovic Gomez[2], Hongxin Wang[3], Amteshwar S. Jaggi[4] and James M. Downey[5]

[1]*Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Science, Tomsk, Russia;* [2]*Inserm UMR-1060, Laboratoire CarMeN, Université Lyon 1, Oullins Cedex, France;* [3]*Department of Pharmacology, Jinzhou Medical University, Jinzhou, China;* [4]*Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala, India;* [5]*Department of Physiology and Cell Biology, College of Medicine, University of South Alabama, Mobile, AL, USA*

Abstract: *Background*: Redox signaling plays an important role in the lives of cells. This signaling not only becomes apparent in pathologies but is also thought to be involved in maintaining physiological homeostasis. Reactive Oxygen Species (ROS) can activate protein kinases: CaMKII, PKG, PKA, ERK, PI3K, Akt, PKC, PDK, JNK, p38. It is unclear whether it is a direct interaction of ROS with these kinases or whether their activation is a consequence of inhibition of phosphatases. ROS have a biphasic effect on the transport of Ca^{2+} in the cell: on one hand, they activate the sarcoplasmic reticulum Ca^{2+}-ATPase, which can reduce the level of Ca^{2+} in the cell, and on the other hand, they can inactivate Ca^{2+}-ATPase of the plasma membrane and open the cation channels TRPM2, which promote Ca^{2+}-loading and subsequent apoptosis. ROS inhibit the enzyme PHD2, which leads to the stabilization of HIF-α and the formation of the active transcription factor HIF.

Conclusion: Activation of STAT3 and STAT5, induced by cytokines or growth factors, may include activation of NADPH oxidase and enhancement of ROS production. Normal physiological production of ROS under the action of cytokines activates the JAK/STAT while excessive ROS production leads to their inhibition. ROS cause the activation of the transcription factor NF-κB. Physiological levels of ROS control cell proliferation and angiogenesis. ROS signaling is also involved in beneficial adaptations to survive ischemia and hypoxia, while further increases in ROS can trigger programmed cell death by the mechanism of apoptosis or autophagy. ROS formation in the myocardium can be reduced by moderate exercise.

Keywords: Reactive oxygen species, intracellular signaling molecules, heart, exercise, cardiovascular system, autophagy.

1. INTRODUCTION

In the 1980s and 1990s, it was generally accepted that free radicals and Reactive Oxygen Species (ROS) play an extremely negative role in the body [1-5], causing organ and tissue damage in ischemia-reperfusion, stress, Alzheimer's disease, diabetes mellitus [2-5]. Indeed, both in experimental animals [6, 7] and in the course of clinical observations [8, 9] it was reported that antioxidants seemed to increase the resistance of the heart to the impact of ischemia-reperfusion. However, gradually the attitude towards free radicals began to change. Now we know that they can also act as intracellular signaling molecules which are involved in the homeostatic equilibrium and can participate in beneficial adaptation

of organs and tissues to various stresses such as hypoxia or ischemia [10-17], or may be intracellular messengers of pathological processes [18-23].

2. WHICH REACTIVE OXYGEN SPECIES ACT AS MESSENGERS?

The family of ROS includes small molecules, characterized by high reactivity and biological activity. These include the free radicals: superoxide radical anion ($O_2^{\bullet}$) and the hydroxyl radical ($OH^{\bullet}$). In addition, ROS include: hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl) [17]. The half-life of $O_2^{\bullet}$ in tissues is brief, 10^{-6} -10^{-3} s, as a result of spontaneous dismutation or dismutation catalyzed by superoxide dismutase (SOD) [5, 24] making it an unlikely signaling molecule. $O_2^{\bullet}$ is converted to H_2O_2 [17] whose half-life in tissues is also brief: 10^{-9} to 10^{-6} s [5, 24, 25]. $^1O_2$ in aqueous solutions is no better with a half life of 10^{-12} - 3×10^{-6} s [5, 26, 27]. The

*Address correspondence to this author at the Cardiology Research Institute, Tomsk National Research Medical Center of the Russian Academy of Science, Tomsk, Russia; Tel: 3822 262174; Fax: 3822 555057; E-mail: maslov@cardio-tomsk.ru

half-life of H_2O_2, however, is a few seconds [24]. In addition, H_2O_2 dissolves in lipids and, due to lack of charge, can easily pass through membranes [28]. Therefore hydrogen peroxide is the most likely candidate for an intercellular and intracellular signaling molecule. The superoxide radical, in our opinion, can only function as an intracellular messenger, acting on protein molecules near the site of its formation such as the inner mitochondrial membrane, NADPH oxidase, xanthine oxidase, or NO-synthase.

It has been a matter of debate as to whether $O_2^{\bullet}$ can cross the outer membrane of the mitochondria [24]. but a number of studies have shown the ability of intact mitochondria to spontaneously release this radical [29, 30]. Due to its short half-life, hydroxyl radical would seem to be an unlikely participant in intracellular signaling [25]. However, in the presence of Fe^{2+} $OH^{\bullet}$ can be a intermediate between H_2O_2 and its sensor molecule due to the Haber-Weiss reaction: $H_2O_2 + Fe^{2+} = Fe^{3+} + OH^{\bullet} + OH^-$. Peroxynitrite ($ONOO^-$) is formed as a result of the interaction of $O_2^{\bullet}$ with $NO^{\bullet}$ [17]. The half-life of $ONOO^-$ is ~1 s [31] and it can penetrate cell membranes [31] making it a possible intracellular messenger. Hypochlorite: $H_2O_2 + Cl^- \rightarrow H_2O + OCl^-$ [17] is a bactericidal molecule produced in mammals by the myeloperoxidase enzyme, which is almost exclusively confined to neutrophils. Thus, the most likely candidates for the role of intracellular signaling molecules are: H_2O_2, $O_2^{\bullet}$, and $ONOO^-$.

3. SOURCES OF REACTIVE OXYGEN SPECIES IN THE CELL

3.1. Mitochondria

The main source of superoxide radicals in some cells is mitochondria [24, 32]. It has been reported that as much as 4 - 5% of the oxygen consumed by mitochondria is diverted to $O_2^{\bullet}$ synthesis [33] while others estimate the leak as only 1 - 2% [34]. The main source of $O_2^{\bullet}$ is NADH dehydrogenase in complex I of the mitochondrial respiratory chain [33, 35]. Blockade of complex I with rotenone transfers electrons from complex I to ubiquinone and leads to a several-fold increase in $O_2^{\bullet}$ production [33]. The free radical of ubisemiquinone Q and complex III (cytochrome-bc1 complex) of the mitochondrial respiratory chain can also be a source of $O_2^{\bullet}$ [24, 32]. The superoxide anion formed in complex I enters the matrix, while $O_2^{\bullet}$, formed in complex III, enters both the intermembrane space and the matrix [36, 37]. Mitochondria are the main source of $O_2^{\bullet}$ for those cells in which the mitochondrial content is high such as cardiomyocytes where the occupy 22 to 37% of the cell's volume [38]. Mitochondria are also the main source of $O_2^{\bullet}$ in mitochondria-rich brain cells [39]. The opening of ATP-sensitive potassium channels in the mitochondrial inner membrane also generates ROS [40].

3.2. The Nox Family of NADPH oxidases (Nox)

In other cells such as endothelial cells, these organelles occupy only 2 - 5% of the cytoplasmic cell volume [41]. In these cells, the main source of $O_2^{\bullet}$ is NADPH oxidase, which catalyzes the reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet} .$$

It was originally thought that these NADPH oxidases were exclusive to phagocytic cells, but then Nox homologs were found in the plasma membranes of many mammalian cells including kidney cells, epithelial cells, endothelial cells, smooth muscle cells, lymphoid cells, and macrophages [24]. Both Nox2 and Nox4 isoforms are expressed in cardiomyocytes [42].

The activity of Nox isoenzymes is enhanced by leukotrienes, tumor necrosis factor-α (TNF-α), toll-like receptor agonists, hepatocyte growth factor (HGF), leptin, interleukin-6, and granulocyte colony-stimulating factor (G-CSF) [43]. There is also evidence that Nox isoenzymes may be stimulated by certain G-protein-coupled receptor agonists such as angiotensin II and morphine [44, 45]. It is believed that protein kinase C (PKC) is involved in the signaling from the angiotensin II receptor to a Nox [43]. Morphine signals differently. In Nox1 knockout mice, the analgesic effect of morphine weakens and translocation (activation) of PKC to the cell membrane is disrupted [44]. The following chain of events is thought to describe the signaling:

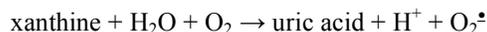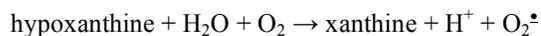$$morphine \rightarrow OR \rightarrow Nox1 \rightarrow PKC \rightarrow analgesia$$

OR is the opioid receptor. The fundamental difference between the angiotensin and morphine receptors is that the angiotensin receptors are coupled to G_q proteins, and the ORs interact with $G_{i/o}$ proteins. Apparently this feature determines the nature of their interaction with Nox.

3.3. NO Synthase

In pathological conditions, the source of $O_2^{\bullet}$ can be from the so-called "uncoupling of NO synthase" [45], which, in the absence of its cofactor tetrahydrobiopterin, or as a result of lack of L-arginine substrate, synthesizes $O_2^{\bullet}$ instead of $NO^{\bullet}$.

3.4. Xanthine Oxidase

Xanthine oxidase can be a source of $O_2^{\bullet}$ in cells of the intestine, lungs, skin, brain, skeletal muscles, liver, pancreas, testes, kidneys [39]. During ischemia most cells release adenosine which is quickly converted to hypoxanthine. Then during reperfusion xanthine oxidase catalyzes the two-step reaction [17]:

$$hypoxanthine + H_2O + O_2 \rightarrow xanthine + H^+ + O_2^{\bullet}$$

$$xanthine + H_2O + O_2 \rightarrow uric\ acid + H^+ + O_2^{\bullet}$$

While xanthine oxidase is found in the hearts of many animals and is a source of ROS during ischemia-reperfusion, the enzyme is undetectable in human myocardium [46].

Flavoprotein NADPH-cytochrome-P450-reductase is yet another source of ROS ($O_2^{\bullet}$, $OH^{\bullet}$, H_2O_2) [17]. The highest activity of NADPH-cytochrome-P450 reductase and NADH-cytochrome-P450 reductase is observed in cells of the lungs, liver, heart, kidneys [39]. A small contribution to the production of ROS is made by monoamine oxidases (heart, kidney, brain) or lipoxygenase and cyclooxygenases (brain, pancreas) [39].

4. TARGETS OF REACTIVE OXYGEN SPECIES

Cysteine Residues. ROS acts through post-translational modification of numerous proteins, such as receptors,

kinases, phosphatases, ion channels and transcription factors. An extensive range of oxidation-reduction modifications can be involved in redox signaling, among which the most studied is the H_2O_2-mediated oxidation of the amino acid residues of cysteine in proteins. Cysteine is sensitive to oxidation and usually forms reversible intra- or intermolecular disulfide bonds [47, 48]. ROS-mediated oxidation of thiol groups can modulate the activity of proteins in which cysteine fragments are critical for substrate binding *e.g.* glyceraldehyde-3-phosphate dehydrogenase, p38 kinase, tropomyosin, and many of the tyrosine phosphatases [49-51]. Oxidation of cysteine can occur by binding with other chemicals such as NO (nitrosylation), nitroxyl (HNO) [52] or glutathione (glutathiolation) [47]. It is important to note that the effect of ROS is strongly modulated by the presence of antioxidants. Glutathione, which is the main cellular antioxidant, can remove disulfide bonds in oxidized proteins. The thioredoxin system may have a similar effect [17].

Ca^{2+}/Calmodulin-dependent Kinase II (CaMKII) is a known target of ROS [42]. CaMKII is a supramolecular complex consisting of 12 monomers assembled as hexamers, each monomer having catalytic, autoinhibitory and Ca^{2+}/calmodulin-binding domains. At rest, the autoinhibitory domain binds to the catalytic site and blocks the kinase activity [53]. The binding of Ca^{2+} to the Ca^{2+}/calmodulin domain induces autophosphorylation and removes the autoinhibitory domain to expose the catalytic site and commence its enzymatic function. Anderson's laboratory found that after the initial Ca^{2+}-dependent activation of CaMKII oxidation of methionine residues 281/282 in the regulatory domain further increases CaMKII activity [54]. Oxidation of methionines can be eliminated by methionine sulfoxide reductase A, which regulates the physiological role of this pathway. Thus, CaMKII through this mechanism combines two key signals, namely Ca^{2+} and ROS.

Studies in mice with modified genes have shown that the oxidation of CaMKII increases the death of cardiomyocytes and the development of heart failure following myocardial infarction or angiotensin II-induced stress [54]. In subsequent studies, the important role of increased oxidation of CaMKII in the pathogenesis of atrial fibrillation was also demonstrated [55]. It also has been shown that H_2O_2 can activate CaMKII in T-lymphocyte cultures [56]. All these facts indicate that CaMKII is a pathological target for ROS.

Protein Kinase G (PKG) is a cGMP-dependent protein kinase that is also a target for ROS [42]. PKG is a cytosolic protein that forms a homodimer of two identical subunits. This enzyme plays an important role in mediating the cardioprotective effect of NO [57]. The binding of cGMP to PKG's regulatory domain activates its catalytic kinase domain. PKG type Iα has at least 5 Cys residues that can be oxidized. Oxidation of Cys42 leads to the formation of a disulfide bridge between the two monomers that activate the kinase independently of NO [58]. Eaton's laboratory substituted PKGIα's Cys42 with a serine and found that these animals developed a moderate degree of hypertension, which suggested that ROS-mediated vasodilation from PKG might be involved in the physiological regulation of blood pressure [59]. But Nakamura *et al.* demonstrated that PKGIα is oxidized both in patients with heart disease and in rodents with

models of heart disease [60]. Moreover, this oxidation promoted unfavorable cardiac remodeling after prolonged pressure overload or stimulation of Gq-coupled receptors. Compared to the wild-type hearts and cardiomyocytes, the hearts and myocardial cells that express the PKGIα with the $C^{42}S$ substitution are better adapted to the functional stresses on the heart. Redox-dependent changes in PKG1α control intracellular translocation. While the activated, oxidized PKGIα was exclusively in the cytosol, the PKGIα $C^{42}S$ was translocated to the plasma membrane [60]. This observation suggests that oxidation of PKG with ROS can negatively affect myocardial response to increased loads. The source of the ROS oxidizing PKG has not yet been identified.

Protein Kinase A (PKA) is a cAMP-dependent protein kinase and is also activated by ROS [42]. PKA is a heterotetramer with a dimeric regulatory subunit and a dimeric catalytic subunit. It was found that the oxidation of PKA leads to the formation of a disulphide bond between the two regulatory subunits and that activates the enzyme independent of cAMP [61]. This modification of the PKA caused subcellular translocation of PKA to the myofilament components troponin I and myosin binding protein C in heart muscle which directly increased its contractility. The important role of PKA oxidation has recently been demonstrated using a model in mice, in which PKA became "redox dead" due to the mutation of $C^{175}S$ in the regulatory subunit [62]. The authors showed that this disrupts the PKA-dependent Vascular Endothelial Growth Factor (VEGF)/Extracellular Regulated Kinase (ERK) signal ratio. It was found that the functional effect of PKA oxidation is the enhancement of angiogenesis in models of ischemia in the hind limbs but, unfortunately, it also promotes angiogenesis in tumors [62].

ERK kinase is a member of the mitogen-activated protein kinase family which also includes JNK and P38 (see below). Erk is involved in cell division and survival and its activity is enhanced by activation of the EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) receptors. These receptors can also be activated by ROS [63-65], which can lead to a phosphorylation-dependent activation of ERK. Activation of ERK by physiological concentrations of H_2O_2 was observed by Preston *et al.* [66]. Simultaneously, they noted an increase in cell proliferation. This activation of ERK by ROS was confirmed by other investigators [67-70]. Conversely, in 2013 Wang *et al.* [71] found that antioxidants could suppress the proliferation of HeLa cells along with a decrease in ERK activity. Adding hydrogen peroxide increased the phosphorylation and thus activation of ERK.

Phosphatase PTEN and PI3K/Akt. The phosphoinositide-3 kinase, PI3K, acts to phosphorylate inositol in its 3 position. The inositol-3-phosphate then causes a Phosphoinositide-dependent Kinase (PDK) to activate a kinase named Akt through phosphorylation. Akt was first identified as the oncogene for the retrovirus AKT8, hence the name. PTEN (Phosphatase and tensin homolog deleted on chromosome TEN) is a phosphatase that acts to remove phosphates in inositol's 3 position which opposes Akt's activation. In experiments on cell culture RAW264.7, it was shown that H_2O_2 causes phosphorylation of Akt and an increase in its activity [72]. Simultaneously, the inactivation of PTEN by oxidation was noted. The authors proposed that the oxidation of PTEN
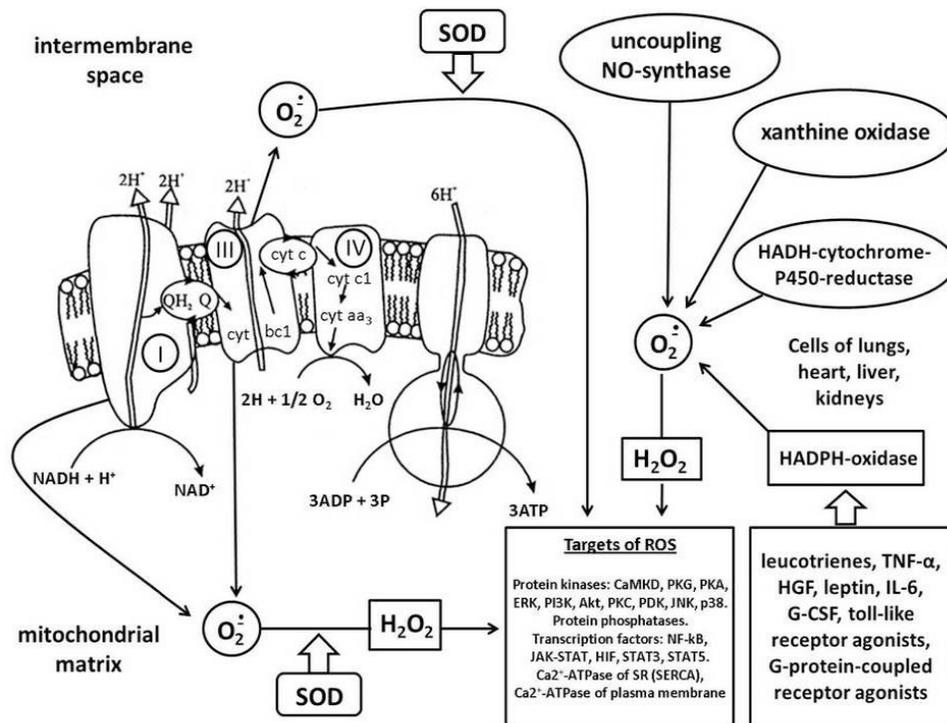
Fig. (1). An involvement of reactive oxygen species in intracellular signaling. ROS-mediated signaling pathway is involved kinases (CaM-KII, PKG, PKA, ERK, PI3K, Akt, PKC, PDK, JNK, Kinase p38), phosphatases (PTEN, PP1, other protein phosphatases), transcription factors (HIF, STAT, NF-κB), Ca^{2+} transport system.

disrupts Akt's dephosphorylation resulting in a net activation of Akt [72]. Analysis of various cysteine mutants showed that the main Cys^{124} residue in the active site of PTEN specifically forms a disulfide bond with Cys^{71} during oxidation with hydrogen peroxide (200 μM) [73]. Activation of Akt by ROS was also reported by other investigators [68].

Reduction of the level of H_2O_2-oxidized PTEN in cells appears to be mediated predominantly by thioredoxin because thioredoxin was more effective than glutaredoxin, glutathione, or a 14-kDa thioredoxin-like protein for the reduction of oxidized PTEN in vitro [73]. In experiments on H9c2 cell culture, it was shown that H_2O_2 (100 μM) can cause the formation of disulfide bonds between the Cys^{297} and Cys^{311} residues in the Akt molecule, which is accompanied by dephosphorylation and degradation of this enzyme [74]. Therefore, the ROS can not only activate, but also inactivate the PI3K/Akt tandem depending on its concentration. The final physiological effect apparently also depends on the type of cells studied. In experiments on cultured A549 lung cancer cells, it was shown that $O_2^{\bullet}$ and H_2O_2 promoted cell survival by activating the PI3K/Akt [75]. Akt along with ERK is known to play a prominent role in mediating the protective effect in the Ischemic Preconditioning (IPC) pathway (see below) [76].

Protein Kinase C (PKC). In the inactive state, PKC only weakly binds to membrane lipids and is located mainly in the cytosolic fraction, whereas activation of PKC by diacylglycerol or Ca^{2+} increases the affinity of the enzyme to the membrane lipids which stabilizes its association with the membranes. This translocation causes a conformational transition to its catalytically active form [77, 78]. Both regulatory and catalytic domains of PKC contain cysteine-rich regions,

which makes PKC a very vulnerable target for oxidation-reduction regulation. Depending on the concentration, oxidants play a dual role in stimulating or inactivating PKC. IPC is a phenomenon whereby a brief transient ischemic insult causes the heart to become resistant to infarction from a subsequent extended period of ischemia. This cardioprotection is the result of protective signaling originating from the occupation of several G_i-coupled receptors during the conditioning ischemia and PKC plays a prominent role in this signaling [79].

The first evidence of ROS involvement in IPC was when combined SOD and catalase blunted the infarct-limiting effect of IPC in canine hearts [80]. Treating rabbits with SOD or N-2-mercaptopropionyl Glycine (2-MPG) did not affect the infarct size in rabbits without IPC but eliminated IPC's infarct-reducing effect [81]. The involvement of ROS in the cardiac IPC signaling pathway was confirmed in later studies [82-84]. It has been established that ROS can act as triggers of the infarct-reducing effect of IPC [85-87]. PKCε is thought to be the target for ROS and inhibition of the Mitochondrial Permeability Transition Pore (MPTP) is thought to be the final effector for the cardioprotective effect of IPC [88-90].

The critical time for redox signaling is the brief period of reperfusion between the IPC coronary occlusion and the prolonged ischemic insult. Perfusing the heart with oxygen-free buffer during that period blocked the protection and the ROS scavenger only blocked IPC's protection when it was present during that brief reperfusion period [91]. During the conditioning ischemia the signaling is initiated by G_i-coupled receptor (*e.g.* adenosine) occupation, but cannot proceed past the redox step until oxygen becomes available to fuel the

signaling during a brief period of reperfusion. Ischemic post-conditioning is observed when short occlusion/reperfusion cycles starting with the onset of reperfusion also protect against infarction. The occlusion cycles keep the pH of the tissue low by reducing coronary flow during the first minutes of reperfusion which inhibits MPTP opening. At the same time, enough oxygen is delivered to fuel ROS signaling as occurs in IPC and that keeps the MPTP closed thereafter [92].

At high concentrations, ROS react with catalytically important cysteine residues and inactivate PKC. However, at low concentrations, oxidants induce activation of PKC [78]. In addition, it has been found that H_2O_2 stimulates the activation of tyrosine kinases and can indirectly regulate the phosphorylation of PKC-δ in tyrosine residues Tyr^{512} and Tyr^{523} [93]. The observation that MPG can block protection from IPC is an example of how beneficial physiological redox signaling in a microcompartment can be interfered with by systemic administration of a ROS scavenger

PDK1. Phospholipid-dependent kinase-1 is dependent on the phospholipid phosphatidylinositol-3-inositol and is the intermediate link between the kinases PI3K and Akt. PDK1 can also be phosphorylated and activated by PKC, which phosphorylates the residues of Ser^{744} and Ser^{748} [94]. It was found that PKCδ activates PDK1through the phosphorylation of Ser^{738} and Ser^{742} residues [95]. As we noted above, PKC can be activated by ROS. Therefore, the following chain of events can be built: ROS → PKC → PDK1 → Akt. Indeed PI3K and Akt are downstream of PKC in the protective signaling pathway of IPC [96]. It has also been reported that ROS can provide activation of PDK1 by phosphorylation of Tyr^{463} with tyrosine kinase Abl. Tyrosine kinase Abl, in turn, is activated by cytosolic tyrosine kinase Src [95]. According to Storz *et al*. ROS activates PKC and Src through the inhibition of protein phosphatase [95].

JNK (c-Jun N-terminal kinase). This kinase is known to promote transcription and thus protein synthesis. Preston *et al*. [66] showed physiological concentrations of H_2O_2 caused activation by phosphorylation of JNK in cultured fibroblasts. Simultaneously, they noted an increase in cell proliferation. In experiments with malignant cells of the large intestine, it was shown that H_2O_2 can stimulate cell proliferation by activating JNK [97]. In 2005, Kamata *et al*. [98] showed that oxidative stress leads to increased phosphorylation of JNK, not due to increased kinase activity but rather to the oxidation and inactivation of the phosphatases that target JNK.

p38 MAP kinase. This mitogen-activated kinase is activated by cell stress. Physiological concentrations of H_2O_2 increased the phosphorylation of p38 in cultured fibroblasts [66]. Simultaneously, they noted an increase in cell proliferation. In experiments with cultured A549 lung cancer cells, it was shown that $O_2^{\bullet-}$ and H_2O_2 promoted cell survival through activation of p38 [75]. Pacing-induced heart failure increased ROS production and inhibition of p38 kinase by SB281832 counteracted it suggesting that p38 kinase activation was upstream rather than downstream of the ROS formation [51].

Protein phosphatases are a large diverse group of enzymes that remove the phosphate group from the phosphorylated amino acid residue of the substrate protein. Each only

removes phosphate groups from a specific amino acid sequence. Phosphatases are often the target for inactivation by oxidation from ROS [56, 72, 95, 98, 99]. Inactivation of a phosphatase will have the effect of increasing the phosphorylation level of its target similar to activating the target's kinase.

EGF and PDGF receptors are coupled to Nox. It was found that their activation leads to the production of ROS and increased cell proliferation [100].

Ca^{2+} transport system. During the contraction of cardiomyocytes, the initial influx of a small amount of Ca^{2+} through L-type sarcolemmal Ca^{2+} channels during an action potential triggers the opening of the ryanodine receptor (RyR) channels that are located in the Sarcoplasmic Reticulum (SR) where Ca^{2+} is stored. These high conductance channels allow the rapid release of Ca^{2+} into the cytoplasm (Ca^{2+}-induced Ca^{2+} release) to cause contraction as it binds to the myofilament protein troponin C. During relaxation of the heart, Ca^{2+} is translocated back into the SR by the Ca^{2+}-ATPase Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase, (SERCA) and out of the cytosol *via* the sarcolemmal Na^+/Ca^{2+} exchanger. The affinity of SERCA to Ca^{2+} is regulated by its interaction with phospholamban [101]. Recently, indirect redox modulation of SERCA activity mediated by Nox2 has been observed [98]. In hearts overexpressing Nox2, SERCA activity and contractility increased as a result of increased phosphorylation of phospholamban. This increase in phospholamban phosphorylation was associated with a decrease in Ser/Thr Protein Phosphatase-1 (PP1) activity, which normally dephosphorylates phospholamban. Some of the proteins involved in the contraction, such as RyR and SERCA, are also direct targets of the ROS [42].

Recently, the structure of RyR has been clarified. It was found that this protein forms a tetrameric complex [102]. Each subunit has about 20 free Cys residues that can undergo various redox modifications. In addition, it was shown that mechanical stretching of cardiomyocytes induces cyclic activation of Nox2 in T-tubules, which leads to an increase in the release of Ca^{2+} from SR via RyR (Ca^{2+} spikes), thereby contributing to a fundamentally important physiological pathway for a stretch-induced increase in cardiac output known as "Starling's law of the heart" [103, 104]. At the molecular level, this is proposed to be mediated by the reversible oxidation of the RyR. In pathological conditions, more extensive and often irreversible oxidation or S-nitrosylation of RyR contributes to the release of Ca^{2+} from the SR followed by rhythm and contractile dysfunction [105].

The exact source of the ROS that participates in the oxidation of the RyR remains unclear. Mitochondria and Nox2 are probably involved [103]. In skeletal muscle, it was reported that Nox4 can oxidize RyR1 and cause an increase in Ca^{2+} leakage during hypoxia [106]. Whether Nox4 influences similar effects in cardiomyocytes is unknown. The physiological redox regulation of SERCA in the heart remains poorly studied. The isoform SERCA2's activity can be enhanced by HNO-induced phospholamban oligomerization [52]. Irreversible oxidation of cardiac SERCA in pathological conditions such as hemodynamic overload and

chronic neurohumoral activation reduces the activity of SERCA and causes contractile dysfunction [107, 108].

All of the above apparently can be extrapolated to the Ca^{2+} transport system. In other cells, it has been established that H_2O_2 can oxidize and inactivate the purified Ca^{2+}-ATPase from the plasma membrane [109]. In experiments on neuronal culture, it was shown that H_2O_2 can kill cells by the opening of the TRPM2 (transient receptor potential M2) channel with subsequent Ca^{2+} overload [110]. The ability of ROS to activate TRPM2 has been confirmed by others [111, 112]. The important role of TRPM2 in H_2O_2-induced apoptosis and cell death has also been corroborated by others [113, 114]. There is also the reverse situation where TRPV1 (transient receptor potential vanilloid) channel opening with capsaicin results in increased ROS production and autophagy [115].

Hypoxia Inducible Factor (HIF). Cells have developed complex mechanisms for adapting to hypoxia. One such mechanism is the activation of HIF [116]. When activated during hypoxia HIF binds to the hypoxia response elements. These promoters contain the sequence NCGTG to which HIF binds and control the expression of genes that cause a cellular adaptation to hypoxia. HIF proteins are heterodimers consisting of a HIF-α and a HIF-β subunit, each of which is constitutively expressed. At normal levels of O_2, that is, with normoxia, HIF-α rapidly undergoes hydroxylation with Prolyl Hydroxylase 2 (PHD2) [117] and is subsequently degraded in the proteasomes [118]. Conversely, under hypoxic conditions, PHD2 becomes inactivated, which allows HIF-α to accumulate and bind to HIF-β to form active HIF. More than 70 genes are known to be activated by HIF-1 [119].

Under hypoxic conditions, mitochondria paradoxically increase their ROS production by complex III [42, 120]. The ROS formed in complex III are necessary for the stabilization of HIF-α as cells lacking the ability to generate ROS by complex III do not stabilize HIF-α during hypoxia [121-125]. Recent studies have shown that small molecules that selectively remove superoxide from complex III reduce hypoxic stabilization of the HIF-α protein [125]. Together, these data reveal that mitochondrial ROS, especially from complex III, plays a key role in this system. It has been shown that this ROS inhibits the activity of PHD2, but the exact mechanism by which they inactivate PHD2 is unknown [121]. There is indirect evidence that the signaling molecule between the complex III and PHD2 is H_2O_2 [122, 124]. Thus, the physiological adaptation appears to be affected by hypoxia-induced release of ROS (probably H_2O_2) that inhibits PHD2. That, in turn, leads to the stabilization of the HIF-α protein. However, there are also data that HIF-1α deficiency can abrogate mitochondrial ROS formation and prevent the oxidation of the phosphatase PTEN [126, 127]. It was proposed that HIF-1α is actually upstream of mitochondrial ROS formation and PTEN oxidation [126, 127].

Transcription factors STAT. The family of highly conserved signal transducer and activator of transcription (STAT) include STAT1-4, 5A, 5B and 6 [128, 129]. In unstimulated cells, inactivated STATs are localized in the cytoplasm together with Janus Kinase (JAK), and are induced by the binding of ligands to the receptors on the cell membrane which leads to phosphorylation of the corresponding proteins. STATs are activated by tyrosine phosphorylation followed by homo- or heterodimerization and then translocated to the nucleus where they directly bind to specific DNA recognition elements or interact with other transcription factors to regulate gene expression [130-132]. As an example, interleukin 6 and prolactin activate STAT3 and STAT5, respectively, to enhance the transcription of target genes in their target cell types [133, 134]. STAT3 and STAT5 become constitutively activated in human cancer cell lines obtained from the chest, head and neck, lungs, ovaries, pancreas, lymphoma, *etc*. [135]. STAT3 and, apparently, STAT5 also contribute to heart's tolerance to ischemia-reperfusion during postconditioning and remote postconditioning [136, 137].

In the healthy individual ROS function as important signaling molecules to maintain normal tissue homeostasis. For example, cytokines rapidly increase the ROS level in resting hematopoietic cells, which is associated with an increase in tyrosine phosphorylation followed by activation of JAK2 and STAT5. This leads to induced expression of the early response gene c-FOS, and G1- to S-phase transition in the cell cycle [138, 139]. The use of antioxidants in assessing the effect of cytokines on cells or stimulating cells with hydrogen peroxide without cytokines demonstrates that ROS plays an important signaling role in the control of JAK, STAT3 and STAT5 [140]. Activation of STAT3 and STAT5 by ROS induced by cytokines or growth factors may also include activation of Nox [141, 142].

ROS interact with cysteine residues in the catalytic domain of JAK2, which, when oxidized, inhibit its kinase activity [143]. STAT3 can also interact directly with intracellular oxidants. In cells treated with hydrogen peroxide, STAT3 reversibly forms crosslinked monomers, dimers, trimers and tetramers through oligomerization associated with the formation of disulfide bonds in conserved cysteine residues. This inhibits STAT3 binding to DNA and transcriptional activity without affecting its activation by tyrosine phosphorylation [144, 145].

The function of oxidized STAT3 complexes still needs to be resolved. STAT3, in addition to its cytoplasmic and nuclear distribution, is also found in the mitochondria. One possibility is that oxidation of STAT3 by H_2O_2 in the mitochondria might cause it to modulate cellular respiration through its possible interaction with the electron transport chain [146]. Oxidation of STAT5 is observed in macrophages of aging mice. The oxidation blocks the phosphorylation of its tyrosine residues in response to the stimulation of cytokine receptors [147]. Thus, physiological ROS production activates the JAK/STAT tandem in response to cytokines while excessive ROS production leads to inhibition of JAK/STAT.

Transcription factors NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells). The family of NF-κB transcription factors consists of 5 proteins: p65 (RelA), RelB (RelB), c-Rel (Rel), p105 / p50 (NFκB1) and p100 / p52 (NFκB2). NF-κB exhibits activity only in dimeric form (both hetero- and homodimers are possible) There are 15 known active combinations of these dimers [148]. The most common forms are the dimers of the subunits NF-κB1 or NF-κB2 with the p65 subunit. Members of the NF-κB family all have

an N-terminal RHD (Rel-homology domain) which is necessary for homo- and heterodimerization, nuclear translocation, and association with IκB group proteins that inhibit NF-κB and prevent its binding to DNA. The NF-κB subunits can bind to a DNA sequence that contains the nucleotide sequence 5-GGGRNWYYCC-3 (wherein R is any purine, N is any nucleotide, W is adenine or thymine, and Y is any pyrimidine) [148]. In addition, p65, RelB and c-Rel contain the C-terminal TAD (transcription activation domain) which is necessary for the activation of transcription of target genes. In unstimulated cells, the NF-κB dimers are inactivated by being bound to IκBs which sequester NF-κB in the cytoplasm. There are 8 known IκBs: IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3, p100 and p105 which bind to NF-κB *via* ankyrin repeats consisting of 33 amino acid residues [148]. Cytokines activate the NF-κB signaling pathway by activating IKK (IκB kinase). IKK phosphorylates the IκB marking it for proteolytic degradation by the proteosome. When NF-κB is released from its inhibiting complex it translocates into the nucleus and activates the transcription of its genetic program.

ROS can affect NF-κB's activation by affecting the phosphorylation of IκB. Exogenous H_2O_2 causes phosphorylation of Tyr^{42} and other tyrosine residues in the IκB resulting in NF-κB activation [149, 150]. EL4 cell culture experiments have shown that H_2O_2 causes IκB phosphorylation, but these phosphorylated amino acid residues differ from those phosphorylated by cytokines. Their phosphorylation catalyzes casein kinase II rather than IKK [149]. Treatment of KBM-5 cells with H_2O_2 resulted in the activation of NF-κB [150], but without the enhancing IκB degradation. Hydrogen peroxide induced serine phosphorylation in the p65 subunit, followed by translocation of p65. In addition, it was found that H_2O_2 causes tyrosine phosphorylation of IκBα, which is necessary for the activation of NF-κB. It was also found that Syk tyrosine kinase is involved in the H_2O_2-induced activation of NF-κB as stimulation of NF-κB is observed only in those cells that express Syk tyrosine kinase [150]. Depending on the cell type, H_2O_2 induced NF-κB activation may occur with the aid of casein kinase II or Syk-tyrosine kinase. In experiments on MCF-7 cells, it was shown that H_2O_2 (1-10 μM) activates the NIK kinase (NF-κB-inducing kinase), which leads to phosphorylation (activation) of IKKα, followed by phosphorylation of IκB and activation of NF-κB [151]. Okadaic acid, which is a Ser/Thr phosphatase inhibitor, has an effect similar to that of H_2O_2, so the authors [151] proposed that H_2O_2 might be inhibiting a Ser/Thr-phosphatase, thereby promoting NIK phosphorylation. In 2008, Kim *et al.* [72] reported that H_2O_2 causes redox activation of the signaling pathways: PI3K/PTEN/Akt and NIK/IKK both of which contributed to the IKK-dependent activation of NF-κB.

5. EFFECT OF PHYSICAL EXERCISE ON ROS PRODUCTION IN THE HEART AND SKELETAL MUSCLES

Physical exercise is an important regulator of redox signaling in the heart [152]. In animal models, physical activity has been shown to increase the heart's tolerance to ischemia-reperfusion [153, 154]. Knocking down Mn-SOD by pretreatment with an antisense oligodeoxyribonucleotide elimi-

nated the exercise-induced decrease in experimental infarct size indicating that Mn-SOD induction was involved in the protection [153, 154]. Aerobic exercise prevents pressure overload-induced cardiac dysfunction and hypertrophy [155]. Aerobic exercise training also decreased the myocardial ROS and Malonyldialdehyde (MDA) content and increased myocardial SOD content [155]. Physical exercise is reported to reduce ROS production in aging heart by increasing the expression of antioxidant enzymes [156]. Physical exercise also attenuates aortic stenosis-induced heart failure [157]. Acute heavy exercise induces mitochondrial stress and enhanced ROS generation in cardiomyocytes [158]. Those ROS, apparently, play the role of an intracellular messenger and mediate the beneficial adaptation from physical training on the heart. It has been shown that tocopherol-enriched diet in trained rats prevents this increase in tissue content of mitochondrial proteins, and cytochrome c expression in the heart [159]. However, physical exertion not only induces the formation of ROS, but paradoxically it also combats oxidative stress in the heart. It has been found that swimming training attenuates isoproterenol-induced fibrosis and isoproterenol-induced ROS production in the myocardium [160]. At the same time, physical training's reported to reduce OH$^•$ production of skeletal muscle mitochondria, but not that of the heart [161].

One month after experimental infarction in rat hearts, an increase in cardiac H_2O_2, other ROS, and MDA were observed. But after two months of subsequent exercise training, these indicators returned to their control values [162]. After exercise training signs of heart failure were also reduced in these rats. A reduction in skeletal muscle atrophy and skeletal muscle Nox activity were also reported [163]. Physical training increases the activity of myocardial Aldehyde Dehydrogenase 2 (ALDH2), which eliminates toxic aldehydes including MDA [164]. It is possible that the increase in ALDH2 contributes to the exercise-induced decrease in MDA formation [162].

Above we have written about the cardioprotective effects of physical training. However, it should be remembered that exhausting exercise itself can also cause heart damage. Intensive exercise in mice causes an increase in the cardiospecific marker of necrosis, creatine kinase MB and ROS production by cardiac mitochondria. The flavonoid quercetin, which has antioxidant properties, opposed those negative manifestations [165]. Thus, while moderate exercise exerts a positive effect on redox signaling in the heart that ameliorates cardiac injury, exhausting physical exercises can also cause oxidative stress.

CONCLUSION

In this review, we note only that the normal physiological level of ROS controls cell proliferation [66, 71, 100, 166, 167] and angiogenesis [58, 166-168]. It is believed that an elevated level of ROS exceeding physiological values triggers programmed cell death by apoptosis [169] or autophagy [169, 170], the occurrence of arrhythmias, and contractile dysfunction of the heart [101]. On the other hand, it may put the heart or other organs into a protected (conditioned) state.

We have reviewed a large number of reports in which ROS have the clear ability to affect signaling pathways. Most of these were discovered by exposing cells to exogenous ROS or scavengers. In a few cases, such as the control of HIF, a clear physiological signaling role has been revealed. In many cases, a biphasic response is seen: Low levels of ROS production, as might be generated by physiological signaling, seem to stimulate a pathway while higher levels capable of oxidizing amino acids within a signaling protein appear to inhibit it. But in most cases it is largely unknown, how extensively the body actually utilizes these signaling mechanisms in its normal physiology or whether they only come into play under pathological conditions.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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