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Involvement of plasma membrane redox systems in hormone action

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Abstract

Reactive oxygen species (ROS) is the common name used to describe the partially reduced forms of molecular oxygen that may be generated in cells during oxidative metabolism. They are normally considered to be toxic, and cells possess various defence systems to protect themselves including antioxidant enzymes and low molecular weight antioxidants like vitamin C and vitamin E. However, it is now clear that small amounts of ROS also act as messenger molecules in cell signal transduction pathways; the plasma membrane of eukaryotic cells in particular contains a variety of different ROS-producing oxidases and reductases, of which the best characterized are the superoxide-producing NADPH oxidases. It has been known for many years that membrane redox activity can be changed rapidly by various hormones and growth factors, but the molecular mechanisms involved and the physiological importance of this phenomenon have only recently begun to be unveiled. This review summarizes the state of the art on plasma membrane-based ROS signalling in the pathways of insulin, steroid and thyroid hormones and growth factors. The apparent paradox of ROS being essential biomolecules in the regulation of cellular functions, but also toxic by-products of metabolism, may be important for the pharmacological application of natural and synthetic antioxidants.

The good and the bad of oxygen

Higher eukaryotic organisms cannot exist without oxygen, but oxygen is also dangerous to their life, a situation often referred to as the 'Oxygen Paradox'. The 'bad side' of oxygen relates to the fact that each oxygen atom has one unpaired electron in its outer valence shell, and molecular oxygen has two unpaired electrons; therefore both atomic oxygen and molecular oxygen are radicals. The bulk of the oxygen consumed during metabolism is accounted for by the respiratory electron transport chain in the mitochondria. The reduction of oxygen to produce water in the final step of this process is considered to be a relatively safe process; however, the incomplete reduction of oxygen generates reactive intermediates. The reductive intracellular environment provides a variety of possibilities for oxygen to undergo partial reduction, resulting in the formation of the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) or the very reactive hydroxyl radical ($HO^\bullet$). These compounds, all common products of the everyday life in an aerobic environment, are collectively known as reactive oxygen species (ROS) and are responsible for oxygen toxicity (Davies 1995). To survive in such an environment all aerobic organisms are endowed with a variety of water- and lipid-soluble antioxidant compounds, together with specialized antioxidant enzymes whose role is to detect and eliminate the different types of ROS. If the oxidative status in the cell exceeds the capacity of the antioxidant defence to neutralize the reactive species produced, a situation of acute oxidative stress occurs, which, if not counteracted, leads to damage to the cell structures and molecules such as lipids, proteins and nucleic acids. However, it is also possible that this type of damage develops even under normal circumstances; O_2^- or H_2O_2 are in fact produced by various enzymes under physiological conditions, and much evidence now indicates that ROS are not only noxious by-products of cellular metabolism, but also essential participants in cell signalling and regulation (Dröge 2001; Halliwell & Gutteridge 2007). Transient bursts of small amounts of O_2^- or H_2O_2 can be detected following stimulation with a variety of growth factors and hormones, and these ROS can subsequently trigger reactions elsewhere in the cell, behaving as true second messengers. This obviously requires that these species must be able to survive inside the cell, if only for a very short time and at very low concentrations, in spite of all the antioxidant mechanisms

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involved in preventing or counteracting the oxidative damage. Therefore a basal low level of intracellular oxidative stress is likely to be inevitable, and under certain conditions the damage caused may accumulate with time, even over years, until it eventually manifests as some kind of disease. It is currently believed that oxidative damage may be involved in the development of age-related pathologies such as atherosclerosis and neurodegenerative disorders (Dai et al 2006).

A very hot topic in this context is the role of natural antioxidants in the diet, which may counteract the reactive oxygen species produced by our cells. The importance of the quintessential antioxidants vitamins C and E has long been beyond discussion, but the functions of the myriad of natural polyphenol antioxidants, particularly in fruits and vegetables, have turned out to be much less clear-cut to interpret. Although many of these compounds show excellent antioxidant properties in-vitro, they also give rise to a plethora of unexpected and seemingly unrelated effects in cells and tissues, and in many cases these effects cannot be connected to an antioxidant response. One possible explanation could be that these compounds not only act against the oxidative stress induced by ROS, but also interfere with the signalling mechanisms mediated by superoxide and hydrogen peroxide, particularly the ones involving redox enzymes of the plasma membrane.

Plasma membrane redox systems

Plasma membrane redox enzymes have been found in all cell types, but in most cases their physiological function has not been well established (Crane et al 1985; Bedard & Krause 2007). In response to hormones, growth factors or other physiopathological stimuli, these enzymes transfer electrons from reducing agents in the cytoplasm (normally NADPH or NADH) to external oxidants such as molecular oxygen, ferricyanide, ascorbyl radicals or other suitable electron acceptors (Crane et al 1991). Plasma membrane redox activity is known to be important for several cell functions, including the control of cell growth, iron uptake, and defence against bacteria, and it is now becoming increasingly clear that hormone effects at the plasma membrane, but maybe also at the nuclear level, are related to that redox activity. This review deals with such systems found in the plasma membranes of animal cells, mainly the nucleophil and non-nucleophil NADPH oxidases, but including a summary of other oxidoreductase enzyme activity, for which solid and detailed information has finally become available after decades of a shadowy existence as hypothetical players.

NADPH oxidases

The NOX family of NADPH oxidases are a family of enzymes that catalyse the formation of superoxide from oxygen and NADPH, according to the reaction:

$$NADPH + 2O_2 \Rightarrow NADP^+ + H^+ + 2O_2^-$$

To date seven members of the NADPH oxidase family have been identified, five named NOX1 to NOX5 and two somewhat different enzymes called DUOX1 and DUOX2. They are all transmembrane redox complexes that connect the electron donor NADPH on the cytoplasmic side of the membrane with the electron acceptor oxygen on the outer side. The most famous and best characterized member of the family is NOX2 (previously called $gp91^{phox}$), which produces large amounts of superoxide in phagocytic cells as an efficient cell defence system against microorganisms. Four homologues of the cytochrome *b* component $gp91^{phox}$ of the phagocyte NADPH oxidase, called NOX1 and NOX3–NOX5, have been found in different tissues (for a very recent and comprehensive review on the NOX family see Bedard & Krause (2007)). These enzymes produce superoxide in much smaller amounts, to be used as a signal system. The ROS generated by NADPH oxidases also include H_2O_2 formed by dismutation of superoxide, either spontaneously or through the intervention of a superoxide dismutase (Fridovich 1975):

$$2O_2^- + 2H^+ \Rightarrow O_2 + H_2O_2$$

The structure of a NADPH oxidase is quite complex and no crystal structures have been described so far. In phagocytes the resting enzyme exists as a dimer of the two intrinsic membrane proteins NOX2 and $p22^{phox}$. Activation initiates by phosphorylation of a cytosolic factor, $p47^{phox}$, which after binding to two other cytosolic proteins, $p67^{phox}$ and $p40^{phox}$, migrates to the cell membrane and binds to the NOX2–$p22^{phox}$ dimer. Final activation requires binding of the GTPase Rac to NOX2/ $p67^{phox}$ in a GTP-dependent process (Babior 2004; Opitz et al 2007). The phosphorylation of $p47^{phox}$ probably involves protein kinase C (PKC) (Iaccio et al 2007), whereas the effect of Rac may depend on the release of a guanine nucleotide dissociation inhibitor, that prevents the exchange of guanine nucleotides from Rac (Schalk et al 1996). Also NOX1 and NOX3 are believed to be activated through the assembly of the complete enzyme at the membrane, but the cofactors involved and the details of the process vary. Rac is required for activation of NOX1 and possibly also NOX3, whereas NOX4 only requires $p22^{phox}$ but no other cytosolic factors. The most recently discovered isoform NOX5 apparently does not depend on other proteins for activity, but seems to be activated through binding of Ca^{2+} with a classic EF-hand mechanism (Bedard & Krause 2007). (The EF hand is a calcium-binding helix-turn-helix structural motif in proteins, consisting of two alpha helices approximately perpendicular to one another and linked by a short loop.) PKC-mediated phosphorylation of NOX5 increases the affinity for Ca^{2+} and greatly enhances its activity (Jagnandan et al 2007).

To a large extent our current understanding of NADPH oxidases is modelled on the behaviour of the NOX2 enzyme, but it is clear from what is written above that the mechanisms controlling the activity may vary considerably among the different isoforms. Activation of NADPH oxidases depends on a complex pattern of highly specialized redox-dependent signalling. It is likely that much of this complex pattern of activation depends on specific interaction of the NOXs with various regulatory proteins, which associate to give rise to the active enzyme. Some of these regulatory subunits have been identified as 'Activators' or 'Organizers' (Opitz et al 2007). Activators are proteins such as $p67^{phox}$, an essential component of NOX2, and NOXA1, an essential component of NOX 1 and perhaps also of NOX3; these two proteins share approximately 28% amino acid identity but show different

tissue distribution (Bedard & Krause 2007). After binding to Rac the activator subunit migrates to the plasma membrane (with the involvement of an Organizer subunit) and docks the NOX protein, this leads to a conformational change and production of ROS. The two Organizer proteins known so far are $p47^{phox}$ that binds to NOX2, and NOXO1 that binds to NOX1 and NOX3. These two Organizer subunits share approximately 25% sequence identity; they have different tissue distribution and are involved in targeting both an Activator to NOX, and NOX to different subcellular compartments (Bedard & Krause 2007). NOX2 was first identified in neutrophils and macrophages, but has later been found in several nonphagocytic tissues. It is possible that the activator protein NOXA1 may substitute the essential regulatory function of $p67^{phox}$ in nonphagocytic cells or that the NADPH oxidase in nonphagocytic cells does not require such Activator and Organizer proteins, and therefore the whole modulation of this enzyme may be different from the phagocytic counterpart (Opitz et al 2007).

In addition to the five NOX isoforms, the NADPH oxidase family also comprises DUOX1 and DUOX2, that apart from the superoxide-producing NOX core region also contain a large ectofacing peroxidase-like domain, which however does not appear to have peroxidase activity (Bedard & Krause 2007). DUOX1 and DUOX2 are found abundantly in the thyroid, where they are believed to supply the H_2O_2 required for the activity of thyroid peroxidase, but they are also widely expressed on tissue surfaces exposed to infection, in particular in air epithelia and throughout the gastrointestinal tract. It has been suggested that their role here is to provide the H_2O_2 necessary for the antimicrobial activity of lactoperoxidase, which generates antimicrobial agents such as hypothiocyanate rather than hypochlorite; hypothiocyanate is considered to be less toxic for the host tissue (Slungaard & Mahoney 1991; Geiszt et al 2003). As in the case of NOX5, the two DUOX enzymes appear to be regulated by Ca^{2+} but otherwise do not depend on the presence of cytosolic factors (Bedard & Krause 2007).

NADPH oxidase, growth factors and hormones

Growth factors

A role for cell membrane redox systems in growth control and development was suggested in a seminal paper by Crane et al (1985) and has been confirmed many times later (Crane et al 1991; Bedard & Krause 2007). The group of Crane gathered a pool of interesting observations from the beginning of the '80s showing that external ferricyanide could stimulate the growth of melanoma cells (Ellem & Kay 1983). Analogously, other oxidants were shown to be able to stimulate growth in fibroblasts and sea urchin eggs. These observations, combined with the fact that some anticancer drugs can inhibit the plasma membrane redox systems, indicated that the redox system might be an important modulator of cell growth, apparently at variance with the fact that ROS are also considered at least partly responsible for senescence (Harman 2003). The mechanism by which ferricyanide could control growth was at that time still to be established, but was related to several mechanisms proposed for growth control: increase of intracellular pH, mobilization of Ca^{2+} ions, turnover of phosphatidylinositol, and changes in the ratio of cyclic nucleotides (Crane et al 1985). The hormones found to affect the redox systems and cell growth were mainly triiodothyronine, insulin and growth factors. What is the state of the art of this very stimulating topic 20 years later?

The production of ROS by NADPH oxidase isoforms in nonphagocytic cells plays a role in signal transduction, and in many cases Rac1 is involved in the activation of NADPH oxidase activity (Bokoch & Knaus 2003). Several growth factors have been reported to give rise to ROS production in nonphagocytic cells after binding to membrane receptors; this ROS production can mediate a positive effect on signal transduction from receptors to intracellular signalling and the physiological response. A role for ROS has been shown for nerve growth factor (NGF) in nerve cells (Suzukawa et al 2000), for epidermal growth factor (EGF) in epidermoid carcinoma cells (Bae et al 1997), for platelet-derived growth factor (PDGF) and also vascular endothelial growth factor (VEGF) in endothelial cells (Bae et al 2000; Ushio-Fukai 2006). All these growth factors give rise to ROS production through the Rac1 protein.

NGF is a growth factor essential for the maturation and growth of neurons (Levi-Montalcini 1987). It has been reported that NGF, through the TrkA receptor, gives rise to ROS production by activation of Rac1/NADPH oxidase pathway; the increase in ROS, in turn, is a prerequisite for the activation of the MAPK pathway, essential for the mediation of the NGF-induced neuronal differentiation and also pain perception in the sensory neurons. Pain perception is regulated by TRPV1 expression in sensory neurons and the authors speculate that the modulation of the expression of these TRPV1 receptors through ROS and activation of the p38 MAPK pathway is a unifying model to control both differentiation and inflammation combined with pain perception (Puntambekar et al 2005). Very recently ROS have been shown to function also as signalling molecules in angiogenesis. VEGF is a key angiogenic factor and stimulates proliferation and migration of endothelial cells through VEGF-mediated receptor type 2. The first event after the binding of ligand is autophosphorylation of VEGFR2, resulting in activation of downstream signalling ERK1/2, Akt, and eNOS (NOS3), leading to angiogenesis stimulation. The ROS derive from NADPH oxidase and the activation process involves Rac1 (Ushio-Fukai 2006).

To summarize, there are now various examples of growth factors that give rise to ROS production in nonphagocytic cells, through activation of their corresponding membrane receptors. This ROS production can mediate a positive feedback effect on signal transduction, since ROS production in turn enhances the intracellular signalling. The molecular details of these oxidative activation mechanisms are still far from being elucidated (Dröge 2001).

Insulin: signal mechanisms

Insulin is at present the hormone that appears to be most connected to ROS, and both its release and its physiological responses have been related to ROS production. Insulin action is initiated by binding to the plasma membrane receptor endowed with tyrosine kinase activity, essential for insulin's growth promoting activity and its metabolic effects. The targets of the tyrosine kinase activity are the different insulin

receptor substrates, IRS-1 and IRS-2. The phosphorylated proteins serve as docking scaffolds for binding and activation of a variety of signals linked to the activation of several insulin responses, including glucose transport, DNA synthesis and gene expression. Insulin is a pleiotropic hormone, since it gives rise to a variety of responses that are extremely differentiated in time onset and quality (Saltiel & Pessin 2002, 2003).

In the classical target cells, e.g. liver, skeletal muscle and adipose tissue, insulin controls many important physiological functions, including glucose transport into the cell, intracellular glucose metabolism, lipid metabolism, and protein synthesis at transcriptional and translational levels. Insulin is a special hormone in many other respects. From the discovery of cAMP and up to now, practically all second messengers have at some point been considered potential second messengers of insulin action. This was reflected in the Black Box concept shown in a very famous cartoon in *Trends in Biochemical Sciences* (Figure 1; Kahn (1979)); despite the wealth of discoveries and data that have been poured into that box ever since, we still do not have a much improved picture when it comes to ROS production. Insulin was the first hormone for which a role of the redox state and H_2O_2 as second messenger was reported (Czech et al 1974; Hayes & Lockwood 1987); the authors concluded that "These data represent substantial evidence for the concept that oxidation of fat key cell sulphydryl in response to insulin-receptor interaction plays a role in mediating the activation of glucose transport" (Czech et al 1974). The insulin-like effects of H_2O_2, pervanadate and thiol reactive agents were found to involve insulin-independent tyrosine phosphorylation of the insulin receptor β chain. The effect of hydrogen peroxide in the millimolar range may be explained, at least in part, by inhibition of tyrosine phosphatases. Lower concentrations of H_2O_2 are not able to trigger autophosphorylation of the insulin receptor in the absence of insulin, but potentiate the response to 100 nM insulin, suggesting that the redox signal may contribute to insulin receptor activation under physiological conditions. Hydrogen peroxide production can also be induced by insulin (May & de Häen 1979; Krieger-Brauer et al 1997), therefore the redox effect appears to be part of a positive feedback mechanism, in analogy to what has been found for growth factors (Dröge 2001).

The above quoted sentence from the paper by Czech et al (1974) refers to the redox modulation of one of the most studied and recognized responses of insulin: the activation of glucose transport. Thirty years later this response is still one of the most known and studied, also with respect to its ROS modulation (Figure 2). The major physiological activators of glucose transport are insulin, exercise/contraction, and hypoxia, and in all three cases a role for ROS has been suggested, even though the mechanisms are still poorly understood, and in particular for physical exercise the role of ROS is widely debated (Katz 2007). In skeletal muscle and adipocytes glucose uptake is mediated by the insulin-sensitive glucose transporter 4 (GLUT4). The identification of NOX4 as a component in the insulin signal transduction pathway suggests that this NADPH oxidase may be the source of the insulin signalling-generated ROS (Mahadev et al 2004), though it has recently been reported that insulin-induced ROS production by NOX3 mediates the expression of VEGF in

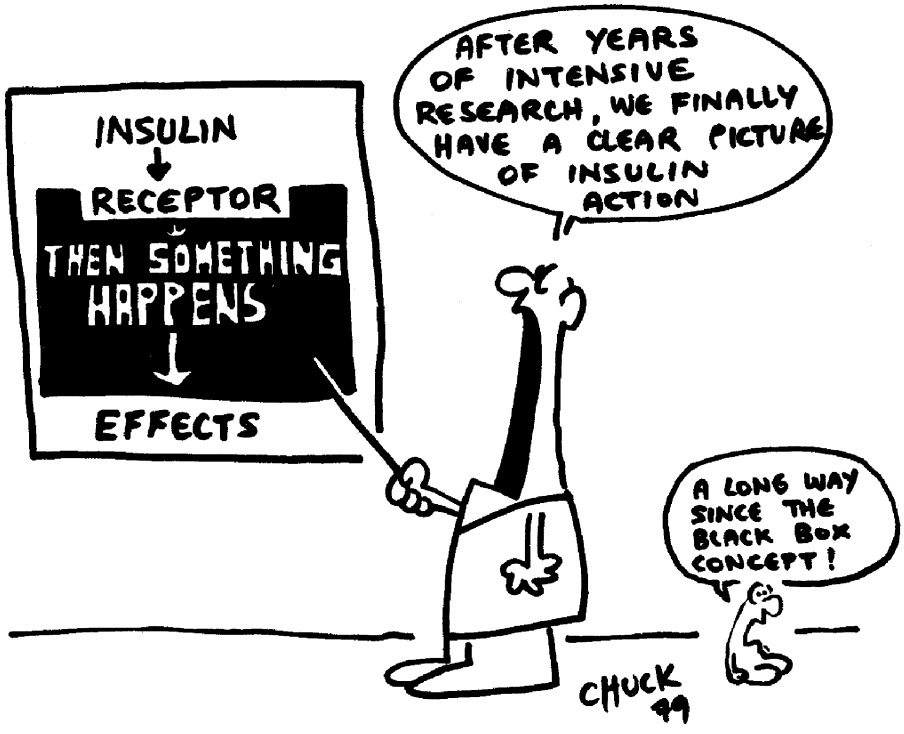

Figure 1 The classic cartoon by Chuck showing the insulin transduction mechanism as a 'black box'. Reprinted from *Trends in Biochemical Sciences*, Vol. 4, C. R. Kahn, What is the molecular basis for the action of insulin?, pp N263–N266, Copyright (1979), with permission from Elsevier.

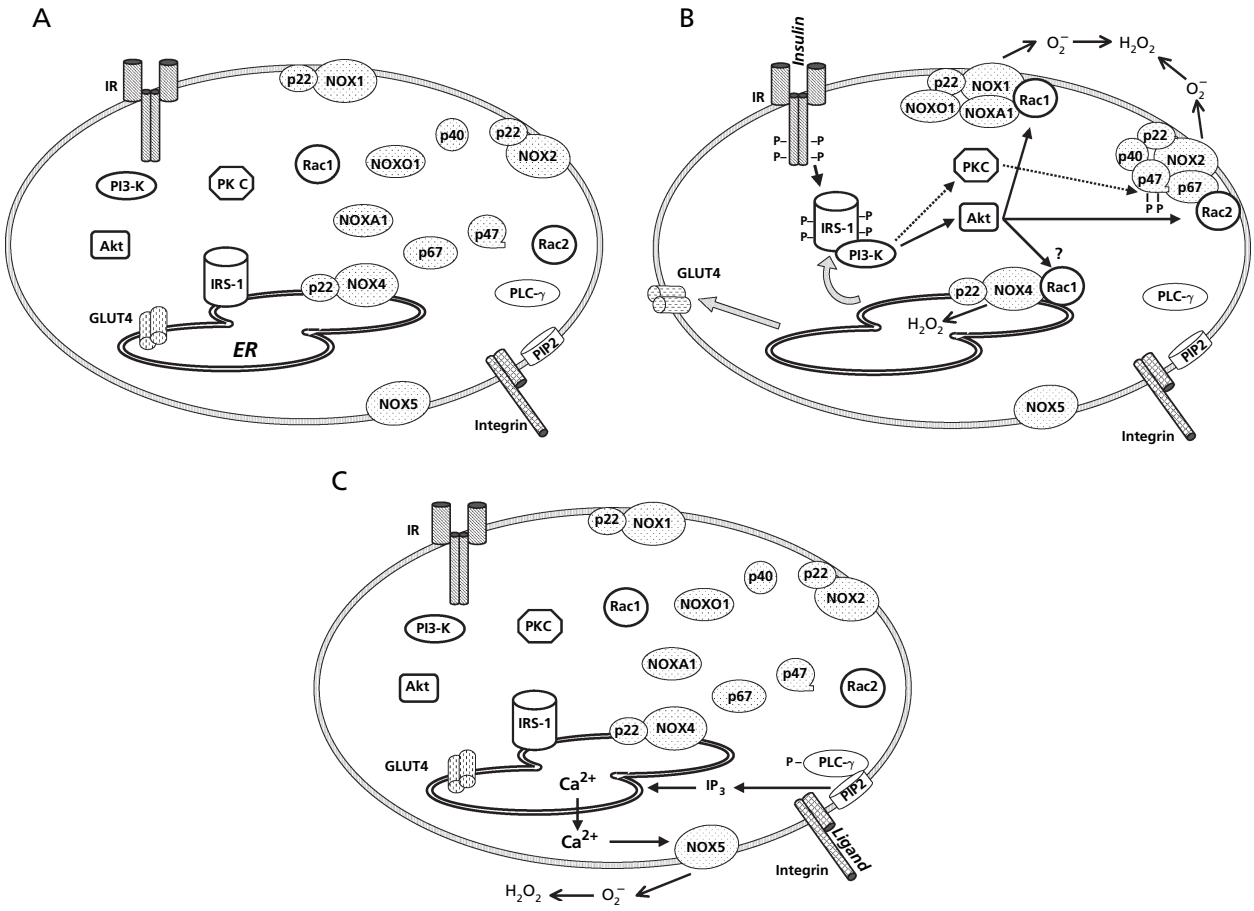

Figure 2 Activation of the different NADPH oxidases giving rise to production of reactive oxygen species in muscle cells after stimulation with insulin or binding of an integrin ligand. NOX1, NOX2, NOX4 and NOX5 are all present in smooth muscle, while only NOX4 and NOX2 have been found in skeletal muscle. Panel A: Resting state before hormone/ligand binding. Most of the protein factors involved are dispersed in the cytosol, but the GLUT4 transporter and the insulin receptor (IR) substrate IRS-1 are bound to internal membranes. Panel B: Insulin-induced activation of IR tyrosine kinase activity leads to IRS-1 migration and phosphorylation, and to phosphatidyl inositol 3-kinase (PI3-K)/Akt-mediated activation of Rac1 and Rac2, which migrate to NOX1 and NOX2 at the plasma membrane, together with other cytosolic components of the NADPH oxidases; in addition NOX2 also requires phosphorylation by PKC. Activation of the NADPH oxidases gives rise to production of O_2^- and subsequently H_2O_2; both may act as second messengers activating glucose transport, but also cell growth and differentiation and gene expression in the long term. Activation of the intracellular NOX4 in the endoplasmic reticulum does not depend on cytosol proteins, with the possible exception of Rac1; the concerted action of several of the enzymes shown results in translocation of GLUT4 to the plasma membrane. For the sake of simplicity the nucleus is not shown, only events taking place at the plasma membrane and described in the text are included. Panel C: Interaction of a ligand with an integrin leads to activation of phospholipase c (PLC)-γ, hydrolysis of phosphatidylinositol bisphosphate (PIP_2) and production of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (not shown), mobilization of intracellular calcium and activation of NOX5 and calcium-dependent physiological responses. Integrin signalling may also activate PKC and Rac1, thus probably stimulating the other NADPH oxidases through an alternative mechanism; direct PKC-induced phosphorylation of NOX5 has not yet been confirmed for smooth muscle cells.

HepG2 cells (Carnesecchi et al 2006). Insulin signalling is a complex process involving multiple pathways and phosphorylation events. The activation system switched on by the phosphorylation of tyrosine kinase is switched off by protein tyrosine phosphatases (PTPs). The pathway currently suggested involves inhibition of the protein tyrosine phosphatases by ROS, via progressive oxidation of their catalytic cysteine thiol moieties, resulting in prolonged activation of insulin signalling (Chiarugi & Cirri 2003; Goldstein et al 2005). This pathway may represent an interesting occasion for therapeutic application in the case of insulin-resistance state diseases. Activation of phosphoinositide 3-kinase (PI3-K) and protein kinase B (Akt) is a key step in skeletal muscle, leading to translocation of GLUT4 from intracellular compartments to the plasma membrane (Stokoe et al 1997; Stephens et al 1998), whereas in adipocytes the relative roles of PI3-K and Akt are debated (Goldstein et al 2005). At the same time insulin also activates Rac1, which is required for GLUT4 translocation, although it is not clear whether Rac1 is an effector of NOX4 in insulin-sensitive cells, and whether it is dependent on Akt activity (Goldstein et al 2005; JeBailey et al 2007). Little is known about specific insulin effects on NOX2, but it seems likely that the activation of PI3-K also may stimulate the PKC-mediated activation of $p47^{phox}$.

Angiotensin II (Ang II) is an important physiological modulator of blood pressure, cardiac function, salt and fluid homeostasis. These effects on blood pressure and growth are mediated by the Ang II receptor 1 (AT1R). Ang II antagonizes insulin effects and thus contributes to insulin resistance, therefore treatment of hypertensive patients with angiotensin converting enzyme inhibitors results in a smaller incidence of Type 2 diabetes. Ang II is known to impair insulin signalling pathways in vascular smooth muscle cells (Rask-Madsen & King 2007). In particular, Ang II inhibits insulin-induced tyrosine phosphorylation of IRS-1, activation of PI3-K and Akt, and GLUT4 translocation from the cytosol to the plasma membrane; but at the same time Ang II was found to stimulate NOX activity, giving rise to ROS formation in L-6 myotubes. This effect was inhibited by ACE inhibitors, and inhibitors of NADPH oxidase (Wei et al 2006). NADPH oxidase is an important source of glucose-induced ROS production in the vascular and kidney cells in the course of diabetes, in agreement with a role of NOXs as mediators of diabetic complications (Li & Shah 2003). It is possible that also in this case Ang II plays a role; in fact high glucose-induced formation of ROS and $p47^{phox}$ phosphorylation can be blocked by Ang II-type receptor antagonists (Wei et al 2006). The NADPH oxidase-mediated production of ROS observed in the vasculature and kidney in diabetes can be suppressed by PKC inhibitors (Valko et al 2007), suggesting the involvement of NOX2 or NOX5.

The signalling mechanisms can be summarized as follows: insulin signalling is initiated by the phosphorylation of tyrosyl residues of the insulin receptor and IRS proteins. Downstream signalling involves the activation of PI3-K, Akt and Rac1, with subsequent stimulation of NOX1 assembly and ROS production; at the same time stimulation of NOX4 with production of H_2O_2 leads to GLUT4 translocation and glucose uptake. ROS production in turn leads to inhibition of PTPs and as a consequence potentiation of tyrosine kinase phosphorylation. Ang II on the other hand inhibits IRS-1 tyrosine phosphorylation, activation of PI3-K and GLUT4 translocation in skeletal muscle cells, but also activates NOX activity. It is difficult to reconcile these contradictory results; both insulin and Ang II stimulate NADPH oxidase activation and ROS formation, but they give rise to opposite responses. At present we have no explanation for this apparent paradox, once more the cellular context appears to be the main determinant of the physiological responses. Interestingly, it was recently reported that insulin and Ang II synergistically stimulated NADPH oxidase activity in vascular smooth muscle cells through an increase in the $NADH/NAD^+$ redox potential; this effect apparently did not involve PI3-K or G_i-protein-dependent pathways (Yang & Kahn 2006), confirming the complexity of these cross-talk mechanisms.

Insulin: from physiology to pathology

The involvement of ROS in insulin action is in any case more complex than the picture outlined in the previous section of this paper. For instance it has been reported that a reduced NOX4 mRNA content is a hallmark in adipocyte differentiation (Mouche et al 2007), whereas increased whole adipose tissue NOX4 expression has been linked to oxidative stress and insulin resistance (Furukawa et al 2004). Endothelial dysfunction, which is just one of the several changes induced in the arterial wall by metabolic impairments connected with diabetes and insulin resistance, involves production of endothelium-derived vasodilators, with activation of eNOS and production of NO, and factors such as endothelin 1, prostacyclin and vasoconstrictor prostanoids, and these processes can also be stimulated through NOX activity. Such complex mechanisms involving several pathways not only at the plasma membrane level are clearly beyond the scope of this review; for that subject we refer the reader to a review by Rask-Madsen & King (2007).

Oxidative stress plays a role in the pathogenesis of β-cell dysfunction and death, and the expression of different isoforms of NOX is changed in tumour pancreatic β-cells compared with normal β-cells (Uchizono et al 2006). Much evidence indicates that oxidative stress is increased in the course of diabetes, and that hyperglycaemia alone can directly increase ROS production. Glucose undergoes autooxidation to generate •OH radicals (Schultz Johansen et al 2005), and glucose can react with proteins to give rise to Amadori products, followed by the formation of advanced glycation end products. ROS are generated at multiple steps during this process. Increased plasma concentration of free fatty acids leads to intracellular lipid accumulation, which has been suggested to play a critical role in initiating insulin resistance and pancreatic β-cell death (Newsholme et al 2007). It seems plausible that activation of NOXs could contribute to oxidative stress in these insulin-related pathologies (Wei et al 2007), but little solid information is available so far. Inspiration for therapeutic intervention comes from Guo et al (2007), where it was shown that the antioxidant N-acetyl-L-cysteine (NAC) downregulated NADPH oxidases, antioxidant enzymes and inflammatory markers in the hearts of diabetic rats. The authors demonstrated that oxidative stress and antioxidant defence systems were upregulated by hyperglycaemia in diabetic rat hearts. This supports the concept that oxidative stress contributes to the pathogenesis of the complications of diabetes. The use of antioxidants may represent a promising therapy to counteract these complications and pathologies where a role for ROS has been reported (Uchizono et al 2006), even though the real benefit of antioxidants in the diet is still widely debated (Halliwell 2007).

The nuclear receptor hormone family

The nuclear receptor hormone family is composed of steroid and thyroid hormones. Both groups act through a cytoplasmic/nuclear receptor, giving rise to modulation of gene transcription and protein synthesis. Recent studies have shown multiple evidence for both steroid/thyroid hormone receptor-dependent and -independent rapid nongenomic effects giving rise to signal transduction pathways (Incerpi et al 2002; D'Arezzo et al 2004; Bergh et al 2005; Davis et al 2005; Wehling & Losel 2006). Since this review deals with plasma membrane redox systems we shall focus on these rapid effects; this of course does not exclude that ROS production at the plasma membrane may affect gene transcription, as reported by Sauer et al (2001).

Steroid hormones and particularly estrogens have been associated with the production of ROS, but normally with the production of ROS at the mitochondrial level and are therefore

not inhibited by the classical inhibitors of the plasma membrane NADPH oxidase, diphenylene iodonium or apocynin. Mitochondrial ROS production is inhibited instead by the respiratory chain inhibitor rotenone or the xanthine oxidase inhibitor allopurinol, as shown in vascular endothelial cells (Felty 2006). Felty et al (2005) dealt with 17β-estradiol-induced ROS production in different human breast cancer cell lines, and an interesting result was that physiological concentrations of estrogens stimulated rapid production of ROS formation via a process that depended on the cytoskeleton and integrins. These effects were nongenomic and were seen also in cells devoid of the nuclear receptor (Felty et al 2005). Many types of cytokines act through integrins (Barillari et al 2001), and it has been shown that ligand binding to integrins at the fibroblast plasma membrane led to reorganization of the actin cytoskeleton, activation of Rac1, and production of ROS in fibroblasts (Kheradmand et al 1998).

For a long time it has been known that thyroid hormones play a role in the modulation of plasma membrane redox systems (Crane et al 1985). Triiodothyronine inhibits NADH indophenol reductase and cytochrome *c* reductase activity of isolated rat or mouse liver plasma membranes. At variance with these data, the NADH oxidase activity of rat liver plasma membrane is stimulated by triiodothyronine and might be the basis for growth stimulation by triiodothyronine, which takes place at both physiological and supraphysiological hormone concentrations. The observation that decreasing environmental oxygen below the normal 20% increases the rate of cell growth dates back to the 1960s and is a well-known phenomenon in nature (Hollenberg 1971; Sauer et al 2001; Bedard & Krause 2007). This is in line with the fact that a redox function of the plasma membrane is connected with amino acid transport, and thyroid hormone has been reported to modulate amino acid transport both at genomic and nongenomic levels (McGivan 1996; Incerpi et al 2002). Stimulation of oxygen uptake by triiodothyronine in the presence of amino acids such as alanine and α-aminoisobutyric acid may be based in part on the stimulation of the plasma membrane NADH oxidase (Müller & Seitz 1983). From these results it appears that thyroid hormone effects might indeed be related to a redox system, but the situation today is no clearer than it was 50 years ago.

Other membrane redox systems

Various other types of plasma membrane oxidoreductase activity have been reported, but for most of these it is only in the last few years that the corresponding enzymes have been characterized to a certain extent. Classic membrane enzymes such as cyclooxygenase (prostaglandin H synthase) and lipoxygenases are not normally counted among the redox enzymes involved in ROS metabolism and will not be considered further here.

A diaphorase is the common name for an enzyme that carries out two-electron reduction of quinones and similar substrates, normally using NADH as an electron donor. Diaphorases are mainly cytosolic enzymes, but a plasma membrane DT-diaphorase has been found in a neuroblastoma cell line. It has been shown to modulate cell growth and differentiation; the enzyme activity varies according to the stages of the cell cycle (Zurbriggen & Dreyer 1994, 1996). One role of this enzyme could be to maintain plasma membrane coenzyme Q in its reduced antioxidant state (ubiquinole). This membrane quinone compound is mainly known for being a component of the respiratory electron transport chain in the inner mitochondrial membrane, but it is also found in the plasma membranes of animal cells and its role there is far less characterized (Villalba et al 1995). It has been reported that the cell plasma membrane may account for approximately 30% of the total cellular diaphorase activity, but it is not known whether a single enzyme is responsible for the bulk of this activity.

One possible source of H_2O_2 at the cell surface is the membrane amine oxidase. It was recently discovered that this enzyme is identical to the vascular adhesion protein-1 (VAP-1); it belongs to the group of copper amine oxidases and is believed to have a reaction mechanism similar to that of the soluble copper amine oxidases (Boomsma et al 2005). These enzymes produce H_2O_2 directly without releasing a superoxide intermediate, and may have a wide range of potential substrates, including polyamines. The physiological role of peroxide production by VAP-1 is not known; both insulin and thyroid hormones have been suggested to regulate enzyme activity and expression but little convincing evidence is available so far (Boomsma et al 2005). However, in contrast to other cell membrane redox systems it should be relatively easy to study the role of VAP-1 because a large number of mechanism-specific inhibitors have already been developed for copper amine oxidases (Padiglia et al 1999).

A special case is represented by the thyroid peroxidase, which is directly involved in the biosynthesis of thyroid hormones (Ruf & Carayon 2006). This enzyme is located on the apical plasma membrane in the functional unit of the thyroid: the follicular cell. The haeme-containing thyroid peroxidase uses H_2O_2, probably obtained from DUOX1 and DUOX2 as mentioned above, to oxidize iodide and produce iodinated tyrosine and thyronines. Clinical problems involving defective peroxidase function are among the most frequent hereditary defects of thyroid hormone formation. Thyroid peroxidase is typically elevated in thyroid tissue from hyperthyroid patients, and frequently diminished in patients with Hashimoto's thyroiditis (Ruf & Carayon 2006).

There is much evidence that plasma membrane oxidoreductase activity may lead to activation of the Na/H antiport or exchanger, a ubiquitous plasma membrane protein exchanging Na^+ and H^+ ions according to the concentration gradient and the main modulator of intracellular pH (Putney et al 2002). This is not surprising since a transmembrane one- or two-electron transfer is different from a simple hydride transfer and actually corresponds to the generation of a proton gradient, in analogy with the process that occurs in the mitochondrial electron transport chain. It has been suggested that the transplasma membrane NADH oxidoreductases could affect intracellular pH through the Na/H exchanger activity, leading to cell proliferation via a pathway involving kinases (Rufini et al 1999). The Na/H exchanger is in fact activated by kinases and calcium ions (D'Arezzo et al 2004), and all hormones and growth factors acting through kinases could, at least in principle, change the Na/H exchanger activity. Intriguingly, the extracellular ferricyanide reduction by

membrane oxidoreductases is known to be inhibited by amiloride, a specific inhibitor of the Na/H antiport, although it should be remembered that amiloride also inhibits copper amine oxidases like VAP-1 (Padiglia et al 1999). Various redox enzymes have been suggested to participate in this type of membrane redox processes, perhaps the most surprising being the mitochondrial porin isoform 1 (VDAC1). This voltage-dependent anion channel is the major protein in the outer mitochondrial membrane, but it is also found in the plasma membrane where it has a second career acting as a transmembrane NADH-ferricyanide reductase (Baker et al 2004). Other NADH oxidases, the so-called ECTO-NOX proteins, are located on the external surface of the cell membrane, but may receive electrons from a NADH-quinone reductase at the cytosolic side of the membrane, with coenzyme Q mediating the transport across the membrane: a complete plasma membrane electron transport chain (Morré & Morré 2004; Scarlett et al 2005). There has been much progress in the characterization of these various redox enzymes in the last few years; however, their physiological importance is still uncertain, and the possible regulatory roles of hormones and other factors are still far from being elucidated.

Conclusions

It is now convincingly documented that reactive oxygen species have important physiological functions as participants in signalling transduction pathways and regulators of cell mechanisms, but at the same time the ROS inevitably generated as a by-product of an oxygen-based metabolism are highly toxic. Cells have to live with this paradox; they have to eliminate dangerous levels of ROS, without interfering with the subtle systems by which a variety of hormones and other bioactive factors trigger transient bursts of small amounts of ROS. Evidently the cellular antioxidant defence is capable of maintaining that delicate equilibrium and removing excessive amounts of ROS without removing too much. However, the existence of this complex and finely tuned mechanism may also explain why treatments with natural or synthetic antioxidants often produce unpredictable effects in-vitro and in-vivo (Dröge 2001; Valko et al 2007). To develop efficient antioxidant therapy for the future it is necessary to appreciate both the good and the bad side of reactive oxygen species.

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