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Commentary

Reactive oxygen species: Destroyers or messengers?

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ABSTRACT

Abundant evidence leaves no doubt that reactive oxygen species (ROS) are not only inevitable by-products of oxygen metabolism but also play a role in cellular signaling. ROS are produced by a family of NADPH oxidases for signaling purposes and mediate or augment the effects of insulin, growth factors, cytokines and G-protein-coupled receptors. Disturbances of ROS signaling leading to overproduction of these intermediates inflict oxidative damage of cell components in the course of various diseases. Restoration of proper ROS signaling, especially inhibition of cellular sources of ROS, may thus provide new ways of therapy.

1. Introduction

Molecular oxygen is relatively not reactive but oxygen derivatives more prone to participate in chemical reactions (reactive oxygen species; ROS) are formed during aerobic metabolism and in the environment (Fig. 1). Superoxide radical ion, the main ROS produced in vivo has both reducing and oxidizing properties, reacting predominantly with metal ions and iron–sulfur clusters. Hydrogen peroxide is a weak oxidant attacking mainly thiols. Reaction of superoxide with nitric oxide produces peroxynitrite, a strong oxidizing, nitrating and nitrosylating agent. Reaction of hydrogen peroxide with Cl^-, catalyzed by myeloperoxidase, produces hypochlorite, an oxidant and chlorinating compound. Reaction of H_2O_2 with transition metal ions yields hydroxyl radical, the most reactive species occurring in vivo (Fig. 1). $^{\bullet}OH$ reacts rapidly and indiscriminately with biomolecules of all classes, including nucleic acids, free nucleotides, proteins, lipids and carbohydrates. ROS inflict oxidative damage which may cause DNA mutations, protein inactivation and cell death.

Historically, three events have had initiated the current biomedical interest in ROS: (i) the postulate that oxygen free radicals may be responsible for hyperoxic injury [1], (ii) the formulation of the free-radical theory of aging [2] and (iii) the

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Abbreviations: ADCG, anchorage-dependent cell growth; AGE, advanced glycation end-products; AMPK, AMP-activated protein kinase; ASK, apoptotic signal-regulating kinase; Cat, catalase; Duox, dual function oxidase; Cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; GPx, glutathione peroxidase; Grx, glutaredoxin; GSH, glutathione; HIF, hypoxia-inducible factor; HNE, H^+/Na^+ exchanger; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MMP, metalloproteinase; Nox, NADPH oxidase; Nrf, nuclear erythroid 2 p45-related factor; PHD, prolyl hydroxylase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; ROS, reactive oxygen species; SOD, superoxide dismutase; Prx, peroxiredoxin; PTP, protein tyrosine phosphatase; Srx, sulfiredoxin; TNF, tumor necrosis factor; Trx, thioredoxin; TrxR, thioredoxin reductase; TXNIP, thioredoxin-interacting protein; UCP, uncoupling protein; UV, ultraviolet; VEGF, vascular endothelial growth factor.

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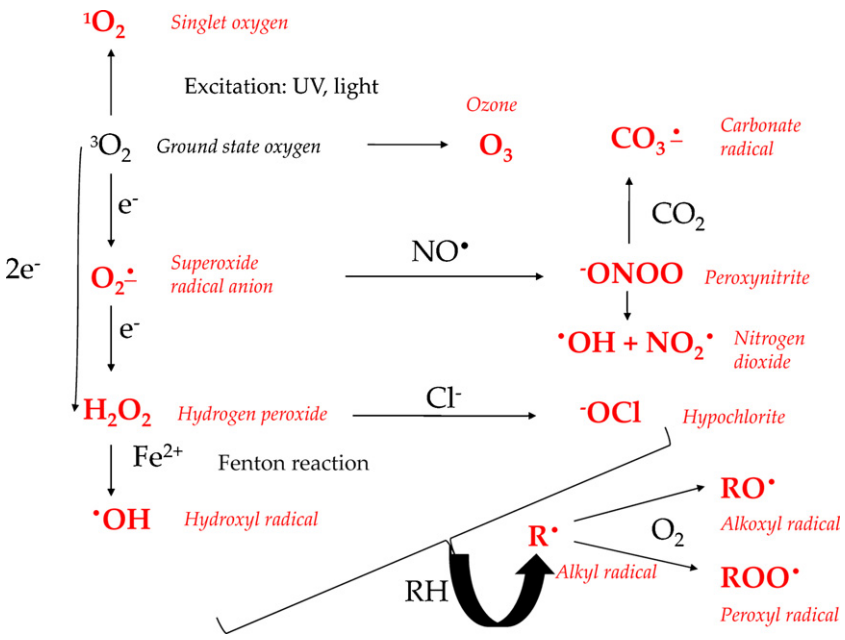

Fig. 1 – Main reactive oxygen species; RH—organic molecule.

discovery of superoxide dismutase (SOD) [3]. All they suggested that ROS are an unavoidable evil of oxygen metabolism. The omnipresence of ROS has forced aerobes to mount complex antioxidant defence which still appears to be inadequate in ROS-mediated aging and in number of diseases. This premise of thinking has stimulated animal and human intervention trials aimed at prevention of oxidative-stress linked diseases and/or prolongation of life span by antioxidant supplementation. These trials, though apparently successful in several cases, overall not found to have encouraging outcomes.

However, a concurrent view has been formulated, according to which the persistence of ROS in cells indicates that ROS production was evolutionarily selected in order to perform some useful role(s) in cellular metabolism [4]. This view has been supported by abundant data demonstrating that ROS have indeed important functions in cellular signaling as participants and modifiers of signaling pathways, essential for the proper development and proliferation of cells, may have mitogenic effects and can mimic and amplify the action of growth factors. Therefore, continuous formation and removal of ROS in our body is not only a threat but also a means of conveying information.

2. Which ROS can take part in cellular signaling?

General requirements for a signaling molecule include: (i) control of its concentration at the level of synthesis and removal, (ii) existence of specific receptors and (iii) reversibility of the signaling effect. A more general approach would also include some irreversible modifications of proteins (subject to turnover anyhow). Since the discovery of the signaling role of nitric oxide (NO), the possibility of signaling by small molecules has been generally accepted. Which ROS can perform a signaling role analogous to NO? For brevity, the arguments for and against the main physiologically formed ROS are given in Table 1. These considerations point to hydrogen peroxide and/or superoxide as the best candidates for signaling purposes. Both species are formed by enzymes and their concentrations are enzymatically controlled (Table 2). Precise identification of ROS involved in a signaling event is not easy, however. Usually, one ROS gives rise to other species and their effects on a target molecule may not be very different from each other. In particular, superoxide always produces hydrogen peroxide via enzyme-catalyzed or spontaneous dismutation, or by reduction. Apart from superoxide or hydrogen peroxide, more aggressive ROS may be used for execution of irreversible protein modifications.

3. Main cellular sources of ROS

Mitochondria are considered to be the main cellular source of superoxide formed by one-electron reduction of oxygen, a side reaction of the respiratory chain. The probability that molecular oxygen is reduced to superoxide rather than to water is increased if the proton concentration gradient at the mitochondrial inner membrane is high and the proper flux of electrons through the respiratory chain is less favored (especially when the availability of ADP is low). Superoxide release from mitochondria depends dramatically on the proton motive force and increases more than 15-fold upon increase of the proton motive force (Δp) from 170 up to 190 mV. The rationale for this is that at high Δp protons are not easily pumped out of the matrix against the electrochemical gradient, so the electron-transport chains slows down, the half-lives of reduced intermediates are longer and the chance for their one-electron reaction with oxygen becomes higher.

Table 1 – Possible suitability of main physiologically present ROS for signaling.

Species	Arguments for	Arguments against
Superoxide $O_2^{\bullet-}$	Enzymatic production by Nox Enzymatic removal Low reactivity thus some selectivity of reactions	Also non-enzymatic formation Difficult penetration through membranes
Hydrogen peroxide H_2O_2	Enzymatic production Enzymatic removal Low reactivity thus some selectivity of reactions Easy penetration through membranes	Also non-enzymatic formation
Organic radicals (alkyl $R^{\bullet}$, alkoxyl $RO^{\bullet}$, peroxyl $ROO^{\bullet}$)		Non-enzymatic formation High reactivity No enzymatic removal
Peroxynitrite $ONOO^-$		Non-enzymatic formation Unspecific reactivity No enzymatic removal
Hypochlorite OCl^-	Enzymatic formation	Non-specific reactivity No enzymatic removal Formation of secondary reactive metabolites
Singlet oxygen $^1O_2$		Rare occurrence inside the body High reactivity No enzymatic removal
Hydroxyl radical $^{\bullet}OH$		Non-enzymatic formation Very high reactivity No enzymatic removal

The main sources of one-electron leakage are Complexes I and III of the respiratory chain. In Complex I, the iron–sulfur centers and the active-site flavin of Complex I have been proposed as the main sites of superoxide production. While Complex III releases superoxide both to the matrix and to the intermembrane space, superoxide production by Complex I is directed towards matrix. Other sources of ROS in the mitochondria have also been considered. Complex II may perhaps generate superoxide when damaged or in hypoxia. Autoxidation of reduced flavins in other mitochondrial flavoproteins may also generate superoxide [5].

Uncoupling of oxidative phosphorylation may decrease the mitochondrial release of superoxide considerably. Uncoupling proteins (UCPs) of the inner mitochondrial membrane seem important in this respect. UCP1 is present in the mitochondria of the brown adipose tissue and functions in adaptive non-shivering thermogenesis. The closely related UCP2 and UCP3 have been found in mitochondria from several other tissues and suggested to play an antioxidant role by decreasing the superoxide release. Ubiquinone was found to be a strong activator of uncoupling. External superoxide has been demonstrated to activate UCPs. This effect seems to be indirect and dependent upon release of iron from aconitase and other FeS proteins. Free iron enables formation of hydroxyl radical and induction of lipid peroxidation. Reactive aldehydes formed in this process, especially 4-hydroxy-*trans*-2-nonenal, are potent activators of proton conductance by all three UCPs, apparently via covalent modification of the proteins. The appearance of hydroperoxy- (or hydroxy)fatty acids liberated by phospholipases from peroxidized phospholipids creates another possi-

Table 2 – Main factors controlling the level of superoxide and hydrogen peroxide.

	Superoxide	Hydrogen peroxide
Formation	Mitochondrial respiratory chain Nox Some other enzymes (e.g. xanthine oxidase) Microsomes Autoxidation of reduced form of flavins, quinones, metals, metalloproteins, thiols Ionizing radiation Photochemical reactions Ultrasound	Non-enzymatic dismutation of $O_2^{\bullet}$ Dismutation of $O_2^{\bullet}$ by SODs Reduction of $O_2^{\bullet}$ Peroxidases Peroxisomal oxidases Some other enzymes (e.g. monoamine oxidase)
Removal	CuZnSOD (cytosol) MnSOD (mitochondria) EC-SOD (extracellular space) Low-molecular mass antioxidants	Catalase Glutathione peroxidases Peroxiredoxins

bility of uncoupling. These modified, more hydrophilic fatty acids may transfer protons across the inner mitochondrial membrane themselves while UCPs (especially UCP2) may mediate their expulsion from the matrix side.

Mitochondrial superoxide is mainly dismutated to H_2O_2 and O_2 by superoxide dismutases: MnSOD present in the mitochondrial matrix and CuZnSOD in the intramembrane space. However, a fraction of $O_2^{\bullet-}$ leaves mitochondria. In contrast to H_2O_2 which can easily penetrate cellular membranes, the charged $O_2^{\bullet-}$ uses mainly the voltage-dependent anion channel (VDAC) to pass the outer mitochondrial membrane.

Mitochondria are responsible for the steady-state ROS production. However, there is evidence that they may be a part of the cellular system of sensing and transduction of ROS signals, apart from NADPH oxidases (Noxs). It has been demonstrated that some agents, like leptin and angiotensin II increase mitochondrial ROS production. S-Glutathionylation of complex I increases superoxide release by this complex [6].

Quantitative estimates of superoxide production by mitochondria vary significantly (0–5% of O_2 metabolized). ROS release from these organelle in vivo may be lower than that found in vitro using isolated and possibly damaged mitochondria [7].

An additional source of ROS in the mitochondria is the 66-kDa isoform of growth-factor adaptor Shc ($p66^{Shc}$) protein which has been implicated in the development of aging and aging-related diseases. $p66^{Shc-/-}$ mice exhibit increased resistance to oxidative stress and a 30% increase in the lifespan. The protein resides mainly in the cytosol where thioredoxin (Trx)1 and glutathione (GSH) keep it in the inactive reduced state. Upon induction by stress factors, including ROS, expression of $p66^{Shc-/-}$ increases. Moreover, the protein is activated by thiol oxidation with induces a dimer–tetramer transition. Activated $p66^{Shc}$ translocates to the intermebrane space of mitochondria and oxidizes cytochrome c producing H_2O_2 as a signaling molecule for apoptosis [8].

Major extramitochondrial cellular sources of superoxide include endoplasmic reticulum, a lysosomal redox chain and plasma membrane Noxs. Smooth endoplasmic reticulum contains enzymes detoxifying xenobiotics, including drugs, among them cytochrome P-450 isozymes, especially the ethanol-inducible CYP2E1. These enzymes are able to reduce molecular oxygen and produce $O_2^{\bullet-}$ and H_2O_2. Nuclear membranes contain cytochrome oxidases and electron transport systems of unknown functions which may also release ROS. Peroxisomes can both produce and scavenge hydrogen peroxide. They contain a number of H_2O_2-generating enzymes (e.g. D-amino acid oxidase, urate oxidase, L-α-hydroxyacid oxidase, fatty acyl-CoA oxidase and glycolate oxidase) but also catalase. Therefore, only a small fraction of H_2O_2 produced in peroxisomes may escape from these organelles under normal conditions [9].

Several enzymes can produce superoxide as a by-product. The most important is xanthine oxidase formed from xanthine dehydrogenase after tissue exposure to hypoxia. This list of enzymes includes aldehyde oxidase, dihydroorotate dehydrogenase and tryptophan dioxygenase. Nitric oxide synthase can also produce superoxide in the ''uncoupled'' state which occurs upon deficiency of cofactors (tetrahydrobiopterine or L-arginine). Interaction of eNOS with Hsp90 protects it against uncoupling. 5-Lipoxygenase generates superoxide directly and induces ROS formation indirectly via a leukotriene-induced activation of Nox. Extraperoxisomal oxidases, including amine oxidase, may be direct sources of hydrogen peroxide [10].

Superoxide is also formed by autoxidation of flavins, hydroquinones, thiols and metals (free or protein-bound). Moreover, ROS are formed by interaction of light, UV and ionizing radiation and ultrasound with biologic material.

While for the vast majority the above sources, formation of superoxide is a side effect of their action, there is a family of enzymes, Noxs, designed for generation of $O_2^{\bullet}$ as the main product. The phagocyte enzyme (now classified as Nox2), responsible for the respiratory burst, was the first discovered. Nox2 produces large amounts of superoxide (and, indirectly, other ROS) for defensive purposes as microbicidal agents. The same enzyme (albeit at lower levels) and its analogs are present ubiquitously in various cells and there is little doubt that small amounts of superoxide are produced by these proteins for the sake of cellular signaling.

The active complex of Nox2 consists of several proteins: the heterodimeric flavocytochrome (cytochrome *b*559) composed of two subunits and cytosolic components which associate with them, activating the complex. The cytochrome *b*559 subunits ($gp91^{phox}$ and $p22^{phox}$) are integral membrane polypeptides spanning several times the lipid bilayer. $gp91^{phox}$ has six transmembrane domains, two low-potential hemes located near the internal and external surfaces of the membrane, respectively, and a cytosolic domain containing the FAD-binding region, interacting with the substrate (NADPH). $gp91^{phox}$ constitutively associates with $p22^{phox}$. In the dormant state, the inactive cytochrome *b*559 resides in intracellular vesicles. Its activation is initiated by the Rac protein which, upon exchange of bound GDP for GTP, induces phosphorylation of a cytosolic $p47^{phox}$ subunit. Conformational change of $p47^{phox}$ occurring upon phosphorylation enables it to interact with the ''organizer'' $p22^{phox}$ subunit. The $p47^{phox}$ brings the ''activator'' $p67^{phox}$ subunit and a small $p47^{phox}$ subunit to the membrane. Vesicles with the assembled complex are recruited to the phagosomal membrane or the plasma membrane and Nox2 produces superoxide by transmembrane electron transfer from cytosolic NADPH, via FAD and hemes, to oxygen at the extracytoplasmatic side of the membrane. NADPH is the preferred substrate; affinity of $gp91^{phox}$ for NADH is about 100 times lower. Another GTP-binding protein, $p21^{Ras}$, functions upstream of Rac in oxidant-dependent signaling.

Nox2 analogs differ in the structure of the main subunit and in the composition of and even requirements for the regulating subunits (Table 3). Some of Noxs are called Duoxs (''dual function oxidases'') since, in addition to the Nox domain, they have a domain homologous to that of thyroid peroxidase (lacking a peroxidatic activity but generating H_2O_2 instead). This H_2O_2 is required for thyroperoxidase-mediated iodination of tyrosyl residues of thyreoglobulin in the thyroid gland. In the digestive tract, Duox2 in combination with lactoperoxidase plays a role in bacterial killing. It has been a question of dispute whether Duoxs produce superoxide or directly hydrogen peroxide. The prevalent view is that the

Table 3 – NADPH oxidases, superoxide-generating enzymes. After [11].

Species	Sequence identity with Nox2	Interacting subunits	Remarks	High level of expression/ Lower level of expression
Nox1	60%	Rac, $p22^{phox}$ NOXO1 ($p47^{phox}$ homolog), NOXA1 ($p67^{phox}$ homolog)		Colon/smooth muscle, endothelium, uterus, placenta, prostate (upregulated upon castration), osteoclasts, retinal pericytes
Nox2		Rac, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$		Neutrophils and macrophages/B lymphocytes, neurons, cardiomyocytes, skeletal muscle, hepatocytes, endothelium, hematopoietic stem cells, smooth muscle
Nox3	56%	Rac? $p22^{phox}$, NOXO1, NOXA1	Perhaps constitutively active? (but what for?)	Inner ear/fetal kidney, fetal spleen, skull bone, brain
Nox4	39%	Rac? $p22^{phox}$,	Constitutively active?	Kidney, blood vessels/osteoclasts, endothelium, smooth muscle, hematopoietic stem cells, fibroblasts, keratinocytes, melanoma cells, neurons
Nox5		Does not require $p22^{phox}$ and cytosolic activator or organizer	Activated by increased Ca^{2+} concentration	Lymphoid tissue, testis/endothelium, smooth muscle, pancreas, placenta, ovary, uterus, stomach, various fetal tissues
Duox1	50% (Nox backbone)		Activated by increased Ca^{2+} concentration	Thyroid/airway epithelia, tongue epithelium, cerebellum, testis
Duox2	50% (Nox backbone)		Activated by increased Ca^{2+} concentration	Thyroid/salivary and rectal glands, gastrointestinal epithelia, airway epithelia, uterus, gall bladder, pancreatic islets

primary product is superoxide although rapid dismutation may preclude its detection.

The Rac protein is involved in the activation of most if not all Nox enzymes. In mammalian cells there are three highly homologous Rac proteins: Rac1, distributed ubiquitously, Rac2, expressed mostly in myeloid cells and Rac3, found mainly in the nervous system.

The mere existence of the many Nox isoforms suggests the in vivo relevance of redox-sensitive signaling cascades. Excessive stimulation of Noxs by cytokines and other mediators is implicated in various disease conditions [11].

Upon Nox2 activation, the phagocytic vacuole is alkalinized due mainly to the consumption of protons during dismutation of superoxide and formation of hydrogen peroxide. The transmembrane transport of electrons by Noxs is coupled with the transport of cations (H^+ or K^+) to preserve electroneutrality. It has been postulated that Noxs form proton channels (located in the histidine-rich transmembrane domain) or that Nox proteins are closely associated with cation channel(s). Interestingly, it has been suggested that the main function of Noxs is to provide conditions for cation movements and pH changes necessary for various cell functions and signaling, superoxide production being of secondary (if any) importance. E.g. Nox2-dependent phagosomal alkalinization is important for the cross-presentation of antigens in dendritic cells [12].

4. How may ROS act as messengers?

There are two, not mutually exclusive, ideas concerning the mechanism of the messenger action of ROS: (i) modification of target protein molecules and (ii) changes of intracellular redox state [13].

(i) While some ROS may inflict irreversible damage to macromolecules with low specificity, hydrogen peroxide and superoxide are relatively mild oxidants. In the absence of transition metal ions, the main targets of H_2O_2 are thiol group of protein cysteine residues. Hydrogen peroxide oxidizes cysteines to disulfides formed with another protein cysteines or with low-molecular weight thiols (usually glutathione) or to a sulfenic acids $-SOH$. Sulfenic acids may be converted to disulfides upon reaction with another thiols. The disulfide bonds can be reduced by glutaredoxins (Grxs), thioredoxins (Trxs) or glutathione (GSH) so this modification is reversible. S-Glutathionylation (formation of a mixed protein–glutathione disulfide) is thought to prevent further, irreversible oxidation of cysteine sulfur. It is often viewed as a general reversible protein modification, comparable to protein phosphorylation [14]. Glutathionylation, thought previously to be due to exchange reaction between glutathione disulfide and protein–SH, is now believed to occur mainly in reaction of protein cysteine sulfenic acids with glutathione. Glutathionylation and deglutathionylation are catalyzed by glutaredoxins. Further oxidation of a thiol group leads to a sulfinic acids $-SO_2H$ or sulfonic acids $-SO_3H$. Both these reactions were considered irreversible; however, sulfiredoxins (Srxs), enzymes able of ATP-dependent reduction of sulfinic acid residues have been identified [15]. In addition to Srxs, sestrins are also able to reduce sulfinic acids [16].

Thiol groups are reactive mainly in their deprotonated form, i.e. as thiolates $-S^-$. The pK_a of most cysteine thiols in proteins, and of GSH, is about 8.5 so these groups are mainly protonated. Therefore Cys residues of low pK_a, being ionized at physiological pH, may be selectively oxidized, even in the presence of excess of other Cys residues [17]. Another factor contributing to the selectivity of cysteine oxidation is the topography of potential targets. Although hydrogen peroxide, a small-uncharged molecule, can diffuse quickly within a cell and across cellular membranes, its localized generation will produce a concentration gradient, affecting the probability of reaction with appropriate targets. Moreover, recent findings indicate that cell membranes may be less permeable to hydrogen peroxide than thought initially and the rate of membrane passage by H_2O_2 may be dependent on the presence of aquaporins [18].

Among proteins most susceptible to thiol oxidation are protein phosphatases, especially protein tyrosine phosphatases (PTPs), G proteins, some ion channels, and some transcription factors. In all cases the reactive cysteine is surrounded by positively charged residues (in transcription factors necessary to enable binding to negatively charged DNA). This neighbourhood stabilizes the dissociated form of the thiol group even at low pH. PTPs have a catalytically important low-pK_a (4,7-5,4) Cys residue which at neutral pH resides as a thiolate anion in a signature active-site motif His-Cys-X-X-Gly-X-X-Arg-Ser/Thr (where X is any amino acid). This low-pK_a Cys is crucial for the enzyme activity and, at the same time, makes this amino acid susceptible for oxidation.

Cysteine oxidation may inactivate proteins (e.g. PTPs) but sometimes may activate them (ryanodine receptors), change their conformation to induce dimerization (chaperone proteins) or release a complexed molecule (ASK-1 from oxidized thioredoxin) [19]. The list of phosphatases consistently reported to be regulated by reversible thiol oxidation includes PTP1B, a phosphatase dephosphorylating, i.e. insulin receptor and mediating insulin resistance and phosphatase, PP2B (calcineurin) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a dual function phosphatase acting on proteins on inositol-3-phosphate (IP_3). Since the level of phosphorylation of substrates is a resultant of the rates of phosphorylaton and dephosphorylation, oxidative inactivation of PTPs increases the level of tyrosine phosphorylation of protein substrates. Inactivation of PTEN amplifies the effects of IP_3 signaling [19].

The stimulation of net kinase activities by ROS seems to be mainly indirect, due to inactivation of protein phosphatases. It has been claimed not infrequently that ROS may also directly activate kinases. However, in most cases such conclusions are based on whole-cell data and the evidence is not always convincing.

Signaling by methionine oxidation (reversible thanks to methionine sulfoxide reductases) is also possible and has been reported for calmodulin and membrane transporters.

Irreversible protein modifications by ROS evades the strict definition of signaling but nevertheless may serve signaling functions by modifying their activities. Reaction of superoxide with iron–sulfur cluster usually inactivate proteins but may also the activate the target protein as demonstrated for Grx2 [20]. Activation of phenylalanine hydroxylase by hydrogen peroxide has been documented and suggested to be due to oxidation of a Trp residue leading to a conformational change. Protein carbonylation (oxidation leading to formation of carbonyl groups), the most commonly studied oxidative protein modification, is considered irreversible and not employed for signaling purposes but both these views have been recently challenged [21].

(ii) The term ''redox state of a cell'' is used commonly and rather imprecisely. It can be defined by a set of values of actual redox potentials of individual redox couples (e.g. GSSG/GSH) in a given cellular compartment. It is well documented that the redox state of a cell, as probed by the redox potential of the glutathione couple, is different for actively growing, confluent, differentiated and apoptotic cells. Production of ROS which are consumed in redox reactions must change the redox state of a compartment in which this production occurs. This change may be reversible as the cell attempts to keep the redox homeostasis. Is the action of ROS mediated by changes in the redox state of a cell? Oxidation of protein thiols means, of course, a change in the redox potential of a thiol redox couple of individual protein species. However, what is meant by this term is usually a change of redox potentials of main intracellular ''redox buffers''(GSSG/GSH, $NADP^+$/NADPH, thioredoxins, glutaredoxins) and effects of these changes on cellular phenomena. The distinction between (i) and (ii) is not easy and the often used term *redox signaling* is in practice equivalent to *ROS signaling*. The difference between these terms has mainly an instrumental meaning, depending on whether one detects redox signaling by studies of oxidative modifications of individual proteins or by measurements of changes in the ''redox environment'' of cell compartments [22].

5. Dynamics of antioxidant defence

At a first sight, the omnipresence of antioxidants and antioxidant proteins should be a serious obstacle to ROS signaling. Numerous data point, however, to the dynamic regulation of the activities of antioxidant proteins.

Activities of catalase (Cat) and glutathione peroxidase (GPx)1 are regulated by c-Abl and Arg non-receptor tyrosine kinases. The cytoplasmic forms of both kinases are activated by oxidative stress and bind to Cat and Gpx1 phosphorylating the enzymes and thus increasing their activities [23]. However, at higher ROS levels, c-Abl and Arg dissociate from Cat, Cat is dephosphorylated by PTPs, ubiquitinylated and degraded, thus potentiating the increase in ROS levels. In turn, Cat has been reported to bind the SHP2 PTP and the adaptor protein Grb2 upon integrin-ligand binding and protect them efficiently from H_2O_2-mediated oxidation [24].

Peroxiredoxin (Prx) activity can be regulated by phosphorylation. Phosphorylation of Prx1 at Thr 90 results in an over 80% activity decrease. Several cyclin-dependent kinases (Cdk2, Cdk4 and Cdk6) are able to phosphorylate this enzyme; such phosphorylation occurs during mitosis. Prx2 can be

phosphorylated by Cdk5, which similarly decreases its activity [25].

Reaction of Prxs with H_2O_2 leads to oxidation of Cys 51 and formation of a disulfide with a C-terminal conserved cysteine (Cys 172 in Prx1). According to the ''floodgate'' hypothesis, the normal catalytic cycle of Prxs consists in cyclic oxidation to sulfenic acid by hydrogen peroxide, followed by formation of an intracellular disulfide bond which is subsequently reduced by Trx and tioredoxin reductase (TrxR). In this cycle, Prxs act as a peroxide floodgate, preventing H_2O_2 signaling. When a transient burst of hydrogen peroxide is produced, Cys 51 of Prx may react with two molecules of hydrogen peroxide which leads to its hyperoxidation to stable (not transient) sulfinic acid and inactivation. Prx inactivation enables a temporary increase in hydrogen peroxide concentration for the sake of signaling. Subsequently, Srx may reactivate Prx. Therefore, peroxidation of Prxs to sulfinate may occur transiently, rendering the floodgate for peroxide signaling open [26]. The slow rate of Prx reactivation by Srx may be a feature permitting execution of the signaling function of H_2O_2 [15].

Antioxidant protein directly interact with other important proteins so changes of their oxidation state induced by interactions with ROS may initiate other signaling events. Trx, an antioxidant and reductant of disulfide bonds in proteins, performs also other signaling functions. Trx1 (cytosolic) forms a complex with apoptosis signal-regulating kinase-1 (ASK1), a mitogen-activated protein kinase kinase kinase (MAPKKK) which is involved in the activation of the c-Jun N-terminal kinase (JNK) and p38 MAP kinase (both inducing apoptosis). This signaling cascade is important for the initiation of apoptosis in cells exposed to strong oxidative stress (e.g. for TNFα-induced apoptosis). Complex formation with Trx1 inactivates ASK1. ROS oxidize Trx and disrupt the complex making ASK1 active. The stress-responsible thioredoxin-interacting protein (TXNIP) is also important for the Trx control of ASK1; binding of TXNIP to Trx attenuates the Trx-ASK1 interaction. Trx2 binds ASK1 in the mitochondria which inhibits apoptosis. Similar to Trx1, human Grx1 binds to ASK1 suppressing the kinase activity of ASK1. In the nucleus, Trx associates with Ref-1, a protein reducing critical cysteine residues of various redox-sensitive transcription factors like NF-κB, AP-1 and p53. Two critical cysteine residues (Cys65 and Cys93) in the N-terminal region of Ref-1 are maintained in the reduced state by Trx [27,28].

Trxs are also subject to redox control at the level of expression. In endothelial cells, low concentrations of H_2O_2 (10–50 μM) augment Trx expression while high concentrations (100–500 μM) promote their degradation [27].

6. ROS signaling

6.1. *Insulin action and secretion*

It has been found already in the 1970s that oxidants can mimic or facilitate insulin action. Low, physiologically relevant concentrations of hydrogen peroxide are not sufficient to trigger the autophosphorylation of the insulin receptor in the absence of insulin. However, they may enhance the response to insulin thus playing a co-regulatory role. Antioxidant treatment of target cells inhibits insulin responsiveness. ROS are generated upon insulin stimulation of target cells by Nox4. They transiently inactivate PTPs, especially PTP1B and thus enhance the tyrosine kinase activity of the insulin receptor.

Oxidative stress has been associated with beta cell destruction in type I diabetes. In an animal model of type II diabetes, increased expression of Nox1 was found in the islets [29]. However, Nox1, 2 and 4 were detected in pancreatic islet cells and a Nox inhibitor suppressed the glucose-stimulated insulin secretion. This finding suggests a role of Noxs in normal beta cell function [30].

Exercise and muscle contraction induces increased transport of glucose into the muscle, stimulating the recruitment of glucose transporter Glut-4 to the plasma membrane by a pathway different from that activated by insulin. This effect is mediated by AMP-activated protein kinase (AMPK). H_2O_2 activates AMPK and glucose uptake [31].

6.2. *Growth hormones, cytokines and G-protein-coupled receptors*

The evidence for the involvement of ROS in the signaling by these factors (and in other instances) is based generally on several sets of data: (i) generation of ROS upon action of the stimuli on cells, (ii) inhibition of effects by antioxidants or by overexpression of antioxidant proteins, (iii) mimicking the effects of the stimuli by exogenous oxidants (usually H_2O_2) and (iv) dependence of the effects on the level of ROS-generating enzymes, usually Noxs (assessed using transgenic animals lacking or overexpressing Nox).

Numerous studies have demonstrated that action of peptide growth factors as nerve growth factor (NGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or transforming growth factor beta 1 (TGF-β1) results in a transient increase in intracellular superoxide and hydrogen peroxide levels. Similar phenomenon is observed after the action of cytokines (TNF-α, IL1), agonists of heterotrimeric G-protein-coupled receptors (angiotensin II, bradykinin, thrombin, endothelin, fMetLeuPhe, lysophosphatidic acid, serotonin, ceramide or histamine) [32]. Specific inhibition of generation of ROS or elimination of H_2O_2 by catalase blocks the signaling by PDGF, EGF and angiotensin II demonstrating that H_2O_2 serves as a true messenger [33–36].

How are ROS generated after receptor stimulation? Certain growth factors and cytokines activate 5-lipoxygenase [37], some increase mitochondrial ROS production but vast majority of effects induced by growth factors and other external stimuli is mediated by Noxs.

ROS may be mediators of the signaling pathways of growth factors and other agents or may enhance signal transduction. Such an action can be also important since (i) it may provide a basis for cooperativity between various hormones, (ii) a membrane receptor may function simultaneously as a sensor for the extracellular ligands and a sensor for the inner metabolic state of a cell [36] and (iii) ROS may act as fine regulators of growth factor signaling by modulating cellular responses to the same stimulus [36,38].

6.3. Activation of kinases

In mammals, three major mitogen-activated protein kinase (MAPK) pathways converging on extracellular signal-regulated kinases (ERK, including ERK1 and ERK2 isoforms), c-Jun NH_2-terminal kinases (JNK, including JNK1, JNK2 and JNK3 isoforms) and p38 MAPKs (including p38α, p38β, p38γ and p38δ isoforms) trigger fundamental cell functions such as gene expression, cell proliferation and differentiation by mitogens, growth factors and cytokines, and apoptosis, by phosphorylation of specific Ser or Thr residues on target proteins. ERK activation has been implicated mainly in proliferation in response to growth factors, whereas p38 and JNK activation seems more important for stress responses like inflammation. The combination of magnitude and kinetics of individual members of the MAPK family appears to determine the response of the cell to various stimuli. All these MAPK pathways can be activated by ROS; one pathway of such activation involves activation of the apoptosis signal-regulating kinase (ASK1). ASK1-mediated myocardial cell death is an important mechanism of ischemia-reperfusion injury of the heart. ASK1 is also a mediator of myocardial cell hypertrophy and fibrosis and of proliferation and migration of vascular smooth muscle cells [39].

6.4. Transcription factors

In mammalian cells, numerous transcription factors are thought to be affected by ROS, among them NF-κB, AP-1, Nrf2, HIF-1α, Hic-5 and p53.

Redox control of NF-κB is a complex event. Oxidation by ROS leads to increase in the activity of inhibitory κB kinase, enhanced I-κB degradation of the regulatory subunit and translocation of the factor to the nucleus. However, binding of the activated NF-κB to DNA requires a reducing environment and Cys-62 in the DNA-binding region of the p50 subunit [40].

Nrf2 is the most important regulator of cell defence against chemical and oxidative stress which coordinatively controls expression of cytoprotective genes via the antioxidant responsive element (ARE), also defined as electrophile-reponsive element. The list of genes controlled by Nrf2 includes those coding for detoxification phase II enzymes, glutathione S-transferase and heme oxygenase-1. Under basal conditions, the transcription factor Nrf2 is anchored by its inhibitory partner, Keap1, within the cytoplasm and targeted for ubiquitination and proteasomal degradation. Activation of Nrf2 may occur via two main mechanisms: modification of Cys residues within Keap 1 and phosphorylation of Nrf2. Human Keap1 protein contains 27 cysteine residues, 9 of them with predicted low pK_a values and thus high reactivities. Alkylation or oxidation of Cys residues of Keap1 leads to Nrf2activation. ROS oxidize Cys 273 and Cys 288 on Keap1 which leads to dissociation of Nrf2 from the complex, translocation of Nrf2 to the nucleus and activation of target genes [41].

The T cell (transcription) factor (TCF) has been demonstrated to be activated by ROS via a Wnt/β-catenin signaling pathway. Nucleoredoxin, a member of the Trx family, is a redox sensor, a critical regulator of the Wnt/β-catenin pathway involved in cell proliferation and differentiation. Nucleoredoxin is bound to Disheveled protein; oxidation of reactive cysteines of nucleoredoxin disrupts the complex and initiates the signaling pathway. ROS activation of the Wnt/β-catenin pathway may contribute to cell proliferation and protection from apoptosis [42].

The family of forkhead box (Fox) containing transcription factors (FoxA-FoxS) includes FoxO proteins which have been implicated in the oxidative stress response and regulation of longevity. Mice lacking FoxO were found to have elevated ROS levels and decreased expression of antioxidant proteins. Regulation of the activity of the FOXO family of Forkhead transcription factors is complex. Protein kinase B/c-AKT phosphorylates FOXO3a which results in the translocation of FOXO3a from the nucleus into the cytosol and increase in cellular ROS level. On the other hand, ROS can activate the small GTPase Ral which results in the phosphorylation and activation of JNK. JNK-mediated phosphorylation of FOXO4 on Thr447 and Thr451 enhances FOXO transcriptional activity. FOXO can thus function in a negative feedback loop to control the cellular level of ROS. Moreover, sirtuins (NAD^+-dependent deacetylases) SIRT1 and SIRT2 bind to FOXO3a and reduce its acetylation level thus increasing DNA binding and elevating the expression of FOXO target genes, including MnSOD, Cat and Bim. This interaction is enhanced by ROS action. As a consequence, SIRT2 decreases cellular levels of ROS [43].

HSF1, a transcription factor crucial for the induction of expression of heat shock proteins, is directly activated by ROS. Sensing of oxidative stress requires two cysteine residues within the HSF1 DNA-binding domain which are engaged in redox-sensitive formation of disulfide bonds [44].

p53 Influences the expression of genes governing the redox status of the cells. Under basal conditions, p53 upregulates expression of several genes coding for antioxidant proteins, including Gpx1 and sestrins. Downregulation of p53 level results in augmented production of ROS. Interestingly, overexpression of p53 also leads to oxidative stress. Apart from nuclear translocation, p53 may be translocated to mitochondria where it binds and inhibits MnSOD promoting apoptosis. ROS may in turn modify p53. Oxidation or glutathionylation of its –SH groups (p53 has 10 –SH groups, all located within the DNA-binding domain) abolishes the DNA-binding activity of p53 and inhibits its tumor suppressor action. This transient inactivation of p53 effect may be an important factor in the cancerogenic action of ROS [45].

Transcription response to hypoxia, including the Pasteur effect, is based mainly on the hypoxia inducible factor (HIF) transcriptional complex. The control of HIF activity, consists in hydroxylation on specific prolyl residues by 2-oxoglutarate dependent prolyl hydroxylase (PHD) and hydroxylation of an asparagine residue by 2-oxoglutarate-dependent asparaginyl hydroxylase (factor inhibiting HIF; FIH-1). Activities of these hydroxylases depend on oxygen concentration so ROS are not directly required for the control of their activity. However, numerous data point to the effects of ROS and antioxidants on the activation of HIF. It is by no means clear whether hypoxia increases or decreases ROS formation (divergent results have been published). Perhaps the duration and degree of hypoxia is an important determinant of ROS production. In the lung, hypoxia upregulates Nox1. If ROS production is augmented in hypoxia, ROS may affect mitochondrial enzymes and decrease 2-oxoglutarate levels. Excessive hydrogen peroxide inacti-

vates PHD, perhaps by oxidation of active site iron. Thus, increased ROS formation in hypoxic mitochondria may contribute to the stabilization of HIF. A mitochondria-targeted antioxidant, MitoQ, cancels out the hypoxia-induced activation of HIF, suggesting that mitochondrial ROS are intrinsically involved in the hypoxic induction of HIF [46].

6.5. Regulation of transporters and ion channels

ROS affect the activity of proteins involved in transport, especially K^+ channels and Ca^{2+} channels. Angiotensin stimulation increases Ca^{2+} currents and decreases K^+ currents via the effects of ROS. Exposure of cells to H_2O_2 induces a rapid increase in the cytosolic concentration of Ca^{2+} in various cell types, due both to calcium release from intracellular stores and influx from extracellular space. Oxidation of L-type Ca^{2+} channels by hydrogen peroxide and other agents enhance while thiol-reducing agents decrease the channel activity. However, reverse effects have also been observed suggesting that the redox regulation of the channels depends on the cell type or on the contribution of auxiliary subunits (absent in cloning experiments). Oxidation of P/Q type calcium channels was found to increase their activity. Ryanodine receptors (RyR) are activated by ROS causing calcium release from the endoplasmic reticulum. In addition, thiol oxidation sensitizes RyR to activation by calcium while desensitizing it to inhibition by magnesium. Sarcoplasmic reticulum Ca^{2+}-ATPase has been reported to be both activated and inhibited by Cys oxidation [47]. Activity of plasma membrane Ca^{2+}-ATPase has been found to be attenuated by ROS mainly directly: calmodulin, activated by oxidation of Met144 and Met145, stabilizes the inactive conformation of the pump. ROS were also reported to decrease the Na^+/Ca^{2+} exchanger activity [48]. Superoxide was found to increase Na^+ reabsorption in the thick ascending limb of Henle's loop, apparently via activation of the Na^+/H^+ exchanger [49].

6.6. Control of proteinases

ROS inhibit activation of caspase 3. This effect may contribute to the transition from apoptosis to necrosis at higher intensity of oxidative stress [50]. ROS were demonstrated to stimulate the synthesis of matrix metalloproteinases (MMP) and to activate extracellular matrix pro-metalloproteinases. Induction of biosynthesis of MMP-1 by UV has been attributed to the UV-induced generation of H_2O_2 [51]. Hydrogen peroxide can directly activate TNF-α-converting enzyme by oxidative modification of the inhibitory prodomain which enables autocatalytic cleavage of the prodomain and activation of this MMP. Interestingly, H_2O_2 was unable to activate pro-MMP7 but other ROS (hypochlorite or peroxynitrite in the presence of glutathione to ensure glutathionylation) were effective [52].

6.7. Activation of heat shock proteins

Hsp33 has two interdependent stress-sensing regions located in the C-terminal redox-switch domain of the protein: a cysteine-zinc peroxide-sensing center and an adjacent linker region responding to unfolding conditions. Neither of these sensors works sufficiently in the absence of the other, making the simultaneous presence of both stress conditions a necessary requirement for full activation of the protein. Reactions of ROS with thiol groups, leading to disulfide formation and/or glutathionylation, is also an element of full activation of other stress proteins, including Hsp 25 and Hsp70 [53].

6.8. Cytoskeleton

Actin belongs to cellular proteins most prone to Cys oxidation and S-glutathionylation. Stimulation by growth factors leads to oxidation-mediated decrease in the rate of actin polymerization and changes in the ratio of soluble to polymerized actin. This results in changes of cellular architecture, membrane ruffling and intracellular trafficking of many molecules, affects cellular adhesion and cell-cell interactions. S-glutathionylated actin shows a lower affinity for tropomyosin [14,54].

6.9. Cell adhesion and spreading

ROS are involved in the signaling of anchorage-dependent cell growth (ADCG) and prevention from *anoikis*, a specific kind of apoptosis induced by lack of anchorage. Interaction of integrins with extracellular ligands (fibronectin, vitronectin or laminin) induces a transient increase in intracellular ROS level, even higher than that arising after growth factor stimulation, ascribed mainly to 5′-lipoxygenase. ROS generation in ADCG depends on the signaling by both integrins and growth factors. ROS induce activation the tyrosine kinase Src and modification of cytoskeleton proteins which affect actin polymerization and contractility, and consequently cell spreading. The adherence of leukocytes to endothelial cells induces ROS formation. The reaction involves a non-receptor tyrosine protein kinase, the focal adhesion kinase [38,55].

6.10. Shear stress

Exposure of cells to shear stress induces generation of ROS; this phenomenon is most relevant for endothelial cells. Oscillatory shear stress induces phosphorylation of ERK1/2 and JNK and expression of Nox2 and Nox4 which seem to be responsible for this phenomenon. No increased ROS production was found in knockout mice lacking the p^{47phox} subunit of Nox. Longer (>18-h) exposure to disturbed shear forces (more relevant physiologically) increases the expression of Nox1 and Nox2 while reducing the expression of Nox4 [56].

6.11. Response to noxious stimuli

Bacterial infection induces ROS generation not only by phagocytes but also by other cells. In human aortic endothelial cells, administered lipopolysaccharide (LPS) of gram-negative bacteria co-localizes with Nox4. Downregulation of Nox4 with siRNS prevents the LPS-induced ROS production, production of inflammatory markers and monocyte adhesion [57]. Interestingly, also other noxious factors like hyperoxia, hyperthermia and UV induce activation of Noxs and production of ROS, apparently for signaling purposes [52,58].

6.12. ROS as regulators of cellular pH

An increase in the $H_2O_2/O_2^{\bullet}$ ratio leads to acidification of the cytosol. The possible mechanism of this effect has been linked to inhibition of ATP-dependent pH regulators such as the Na^+/H^+ exchanger HNE. Superoxide has also been shown to stimulate HNE1 promoter activity and gene expression while apoptogenic concentrations of H_2O_2 were found to have an opposite effect on the promoter activity and expression of the exchanger. These data are interesting since the cytosol is usually more alkaline in tumor than in normal cells [59].

6.13. Immune functions

Antigen stimulaton of the B cell antigen receptor, a membrane-bound multiprotein complex containing a ligand binding immunoglobulin, induces phosphorylation and activation of downstream components. A PTK Lyn participates in this signaling pathway recruiting and activating Syk. Stimulation of the B cell induces a burst of ROS production. H_2O_2 scavengers inhibit while exogenous ROS stimulate Lyn phosphorylation. Apparently, Duox1 is critical for the Lyn phosphorylation induced by B cell receptor activation. Activation of the receptor is accompanied by a transient PTP inhibition (maximal at 30 s and disappearing within 1 min) [60].

Superoxide and low micromolar concentrations of H_2O_2 increase the production of interleukin-2 in activated T cells. Such physiologically relevant ROS concentrations are not sufficient for initiation of signaling cascades in T lymphocytes but amplify signaling induced by relatively weak receptor stimulation [61].

Macrophage-derived ROS were found to have an immune signaling role by influencing T-cell selection, maturation and differentiation. Mice and rats with decreased capacity to mount respiratory burst have paradoxically enhanced arthritis dependent on activation of autoreactive T cells. ROS, unexpectedly, have thus also an anti-inflammatory role. If so, agents activating Nox may have a therapeutic effect on arthritis [62].

6.14. Cell proliferation

It has been repeatedly suggested and demonstrated that exposure of cells to low levels of ROS induces division and proliferation, higher levels induce apoptosis and still higher necrosis (for a review see [63]). Such experiments do not match the complexity of situation in vivo where ROS signaling may involve short localized pulses of ROS nevertheless may reflect some general principles of ROS action.

It is interesting to note that several cell cycle regulatory proteins have ROS-sensitive motifs such as Cys residues and metal co-factors in their active sites. So, it has been speculated that ROS signaling may play a role in regulating G0/G1 to S to G2 and M cell cycle progression. It has been reported that the levels of intracellular protein-bound and non-protein thiols and disulfides undergo cyclic changes during different stages of the cell cycle [64].

6.15. Blood pressure

H_2O_2 can modulate vascular tone but the mode of its action is complex. It may elicit contractile or relaxant responses, depending on the type of the vessel, its contractile state, disease condition and even animal species studied. H_2O_2 may activate eNOS expression, which explains the paradoxical increase of eNOS level in atherosclerosis, hypertension and diabetes. In certain, especially small-diameter vessels, H_2O_2 can act as endothelium-derived hyperpolarizing factor (EDHF) mediating vasodilation. However, H_2O_2 may also decrease the availability of the vasodilating NO. It does not appreciably react with NO but may increase superoxide production [65].

6.16. Oxygen sensing

Carotid bodies, sensory organs detecting changes in arterial blood oxygen, contain glomus type I chemoreceptor cells that release neurotransmitters in response to hypoxia. The primary sensor which triggers this reaction is yet unknown. Numerous evidence suggests that changes in oxygen concentration may be sensed by several ROS-producing proteins including a mitochondrial component of a respiratory chain and/or a b-type cytochrome with properties similar to those of the cytochrome b_{558} in the Nox complex in neutrophils [66].

6.17. Wound healing

Impaired wound healing is typical for chronic granulomatous disease caused by Nox deficiency. H_2O_2 was found to be present in micromolar concentrations at the wound site and to support would healing by inducing VEGF expression in human keratinocytes. Low concentrations of H_2O_2 support while higher concentrations inhibit the healing process. ROS not only support angiogenesis but also stimulate collagen production. On the other hand, antioxidants inhibit monocyte and lymphocyte-induced angiogenesis [67].

7. Messengers become destroyers: diseases due to impaired ROS signaling

Signaling via ROS is dangerous as overproduction of reactive signal molecules may be destructive. Oxidative stress has been defined as ''a disturbance in the prooxidant–antioxidant balance in favor of the former'' [68]. Numerous diseases are known to involve oxidative stress, including atherosclerosis, diabetes, hypertension and cancer. In many cases oxidative stress due to overproduction of signaling ROS seems to play an important role in pathogenesis, as illustrated by several examples. Nox3 expressed in the inner ear is important for the perception of motion and gravity. Mice mutants in Nox3 or its regulatory subunit NOXO1 show defective gravity sensing (the ''head slant'' phenotype). Deafness and ototoxicity of some drugs and toxins may be due to Nox3-mediated ROS overproduction. Ototoxic cisplatin induces Nox3 in the inner ear [69].

Many vascular proinflammatory/proatherogenic states (hypertension, diabetes, hypercholesterolemia) are associated with elevated expression of Nox2 and possibly Nox1 in the vessel wall, with no change or downregulation of Nox4. ROS

produced by angiotensin II-induced Nox hyperactivation mediates vascular smooth muscle hypertrophy and hypertension. The role of Nox2 in this process is essential since administration of angiotensin II to $Nox2^{-/-}$ mice increases systolic pressure, but in contrast to wild-type mice, does not induce aortic medial thickening. Increased generation of ROS by Noxs is involved in the hypertension via augmented scavenging of nitric oxide. Expression of Nox1 increases considerably following balloon injury of the vessels.

Amyloid-β peptide was demonstrated to activate ASK-1 in neurons and Nox2 of microglia, increasing ROS generation. Both effects lead to apoptotic neuronal death. In animal models of Parkinson's disease based on administration LPS and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Nox2 knockout mice were protected against loss of nigral dopaminergic neurons. Nox2 was also found to be activated in the spinal cord of patients with amyotrophic lateral sclerosis [70].

NOXs, especially Nox2 may be involved in myocardial infarction as Nox2-deficient mice are protected from the deleterious consequences of infarction. If so, Nox inhibitors may improve cardiac function after infarction. Moreover, Nox2-deficient mice are less susceptible to doxorubicin cardiotoxicity [7].

Oxidative stress in diabetes has been well documented and attributed mainly to the generation of ROS by glycoxidation of sugars. However, derangement of ROS signaling mechanisms is also of considerable importance. Elevated levels of glucose accelerate the formation of advanced glycation end-products (AGEs) which interact with the AGE-specific receptor (RAGE); this interaction leads to generation of ROS [71]. Nox1 and Nox2 were found to play a role in the endothelial dysfunction in diabetes [72] and a dominant negative Rac1 ameliorates endothelial dysfunction in a mouse model of diabetes. Depletion of Nox4 with antisense nucleotides protects rats from diabetic nephropathy.

Nox2, apart from other sources of ROS, seem to contribute significantly to injuries due to ischemic stroke. Stroke size is reduced in Nox2 deficient mice and in gerbils given a Nox inhibitor. Ethanol-induced hepatitis seems to be mediated by Nox activation since expression of Nox4, Duox1 and Duox2 is strongly augmented and Nox inhibitors ameliorate alcoholic liver injury.

Cataract formation is attributed to UV-generated ROS but Noxs are expressed in ocular lens and activated by UV so they may contribute to cataractogenesis. Similarly, activated Nox1 may play a role in the skin damage by UV, apart from the direct physico-chemical generation of ROS by UV [7].

It has been pointed out that overexpression/overactivation of Nox/Duox enzymes which leads to pathologies takes place later in life, usually after reproduction has occurred. So, genes coding for these enzymes may play a beneficial role in the reproductive period of life (thus being subject to positive selection) but have harmful effects late in life thus fulfilling the definition of phenomenon known in genetics as ''antagonistic pleiotropy'' [70].

8. Cancer

Cancer cells were frequently noted to produce increased amounts of ROS which in most cases is due to the augmented activity of Nox enzymes (Nox1, 4 and/or 5) [70]. While altered expression of many genes is common in cancer, a causal role of Nox overexpression or activation has been postulated. Overexpression of Nox1 was found to cause transformation of a variety of cell types, including fibroblasts. In several cases, suppression of Nox expression was shown to slow down the division of malignant cells. Depletion of Nox4 in pancreatic cancer cells induces apoptosis.

ROS are downstream effectors of several oncogenes. Activation of the c-myc increases generation of ROS and DNA damage and partially disables the p53-mediated DNA damage response, enabling cells with damaged genomes to enter the cycle and accelerate tumor progression via genetic instability.

Transformation of fibroblasts with a constitutively active form of $p21^{Ras}$ has been demonstrated to increase considerably the cellular production of superoxide. Ras activates Nox to generate ROS. In turn, ROS activate Ras via an oxidative modification (including glutathionylation) of Cys118. This leads to activation of the GDP-GTP exchange, enhancement of the GDP/GTP exchange activity and conversion of Ras into the GTP-bound, active state. Activation of Ras leads to activation of AKT and p38. Since mutations and over-expression of the oncogene Ras occur in about 1/3 of all human tumors, these data suggest that increased superoxide flux may be responsible for cancerogenic action of Ras.

Transformation of hematopoietic cells by the oncogenic tyrosine kinase BCR-ABL is associated with a chronic increase in intracellular ROS level of mitochondrial origin. Deficiency of tumor suppressor PTEN results also in increased levels of ROS and decreased level of expression of CuZnSOD and Prxs in mouse embryonic fibroblasts. The Jak/STAT pathway has been implicated in a variety of solid tumors and hematologic malignancies. Activated JAk2 has again been associated with increased intracellular ROS levels [73–75].

9. Perspectives

If ROS are important players in cellular signaling and aberrant ROS signaling may cause diseases, new therapeutic approaches can be envisaged, including inhibition of cellular sources of ROS and perhaps targeting of antioxidants. Inhibitors of Noxs, more specific than those currently used as laboratory research tools could be of value whereas in some cases induction of Nox could be useful [62]. Interestingly, inhibitors of hydroxymetylglutaryl-CoA reductase (statins) inhibit vascular Noxs blocking the formation of the geranyl–geranyl moiety and thus isoprenylation of Rac1 and Rac2 at their N-termini. While the effect of dietary supplements on the cancer developments has been found generally disappointing [75], the fact that many cancer cells show elevated production of ROS, which apparently facilitate their proliferation, suggests two strategies for the cancer therapy via effects on ROS production. ROS level can be either decreased, perhaps using membrane-permeable mimetics of antioxidant enzymes, or drastically increased in cancer cells promoting their apoptosis. Understanding of ROS signaling may thus be useful also for pharmacology.

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