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Commentary
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Redox regulation: A new challenge for pharmacology

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Abstract

Redox signaling is evolving as a new field of biochemical and pharmacological research. Unlike oxidative stress which is characterized by a macroscopic shift in cellular redox potentials and usually accompanied by oxygen radical induced damage, redox regulation involves subtle and more chemically defined oxidations of short duration. Most important is the reductive component as a necessary part of a reversible regulatory process. Examples of redox regulation occur during early stages of the immune response, in hypoxia or in endothelial dysfunction. Persistent oxidative events together with a decline in the cellular reduction potential lead to oxidative stress as is seen in the pathophysiology of sepsis, reperfusion damage, atherosclerosis and diabetes. Oxidative signals involve superoxide and nitric oxide as the main players which form a system of oxidizing, nitrating or nitrosating species leading to posttranslational modifications of proteins. Modern techniques of immunohistochemistry and mass spectrometry allow a correlation of protein modification, e.g., disulfide, *S*-oxide, *S*-nitroso or nitrotyrosine formation, with enzyme activities and cellular responses. In this commentary, examples of the control of prostanoid synthesis by the $^{\bullet}NO/^{\bullet}O_2^-$ system are described. Redox regulation represents an interesting challenge for the development of drugs that modulate the oxidative trigger mechanisms or enforce the reductive pathways.

Keywords: Redox regulation; Nitric oxide; Superoxide; Peroxynitrite; Oxidative stress; Vascular inflammation

1. Introduction: aiming at redox biochemistry as a therapeutic basis

For most of the major complex diseases current efforts for therapeutic interventions focus on the cellular signaling network. A mechanism-based rational approach has become the method of choice for drug discovery, as large libraries of compounds can be evaluated via high-throughput-screening approaches provided that the biochemical target has been identified and methodologies are available to measure its activity. Many successful examples have emerged using this strategy but also many failures probably due to the complex nature of cellular signaling processes. One example, pertinent to this review, is the development of thromboxane A_2 (TxA_2) synthase inhibitors. The compounds discovered in this area failed to produce the expected inhibition of platelet aggregation, as in the presence of such compounds, the unmetabolized prostaglandin (PG)-endoperoxide precursor (PGH_2) had the same agonistic action as TxA_2 at the corresponding TP-receptor [1]. This unexpected finding, which initially appeared to lack any physiological significance, has led to an interesting discovery in vessel tone regulation based on intra- and intercellular redox signaling [2,3].

Redox chemistry is fundamental to life since only electron or hydrogen transfer is associated with changes in free energy sufficient to drive the vast demand for ATP in higher living organisms. It therefore was not a surprise to find redox reactions also involved in the regulation of oxygen supply and in the control of mitochondrial and glycolytic pathways. Current perspectives favor evidence for the existence of a redox-based network of regulatory mechanisms that are intimately linked to cellular function and, in diseased states, to malfunction of these mechanisms. The concept of disturbed redox equilibrium being pathophysiologic is not new having been described as the phenomenon of ''oxidative stress'' [4].

This term describes situations in which the physiological redox state of the main cellular redox systems, e.g., glutathione, ascorbate, vitamin E, lipoic acid, NADPH or NADH undergo shifts to the oxidized state. Cells do not tolerate such changes over longer time periods resulting in pathophysiological events such as lipid peroxidation, DNA strand breaks or protein oxidation. The fact that such parameters become measurable indicates persistent, and

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mostly irreversible damage due to oxidative mechanisms. Consequently cells either have the capacity to repair or to die, the latter being apoptotic or necrotic in nature. In contrast, ''redox regulation'' or ''redox signaling'' describes a reversible phase of physiological regulatory reactions occurring over shorter time periods. In such circumstances, the oxidative reactions leading to posttranslational protein modification (glutathiolation, *S*-nitrosation, methionine sulfoxidation, zinc finger oxidations with disulfide formation) or to changes in the oxidation state of metals (prostaglandin endoperoxide synthase [5], calcineurin [6], guanylyl cyclase [7]) are returned to the resting state by reductive pathways. The requirement for reduction and the implication that the oxidative event has regulatory consequences delineates redox regulation from ''oxidative'' stress, where the latter is not reversible and where the term ''stress'' indicates a deviation from the normal physiological state. For the purpose of this commentary, the term ''oxidative stress'' will be reserved for a persistent oxidative shift that characterizes a pathophysiological state. Diabetes, atherosclerosis, hypertension, ischemia/reperfusion and neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS) or Parkinson's disease all have a strong component of oxidative stress. It is unclear however, whether the oxidative stress is causal in disease progression or the result of the cell death associated with cells dying by necrosis.

Analysis of the biochemical events prior to oxidative stress may answer this question. If the disease can be linked to a prolonged disturbance of redox regulation then oxidative stress can be determined as causal. In such instances pharmacological interventions would be optimal when the corresponding redox events are still reversible. This, however, requires detailed knowledge on the biochemical mechanisms involved. This commentary describes emerging insights into key events in redox signaling and describes a rationale to modulate these events from a drug discovery perspective.

2. The basics: nitric oxide and superoxide

Until the discovery of nitric oxide ($^{\bullet}NO$) as an intra- and intercellular messenger [8,9], knowledge regarding cellular redox chemistry was confined to the electron transport chain, the formation of reactive oxygen species (ROS) and their interaction with cellular macromolecules and antioxidant systems [4]. This is sufficient to explain the toxicity of extracellular noxes, like irradiation, carbon tetrachloride intoxication, redox cycling by quinoid compounds, smoking damage, peroxide poisoning or excessive exposure to transition metals. Cell death under these conditions is mainly necrotic in nature but under less severe conditions apoptosis was also evident. This picture changed with the discovery of oxidative stress-elicited by intracorporal events, including inflammation, ischemia/reperfusion or phagocytosis. Under such conditions of ''mild'' oxidative stress cell death mostly occurs as an apoptotic event. The ROS accompanying both necrosis and apoptosis were identified as hydrogen peroxide and hydroxyl radicals derived from superoxide anions under catalysis by iron (Eqs. (1)–(4)).

$$2O_2 + 2e^- \rightarrow 2^{\bullet}O_2^- \quad (1)$$

$$2^{\bullet}O_2^- + H^+ \rightarrow O_2 + {}^-OOH$$

$$k = 7 \times 10^5\ mol^{-1}\ s^{-1} \quad (2)$$

$$^-OOH + Fe^{2+} \rightarrow {}^{\bullet}OH + Fe^{3+}\,OH^- \quad (or\ Fe^{4+}{=}O) + OH^- \quad (3)$$

$$Fe^{3+} + {}^{\bullet}O_2^- \rightarrow Fe^{2+} + O_2 \quad (4)$$

There current consensus is that the highly reactive $^{\bullet}OH$-radical can attack all organic matter in a cell in radical chain reactions if levels that are controlled by the cellular antioxidants are exceeded. An obvious discrepancy was, and still is, the designation of $^{\bullet}O_2^-$ as an ROS since the chemical properties of this radical were considered reductive rather than oxidative. Therefore, the role of the superoxide dismutases (SOD) appeared unclear since the chemical disproportionation of two superoxide radicals at pH 7 still occurred at a sufficiently fast rate to keep the levels in the micromolar range. This view changed dramatically after the role of nitric oxide ($^{\bullet}NO$) as a signaling molecule was discovered [8–10]. Its fast rate of reaction with $^{\bullet}O_2^-$ (Eq. (5)), which even exceeds the rate for catalysis by Cu,Zn-SOD ($2.4 \times 10^9\ mol^{-1}\ s^{-1}$) [11], reduces the effective cellular level of $^{\bullet}NO$ below that required for guanylyl cyclase activation ($\approx 10^{-9}$ M) and hence changes the function of $^{\bullet}O_2^-$ to a cellular antagonist of $^{\bullet}NO$ [12].

$$^{\bullet}NO + {}^{\bullet}O_2^- \rightarrow ONOO^- \quad k \approx 9 \times 10^9\ mol^{-1}\ s^{-1} \quad (5)$$

After superoxide had been brought into focus as a selective oxidant for $^{\bullet}NO$, it was also found to inhibit basal guanylyl cyclase activity [7]. Thus, cGMP formation by soluble guanylyl cyclase can be blocked both indirectly and directly by $^{\bullet}O_2^-$, allowing $^{\bullet}NO$ and $^{\bullet}O_2^-$ to switch the enzyme on and off in a yin-yang-type fashion.

A similar phenomenon was found for calcineurin. This Ca^{2+}-stimulated enzyme can be blocked by superoxide and this inhibition can be completely reversed in the presence of $^{\bullet}NO$ or ascorbate [6]. In this case, inhibition was due to oxidation of the ferrous iron at the catalytic site. $^{\bullet}NO$ did not combine with the ferrous enzyme but merely trapped $^{\bullet}O_2^-$ whereas ascorbate acted as a reductant for iron and peroxynitrite ($ONOO^-$).

Prostanoid synthesis by prostaglandin endoperoxide synthases (PGHS) 1 and 2 (or cyclooxygenase 1 and 2), represents one of the first described examples of redox regulation [13–16]. These heme-dependent enzymes require peroxides for activation. Interestingly, the levels of this so-called ''peroxide tone'' are different for the two

isoenzymes (PGHS-1: 21 nM, PGHS-2: 2 nM) and are usually limiting under resting conditions [17]. Peroxynitrite can provide this peroxide tone in vitro [5] but recent data from our laboratory indicate that this also applies in vivo to smooth muscle cells [18].

A completely new aspect emerged from the seminal work of Beckman and coworkers [19], showing peroxynitrite as a nitrating agent for Tyr-residues in proteins. Such modified proteins could be formed in tissues under pathophysiological conditions and mitochondrial Mn-SOD was the first enzyme which was found nitrated and also inhibited by peroxynitrite [20,21]. Subsequently, a highly sensitive inhibition of prostacyclin (PGI_2) synthase by peroxynitrite (IC_{50} = 50 nM) was found in our laboratory. This occurred in parallel with nitration of the enzyme [22,23]. Since substrate analogs could block both inhibition and nitration, this posttranslational modification seemed to down-regulate PGI_2 synthase activity in a physiological process.

Meanwhile many more enzymes have been reported to be Tyr-nitrated [24,25] but a link to regulatory processes remains to be shown. In order to fulfill the definition for redox regulation such nitrations must also be reversible. This prerequisite has been demonstrated for histone nitration [26].

Another posttranslational modification that fully meets the requirements of a redox-regulated process is *S*-nitrosation:

$$-\mathrm{SH} + [\mathrm{NO}^+] \rightarrow -\mathrm{S}-\mathrm{NO} + \mathrm{H}^+ \xrightarrow[-\mathrm{GSNO}]{+\mathrm{GSH}} -\mathrm{SH} \qquad (6)$$

In contrast to many proposals in literature, this reaction cannot proceed directly with $^{\bullet}$NO but for stoichiometric reasons requires NO^+ or the presence of a one-electron acceptor together with $^{\bullet}$NO. Since NO^+ is unlikely to exist at pH 7 it has been proposed that N_2O_3 could be the nitrosating intermediate [27]. Pathways leading to the generation of nitrogen dioxide ($^{\bullet}NO_2$) exist within the $^{\bullet}NO/^{\bullet}O_2^-$ system. This radical combines with $^{\bullet}$NO to give N_2O_3. Since peroxynitrite is a nucleophile which can combine with cellular CO_2, a second pathway for $^{\bullet}NO_2$ formation exists after homolytic cleavage of the O–O bond of the addition product with $^{\bullet}$NO. The multiple pathways originating from $^{\bullet}$NO, $^{\bullet}O_2^-$ and CO_2 are summarized in Fig. 1.

Electron transfer between the two reactants results in the formation of nitrite and dioxygen and should be the preferred reaction, since it allows spin conservation. However, the existence of peroxynitrate (O_2NOO^-) in this system is known and could arise from this interaction as a potent new oxidant. An excess of superoxide will also lead to the production of H_2O_2 by dismutation, and, since $^{\bullet}O_2^-$ can reductively release iron from intracellular stores, the Fenton reaction under formation of the $^{\bullet}$OH-radical (or Fe^{4+}=O) should also be possible (Eqs. (1)–(5)). Hence, an excess of $^{\bullet}O_2^-$ will lead to conditions of oxidative stress.

In summary, the $^{\bullet}NO/^{\bullet}O_2^-$ system provides a rich chemistry that can provide a platform for redox regulation. In the following section, we shall look at a scenario where an $^{\bullet}$NO-producing cell, e.g., an endothelial cell, is exposed gradually to increasing levels of superoxide. Emphasis will be placed on the enzymatic sources of superoxide and the conditions under which these can be activated. It will be evident that $^{\bullet}O_2^-$ release is the crucial event for redox regulation but it will also become clear that more knowledge of the chemistry involved, and its regulatory mechanisms, is required.

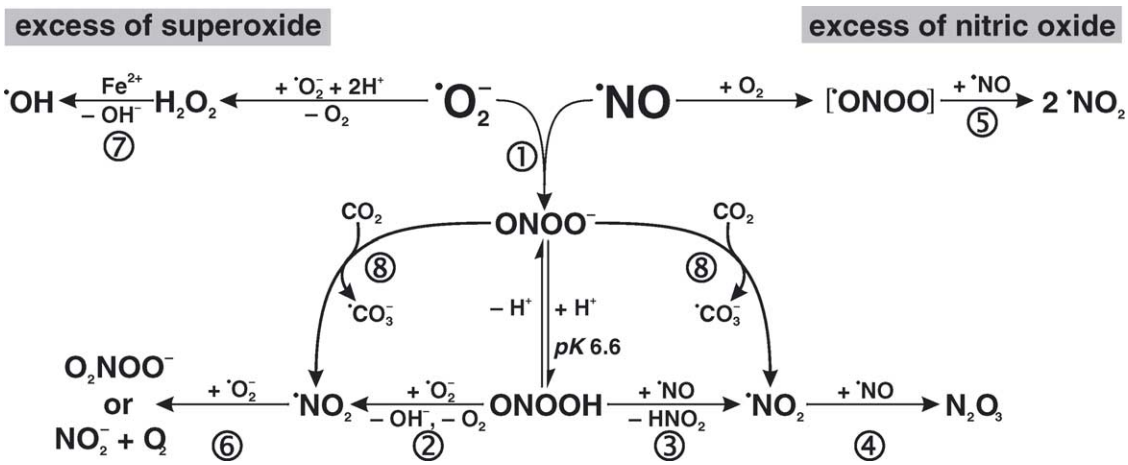

Fig. 1. Reactions between superoxide and nitric oxide. (1) The main product of the reaction between equal amounts of nitric oxide ($^{\bullet}$NO) and superoxide ($^{\bullet}O_2^-$) is peroxynitrite ($ONOO^-$). (2, 3) A slight excess of one species shifts the product pattern towards nitrogen dioxide radicals ($^{\bullet}NO_2$). (4) Greater excess of $^{\bullet}$NO leads to N_2O_3 production and nitrosations. (5) Relatively high concentrations of $^{\bullet}$NO and O_2 are required for autoxidation of $^{\bullet}$NO. (6) If $^{\bullet}O_2^-$ outweighs $^{\bullet}$NO this can cause formation of peroxynitrate (O_2NOO^-) or nitrite. (7) Higher concentrations of $^{\bullet}O_2^-$ over $^{\bullet}$NO will lead to Fenton chemistry and formation of $^{\bullet}$OH-radicals, and thus will initiate toxicity with all signs of oxidative stress. (8) The presence of high concentrations of carbon dioxide (up to 1 mM in biological systems) shifts the decomposition pathways of $ONOO^-$ towards the formation of $^{\bullet}NO_2$.

3. Redox regulation by the $^{\bullet}NO/^{\bullet}O_2^-$ system: origin of nitrosation, nitration, oxidation and peroxidation

Radicals are naturally highly reactive species, due to their unpaired electron spins but in the case of $^{\bullet}NO$ and $^{\bullet}O_2^-$, the unpaired electrons are sufficiently stabilized by resonance and react only with a very limited number of cellular targets. Such targets are either radicals themselves, or are transition metals with unpaired electrons. Even at low levels of $^{\bullet}NO$ and $^{\bullet}O_2^-$, the reactivities with such targets are high, whereas reactions with spin-paired compounds occur only rarely. Interestingly, in models where high concentrations of either radical are employed, two-electron transfers can take place subsequently causing oxidations not normally occurring under cellular conditions (e.g., –SH + 2 $^{\bullet}NO$ → –S–NO + HNO). Considering the usually low cellular levels of $^{\bullet}NO$ and $^{\bullet}O_2^-$, one arrives at the rather simple but fascinating scenario, in which the chemistry described in Fig. 1 can be correlated with cellular redox biochemistry and physiological regulation. The spectrum of redox conditions ranges from states with (i) only $^{\bullet}NO$, (ii) 2–3-fold excess of $^{\bullet}NO$ over $^{\bullet}O_2^-$, (iii) equal levels of both radicals, and (iv) an excess of $^{\bullet}O_2^-$. These states correspond to different redox regulations for which we suggest a chemical basis and physiological relevance in the following sections.

3.1. $^{\bullet}NO$ generation at basal $^{\bullet}O_2^-$ levels: nitrosylation

Resting cells are characterized by low levels of $^{\bullet}O_2^-$ arising from unavoidable spontaneous autoxidations. Superoxide dismutases maintain such $^{\bullet}O_2^-$ levels in the range of 10^{-10} to 10^{-11} M so that a release of $^{\bullet}NO$ in the nanomolar range after activation of nitric oxide synthases (NOS) will not affect the stimulation of soluble guanylyl cyclase at its ferrous heme-containing subunit. The formation of an Fe^{2+}–NO complex is termed ''nitrosylation'' since it is important to differentiate this process from the formally NO^+-dependent nitrosations. The latter require oxidative conditions whereas nitrosylation of guanylyl cyclase will proceed under both resting and reducing conditions. In cells these conditions signal relaxation since cGMP via its corresponding G-kinases lowers Ca^{2+} levels again, and by feed-back inhibition, can switch off Ca^{2+}-calmodulin-dependent nitric oxide synthases. The Ca^{2+}-independent inducible NOS (NOS-2) does not seem to play a major role in resting cells, whereas in contrast, much of the chemistry in diseased states relies on the participation of this isoenzyme [28]. It should be noted that all potential superoxide sources are effectively down-regulated in resting cells.

3.2. $^{\bullet}NO$ generation at elevated $^{\bullet}O_2^-$ levels: Nitrosation

As outlined in Section 2 the oxidizing conditions required to convert $^{\bullet}NO$ to a nitrosating species (NO^+ or N_2O_3) can be best achieved by assuming superoxide formation at an excess of $^{\bullet}NO$ release. The primary product, peroxynitrite, does not react with $^{\bullet}NO$ but the protonated form ONOOH ($pK_a = 6.6$) does to yield $^{\bullet}NO_2$ and nitrite. $^{\bullet}NO$ and $^{\bullet}NO_2$ radicals readily form N_2O_3 which could be the remaining elusive nitrosating species. N_2O_3 reacts with azide, which can therefore serve as an inhibitor for such nitrosations. Interestingly, azide was shown to be a weak inhibitor of the *C*-nitrosation of phenol in the presence of $^{\bullet}NO/^{\bullet}O_2^-$ [27]. This casts some doubts on the function of N_2O_3 as sole nitrosating species in this system. NO^+ and HNO_2, both of which can be formed directly from $^{\bullet}NO$ and ONOOH, are potent nitrosating agents, but are also extremely unstable. In their stoichiometry both require the generation of 2 $^{\bullet}NO$ and 1 $^{\bullet}O_2^-$ whereas N_2O_3 requires 3 $^{\bullet}NO$ and 1 $^{\bullet}O_2^-$. According to data published by Espey [27], the nitrosation of diaminonaphthalene occurs with a stoichiometry of 2:1. Our own data yielded a ratio of 3:1 (unpublished results). Thus, although the exact mechanism for *S*-nitrosation under cellular conditions must be re-examined, there is little doubt that superoxide is required. A most likely source for $^{\bullet}O_2^-$ are the NADPH oxidases (NOX1–5), which are activated by PKC but in many cells produce only little $^{\bullet}O_2^-$. A series of proteins have been found in an *S*-nitrosated state and even a ''nitrosylome'' has also been postulated [29]. Considering the important role of sulfhydryl groups in enzyme catalysis and in the building of protein tertiary structure, an *S*-nitrosation modification could easily influence enzyme activities. The process of trans-nitrosation in the presence of GSH enables the reversal of these reactions which then meet the requirements for redox regulation. Alternatively, GSSG may initially form a mixed disulfide, which is then reduced once the reductive systems have recovered.

An interesting example of *S*-nitrosation has been reported for caspases. In addition to their existence as pro-enzymes, *S*-nitrosation of their essential SH-groups (or S^-) appears to provide a further mechanism of inactivation. Reduction can lead to caspase activation and thus, to apoptosis [30,31]. Similarly, the NF-κB pathway was found to be blocked by *S*-NO formation at the p50 subunit, ready to be converted back under the reducing conditions prevailing in the nucleus [32]. The activity of the *N*-methyl-D-aspartate (NMDA) receptor-associated ion channel is inhibited by *S*-nitrosation of a cysteine on its NR2A subunit [33]. Other known examples for regulatory mechanisms involving *S*-nitrosation are the ryanodine receptor [34], methionine adenosyltransferase [35] and the Ras family of G proteins [36].

Not only signaling pathways are regulated by *S*-nitrosation, also the cellular redox status itself is regulated by thioredoxin and its associated reducing system (thioredoxin, thioredoxin reductase, NADPH). The thioredoxin system together with glutaredoxin and glutathione represent the reductive system of the cell [37]; their reductive power is driven by NADPH. Thioredoxin reduces oxidized

cysteine groups on proteins, scavenges ROS together with thioredoxin peroxidase and acts as a transcriptional activator via NF-κB. *S*-Nitrosation of thioredoxin at cysteine 69 is necessary for its redox regulatory function [38], whereas the inactivating oxidation of cysteine 32 and 35 causes apoptosis via apoptosis signal-regulating kinase 1 [39]. Additionally, thioredoxin gets inactivated by glutathiolation of cysteine 73. Therefore the thioredoxin system is a key regulator of the cellular redox state, regulated by the cellular redox state itself.

Many *S*-nitrosations such as those observed for GAPDH [40] or aldose reductase [41] have not yet been sufficiently correlated with specific regulatory pathways but many have the potential to set the stage for a redox conditioning of the activated cell as outlined in Section 4.

3.3. Equal rates of $^{\bullet}NO$ and $^{\bullet}O_2^-$ generation: thiol oxidation, methionine sulfoxidation and tyrosine nitration

When the rate of $^{\bullet}O_2^-$ generation in an activated cell approaches that of $^{\bullet}NO$, the resulting reaction will be peroxynitrite formation. With a pK_a of 6.6 it mainly exists as an anion but at an intracellular pH of approximately 7.0 it may also act as its more aggressive acid form. In the anionic form it reacts with protein thiols to sulfenic acids which readily form mixed disulfides with GSH. Zinc fingers are the preferred targets and Zn^{2+} is released after disulfide formation between adjacent Cys-residues [42–44]. Since zinc fingers are abundant in transcription factors and required for DNA binding, their oxidation will prevent transcription, which is a meaningful regulation under oxidative conditions when DNA strand breaks prevail.

Methionine can present another target for peroxynitrite, leading to sulfoxidations [45]. Some protease inhibitors can be blocked by this reaction giving rise to protease activation [46]. Since methionine-sulfoxide reductases exist, this forms a link to redox regulation of proteolysis. One of the most intriguing properties of peroxynitrite is its ability to nitrate Tyr-residues [19]. It is now clear that this reaction does not occur with any Tyr-residue at physiological $ONOO^-$ levels but requires catalysis by metal centers [47], allowing the following steps to occur:

$$Me^{red} + ONOO^- \rightarrow [Me^{ox}O + {}^{\bullet}NO_2] \quad (7)$$

$$[Me^{ox}O + {}^{\bullet}NO_2] \xrightarrow[-Me^{red}]{+Tyr} Tyr{-}NO_2 \quad (8)$$

In order to be specific for a given Tyr residue, the attack of the metal-catalyzed $^{\bullet}NO_2$ radical is likely to occur at a neighboring tyrosine. This seems to apply for prostacyclin synthase for which peroxynitrite causes nitration with the consequence that in endothelial cells, the generation of $^{\bullet}O_2^-$ not only traps $^{\bullet}NO$ but also blocks PGI_2 synthesis [3]. In addition, the remaining substrate for PGI_2 synthase, PGH_2, activates the TxA_2/PGH_2 receptor as the antagonistic signal to PGI_2. This sequence of events leads to endothelial activation and dysfunction as a prerequisite for the extravasation of white blood cells into inflamed areas of tissue. A schematic representation of this regulation is depicted in Fig. 2 and will be further discussed in Section 4.

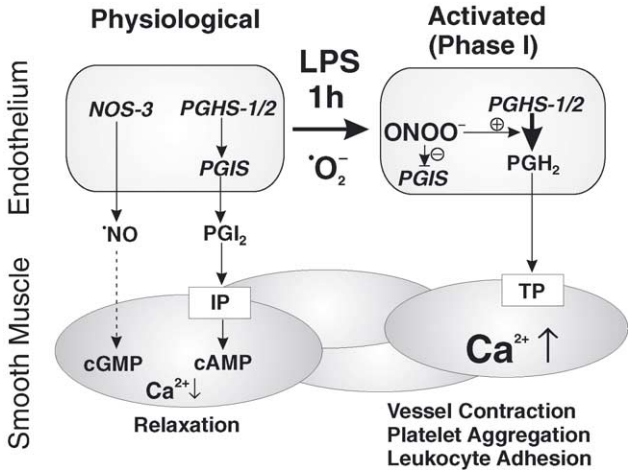

Fig. 2. Endothelial cell activation (ECA). Under physiological conditions, the endothelium maintains vascular tone by a basal formation of endothelial nitric oxide synthase (NOS-3)-derived nitric oxide ($^{\bullet}NO$) and prostaglandin endoperoxide H_2 synthase-1 (PGHS)-1-derived prostacyclin (PGI_2) (left). $^{\bullet}NO$ directly enters the smooth muscle cell and activates soluble guanylyl cyclase resulting in elevated intracellular levels of cGMP. PGI_2 activates its receptor (IP) on the surface of the smooth muscle cell which in a G-protein-mediated way activates adenylyl cyclase to form cAMP. By several intermediate kinase pathways, cGMP and cAMP trigger a decline in the levels of free intracellular Ca^{2+} and therefore smooth muscle relaxation. Activation of the endothelium by agonists like Ang II or LPS leads to an increased generation of superoxide ($^{\bullet}O_2^-$), which reacts in a nearly diffusion-limited reaction with $^{\bullet}NO$ to form peroxynitrite ($ONOO^-$). Peroxynitrite nitrates and inactivates endothelial PGI_2 synthase which can then no longer convert its substrate PGH_2. Since PGHS is still active under such conditions, PGH_2 accumulates and can bind to the TxA_2/PGH_2-receptor on the surface of vascular smooth muscle cells to evoke vessel constriction.

As reversibility of Tyr-nitration in the case of PGI_2 synthase has not yet been demonstrated, and hence, a clear assignment to redox regulation cannot be made at present. On the other hand, a persistent and irreversible Tyr-nitration defines oxidative stress and indeed the results on the possible sources for superoxide support this definition. Allopurinol as a selective inhibitor of xanthine oxidase was able to prevent PGI_2 synthase nitration and inhibition [3]. Superoxide production via this pathway may either involve an oxidative conversion of xanthine dehydrogenase to its oxidase form or a membrane-bound form of xanthine oxidase, secreted from liver, obtains hypoxanthine/xanthine as substrates from AMP degradation products, which only appear after a massive disturbance of energy metabolism. Therefore, mitochondria may be also affected in cases of severe endothelial dysfunction when these are elicited by endotoxin (lipopolysaccharide, LPS) (see Section 4).

In summary, an agonist-stimulated release of $^{\bullet}O_2^-$ by NADPH oxidase leads to $^{\bullet}NO$-trapping as the start of endothelial activation, whereas further release of $^{\bullet}O_2^-$

from mitochondria and xanthine oxidase mark the transition to endothelial dysfunction. The exact biochemical conditions governing the transition between activation and dysfunction still remain to be elucidated.

3.4. Excessive $^{\bullet}O_2^-$ generation: the pathway to oxidative stress and free radical damage

After activation of all potential sources for $^{\bullet}O_2^-$, its rate of formation may far exceed that of $^{\bullet}NO$. Mitochondria can be especially rich regions of superoxide formation but this seems to happen in a controlled process involving Ca^{2+}, ROS and structural changes triggered by the opening of the permeability transition pore (PTP). Reduction of dioxygen to $^{\bullet}O_2^-$ may occur by autoxidation of redox components in complex I or III. A counterregulation by uncoupling proteins probably exists [48] and the combined activity of Mn-SOD and GSH peroxidase reduces mitochondrial $^{\bullet}O_2^-$ to water. Oxidative inactivation of Mn-SOD and GSH depletion eventually would cause superoxide to leave the mitochondrial space and cause an increase in cytosolic $^{\bullet}O_2^-$. These levels of $^{\bullet}O_2^-$ will be much higher than those originating from NADPH oxidase and thus may have severe consequences for the redox state of cytosolic components. Mitochondrial GSH and subsequently cytosolic GSH are oxidized, followed by oxidation of vitamins C and E. Tetrahydrobiopterin (BH_4) is a redox factor for nitric oxide synthases and after its oxidation to BH_2, the synthesis of $^{\bullet}NO$ ceases and NADPH is used by nitric oxide synthases for $^{\bullet}O_2^-$ production instead of for monooxygenation of arginine to $^{\bullet}NO$ [49]. Since $^{\bullet}NO$ acts as an efficient suppressor of $^{\bullet}O_2^-$ the levels of $^{\bullet}O_2^-$ will then rise even more dramatically allowing Fenton chemistry with the associated formation of $^{\bullet}OH$-radicals. Lipid peroxidation resulting from the peroxidative destruction of cellular membranes has cell death as its ultimate consequence. It should however, be mentioned that mitochondrial autoxidations triggered by PTP opening still can be reversible and hence cell death is only a consequence if the rescue mechanisms cannot reverse the process. It is beyond the scope of this review to refer to the complex biochemistry of mitochondrial redox reactions and their control [50]. Instead, we shall take an overall look at the events involved in cell activation by interactions within the $^{\bullet}NO/^{\bullet}O_2^-$ system.

4. Towards a general concept of redox regulation: the endothelial cell as a model

Out of the many details collected so far one can derive a scenario of cellular activation in which redox signaling may play a similar role as Ca^{2+} or phosphorylation. Since these all form a network of signals, interconnections such as Ca^{2+}-triggered oxidations and vice versa or Ca^{2+} and superoxide regulation of calcineurin [6] must exist. PKC stimulates NADPH oxidase and its product $^{\bullet}O_2^-$ causes a feed-forward increase in PKC activity. Ca^{2+} is the main stimulus for PLA_2 activation and the further metabolism of arachidonate requires the ''peroxide tone'' for the function of PGHS, which is involved in cellular communication. Many cells contain either NOS-1 or NOS-3, and, if existence of mitochondrial NOS can be firmly established [51], all cells will release $^{\bullet}NO$ following a Ca^{2+}-stimulus. This would then lead to the activation of multiple pathways in which the $^{\bullet}NO/^{\bullet}O_2^-$-system causes nitrosylations, nitrosations, nitrations and oxidations.

It has been emphasized that, next to $^{\bullet}NO$ release, the formation of superoxide is the crucial step in redox regulation. Therefore all $^{\bullet}O_2^-$ releasing systems must be controlled at multiple check points. Firstly, the systems involved, such as the NADPH oxidases, mitochondria, xanthine oxidase or the oxidase function of nitric oxide synthases, require special activation mechanisms. Secondly, their activations seem to proceed in a cascade, leading to increasing levels of superoxide, reflecting different states of cellular activation. Usually, NADPH oxidases are low-output systems, triggered by receptor-mediated phosphorylations of their cytosolic subunits [52]. Prolonged and heightened $^{\bullet}O_2^-$ release in conjunction with the ceramide pathway may open the mitochondrial PTP, allowing still more $^{\bullet}O_2^-$ being formed [50]. Xanthine oxidase may then also be activated and, in the course of oxidative stress, BH_4 can be oxidized and consequently, NOS is converted to its oxidase form. $^{\bullet}NO$ from four different sources accompanies this release and creates the various conditions of redox regulation. This hypothesis is summarized in Fig. 3.

If then the various mechanisms for posttranslational modifications have taken place, the reversible process of a direct reductive restoration must be initiated. This should be possible at any stage of the oxidative modification unless proteolysis is involved, as, for example, the proteolytic conversion of xanthine oxidase or of the caspases. It should, however, be noted that the mechanisms for the reductive portion of redox regulation are far from being understood and no clear delineation between reversible redox regulation and irreversible oxidative stress exists.

Another unsolved problem is one inherent to redox regulation. A meaningful regulation by oxidation/reduction would demand mechanisms to prevent reductions while oxidations are allowed to continue and vice versa. Otherwise futile cycles can develop, resulting not only in the loss of reducing equivalents, but also in the blurring of on- and off-set of redox regulation. From recent reports, one can derive a hypothesis that also applies redox regulation to the providers of reducing equivalents, since $NADP^+$-dependent isocitrate dehydrogenases can be inhibited by *S*-nitrosation [53]. Considering also the observed activation of aldose reductase by *S*-nitrosation [41] one can propose an effective switch-off for cytosolic NADPH-formation, which serves as a driving force for the reductive restoration pathways (Fig. 4).

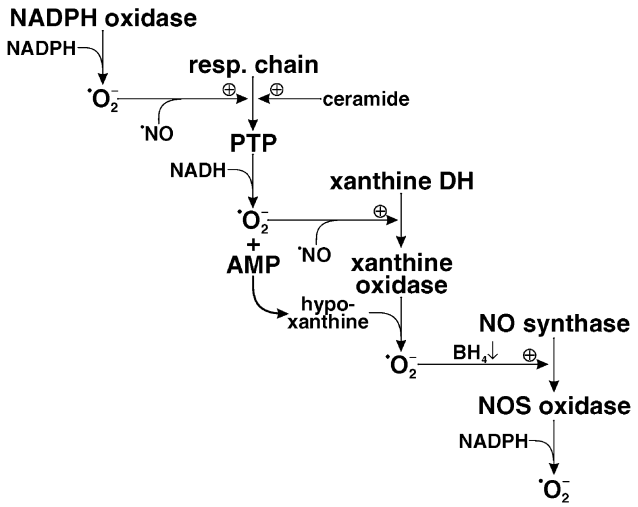

Fig. 3. The consecutive model of superoxide release. Following assembly of the NADPH oxidase complex, elevated superoxide concentrations are leading to peroxynitrite and therefore mitochondrial permeability transition pores (PTP) can be opened, resulting in an uncoupling of the respiratory chain. As a consequence, additional superoxide is released by complex I and ubisemiquinone, cellular Ca^{2+} levels are increased and ATP formation declines. At this point, the activations of superoxide sources are still reversible, but further steps in this activation cascade are unlikely to be. Liver-derived xanthine oxidase or oxidation of xanthine dehydrogenase by peroxynitrite provides another source of superoxide and breakdown of cellular ATP finally yields in hypoxanthine, which serves as substrate of xanthine oxidase. Finally, tetrahydrobiopterin (BH_4) is oxidized, converting NOS-3 to an oxidase, which also contributes to cellular superoxide formation.

The circulatory system provides several examples for redox regulation in pathophysiological events such as atherosclerosis, ischemia/reperfusion or diabetic endothelial dysfunction, all of which are well-investigated diseases even with regard to their biochemical background. Especially the endothelium of the large vessels is able to orchestrate vessel function through a variety of redox mechanisms, of which the nitration of PGI_2 synthase has already been mentioned. This unusual posttranslational modification can be considered the Rosetta stone of endothelial dysfunction, since it explains mechanistically the functions of a vessel during inflammation, infarct, stroke or ageing.

Under physiological conditions vessel endothelium shows a continuous release of $^{\bullet}NO$ by the shear-stress activated endothelial NOS (NOS-3). This characterizes the resting state in which a tightly closed endothelium via $^{\bullet}NO$ relaxes smooth muscle and prevents the adherence of platelets or white blood cells. Many stimuli including angiotensin II, acetylcholine, substance P, TxA_2 or leukotriene C_4 elicit a PI-response by activation of their corresponding receptors, which not only triggers Ca^{2+}-release but also PKC-dependent phosphorylation of the cytosolic NADPH oxidase-subunits, leading to low rates of $^{\bullet}O_2^-$ formation. In an immediate response the basal levels of $^{\bullet}NO$ are trapped (as peroxynitrite) synergizing with the contraction of smooth muscle by the agonists. The contractive phase ceases after several minutes and is replaced by endothelium-dependent relaxation due to Ca^{2+}-triggered $^{\bullet}NO$ synthesis and the liberation of arachidonic acid

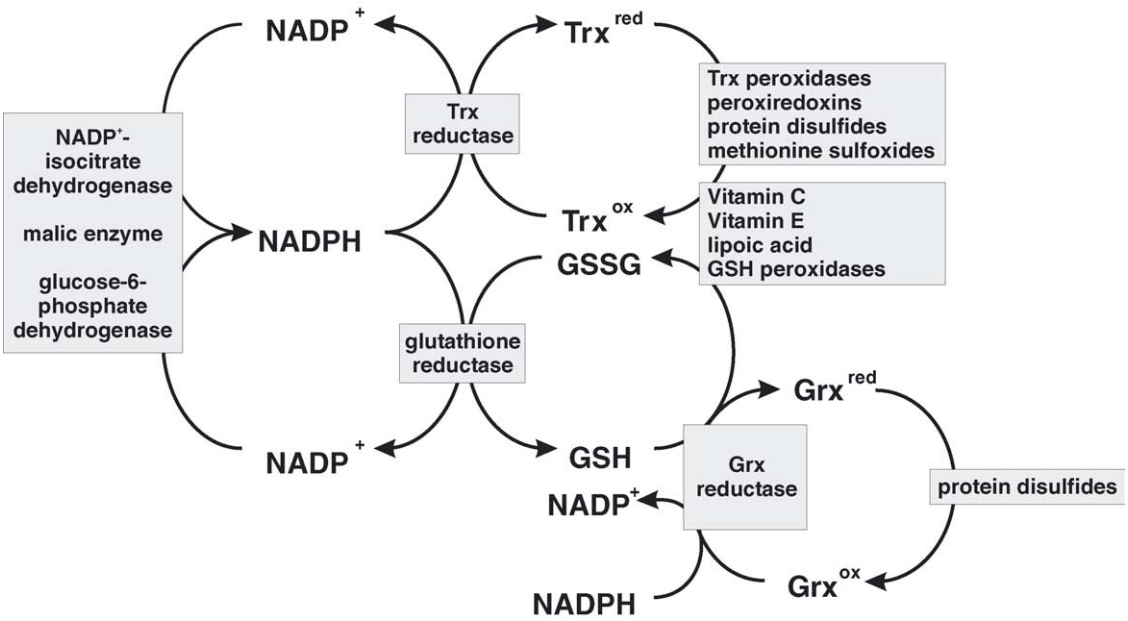

Fig. 4. The reductive components for redox regulation. NADPH represents the major cellular reductant. It is continuously regenerated by $NADP^+$-dependent isocitrate dehydrogenases, the malic enzyme and glucose-6-phosphate dehydrogenase. The thioredoxin (Trx) and the glutaredoxin (Grx) systems, together with the recovery of glutathione (GSH), are directly coupled to cellular NADPH levels. The reduced forms of Trx and Grx provide reduction equivalents for the reduction of disulfides, methionine sulfoxides, peroxiredoxins and Trx peroxidases. Both can regenerate oxidized GSH peroxidases, vitamins C, E and lipoic acid.

with subsequent conversion to PGH_2 and PGI_2. The relative contributions of $^{\bullet}NO$ and PGI_2 to the relaxation process via cGMP and cAMP, respectively, may vary with the particular vessel and species in question. In smaller vessels and in rats the $^{\bullet}NO$ pathway is of major importance; in larger vessels, especially from cattle, humans or pigs, PGI_2 synthesis prevails [54].

The extent, to which PGHS-1 (COX-1) or PGHS-2 (COX-2) are involved in this process is still a matter for debate and this is of particular importance in view of the side effects of PGHS-2 inhibitors (''COX-2 inhibitors''). Since PGHS-2 is present in most endothelia, even under normal conditions, there will be a preference for PGHS-2 activation, based on the peroxide tone of 2 nM required for this isoenzyme compared to 21 nM for PGHS-1. Hence, a mild stimulation of NADPH oxidase will first activate PGHS-2 at the low levels of peroxynitrite formed. PGHS-1 will synthesize PGH_2 and further enhance PGI_2 release only upon massive stimulation of $^{\bullet}O_2^-$ release. The necessary increase in cellular peroxide levels can be reached experimentally by addition of H_2O_2 or by nitroglycerol, which partially exerts its vasodilatation by increasing PGI_2 release [55]. If, however, prolonged exposure to nitroglycerol causes conditions of oxidative stress, this part of the pharmacological effect will be eliminated by causing nitration and inhibition of PGI_2 synthase (''cross-tolerance'' [56], see Section 2). For this reaction a bolus addition of 50 nM peroxynitrite is required for half-inhibition of the isolated enzyme [22], which leaves only a narrow window between a maximal stimulation of PGHS-1 and the onset of PGI_2 synthase nitration. If oxidative conditions are generated by endotoxin (LPS) incubation of vessels ex vivo, the nitration of PGI_2 synthase is complete after 45–60 min [3]. These are the conditions when the unmetabolized PGH_2 occupies and stimulates the TxA_2/PGH_2 receptor which then leads to vasospasm. A number of reports indicating that $^{\bullet}O_2^-$ production under these conditions involves mitochondria have been published. Signals for an opening of the PTP could be NADPH oxidase-derived peroxynitrite [2] together with ceramide [57]. We have also shown that xanthine oxidase contributes to the nitration process and in this respect, it is interesting that xanthine oxidase seems to be associated with NOS-1 [3], which indeed is present in the endothelium, and could provide $^{\bullet}NO$ for peroxynitrite formation.

Since LPS-incubation is an accepted model for inflammation we would like to propose the events of Fig. 2 as phase I of endothelial activation which, if not reversed, ends in endothelial dysfunction. The physiological significance of this phase I seems to be connected with a weakening of the endothelial cell–cell contacts for which NADPH oxidase plays an initial role [2]. The export of P-selectin from the Weibel–Palade bodies for the permeation of white blood cells as an essential step in the innate immune response is another mechanism which seems to be triggered by PGE_2 [58]. Since PGE_2 synthase is present only in smooth muscle it must be assumed that after acting on the TP-receptor, endothelium-derived PGH_2 is converted to PGE_2 in smooth muscle, which then acts on the endothelium for P-selectin expression. A second function of PGE_2 linked to the subsequent phase II of the LPS inflammatory response may also exist. By activation of the EP_2 and EP_4 receptors on smooth muscle, a rise in cAMP and the induction of the early gene products PGHS-2, NOS-2 and heme oxygenase 1 (HO-1) will be triggered [59].

The onset of protein synthesis for the early genes characterizes a second phase of LPS action also involving redox regulation, since the activation of the transcription factor NF-κB requires oxidative activation in the cytosol and reductive conditions in the nucleus [60]. Oxidation seems to be mediated by the inhibition of phosphatases [61] and the activation of kinases [62]. The early gene products are expressed within 2–4 h and then smooth muscle can assume the functions of the endothelium by releasing $^{\bullet}NO$ from NOS-2 or PGI_2 from constitutive PGI_2 synthase, which now can obtain its PGH_2 substrate from newly-synthesized PGHS-2. It was interesting to find that in rat vessels this phase II of LPS action or endothelial dysfunction involves NOS-2, whereas in bovine and human vessels PGI_2 counteracts the vasospasm from PGH_2. This, together with a potent reducing system in LPS-treated smooth muscle which keeps peroxynitrite levels in the nanomolar range [18], is one way of preventing nitration of PGI_2 synthase. The interaction of phase I and II is schematically summarized in Fig. 5.

According to this scheme the physiological process for establishing vessel tone and maintaining it throughout inflammation, starts with $^{\bullet}NO$ formation via nitrosylation of guanylyl cyclase in smooth muscle and ends with an equal or excess formation of $^{\bullet}O_2^-$ under PGI_2-nitration in the endothelium. This loss of endothelial PGI_2 is compensated by transformation of smooth muscle to act as a substitute for the dysfunctional endothelium. Although many details of this scenario are still lacking, the newly-discovered biochemistry of PGI_2 synthase nitration, the active role of PGH_2 and the large reservoir of PGI_2 synthase in smooth muscle becoming active after induction of PGHS-2, allow a rational approach to the complex interaction of the vessel with the innate immune system under the life-threatening conditions of sepsis.

5. Search for pharmacological targets: between Scylla and Charybdis

Pharmacological interventions are most promising when they target the regulatory pathways linked to diseased states. Phosphorylations with phosphodiesterases as targets are successful examples. However, from this example one can also learn that selectivity for a given isoenzyme is required to develop a useful drug. This will also apply for redox regulation and hence, more details on redox-regu-

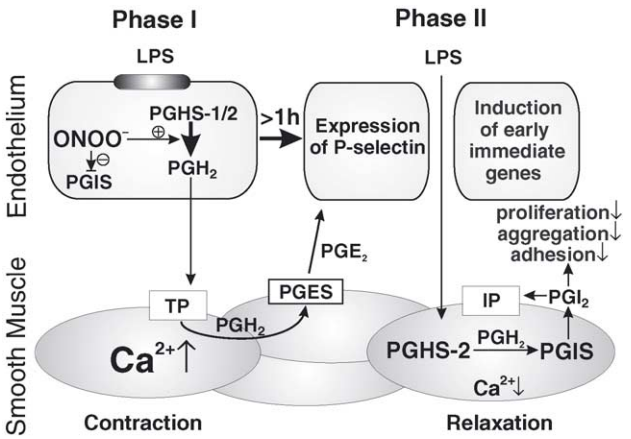

Fig. 5. Involvement of smooth muscle in progressive stages of endothelial activation. In phase I of endothelial cell activation (ECA) elevated formation of superoxide ($^{\bullet}O_2^-$) traps endothelial nitric oxide ($^{\bullet}NO$) to form peroxynitrite ($ONOO^-$), which subsequently nitrates and inactivates PGI_2 synthase. The lack of both vasodilators $^{\bullet}NO$ and PGI_2, together with the activation of the TxA_2/PGH_2 receptor on smooth muscle cells by endothelial PGH_2, results in contraction of the vessel. The events in phase I take place within about 1 h after stimulation and require no de novo protein synthesis. Endothelial PGH_2 can be converted by PGE_2 synthase (PGES) in smooth muscle cells to finally evoke expression of P-selectin on the surface of endothelial cells. Phase II is initiated after approximately 2 h and includes the induction of early immediate genes. The induction of PGHS-2 in smooth muscle cells supplies the constitutively-expressed PGI_2 synthase with substrate and accounts for the PGI_2-mediated autocrine relaxation of smooth muscle cells.

lated enzymes will be necessary and worthwhile since the major diseases of the western world, like atherosclerosis, diabetes, stroke, AIDS or neurodegenerative diseases, have a strong redox component. This, however, is reflected in oxidative stress and it certainly is a misconception that antioxidative treatments will cure such diseases. Certainly, in agreement with the here discussed concept that oxidative stress corresponds to the mostly irreversible phase of the disease, such strategies can have a preventive nature. Promising pharmacological interventions should therefore occur at the level of redox regulation with the aim of shifting oxidative modifications back to the reduced state. This can be achieved by inhibition of the oxidative posttranslational modifications, by enforcing the reductive back reaction or, most effectively, by interfering from both sides. Care has to be taken, however, that a meaningful counterregulation of the body against the development of a disease is not prevented as recently experienced with the prominent example of PGHS-2 inhibitors.

An early attempt to interfere with inflammation as a redox-regulated event leading to oxidative stress has been the use of Cu,Zn-SOD injected into the inflamed tissue [63,64]. The short half-life of this ‘‘drug’’ and its limited access to the $^{\bullet}O_2^-$-sources caused the discontinuation of this innovative idea. Trials with polyethylene glycolated Cu,Zn-SOD seemed to be more successful [65,66] but such attempts remain in the experimental phase. It appeared more suitable to develop lipophilic SOD mimetics for oral intake and Mn-porphyrins are promising in this respect [67,68]. Care must be taken that such treatments only limit the excessive and autoaggressive oxidations and not ‘‘normal’’ cellular activation processes such as the stimulation of transcription factor NF-κB which requires an oxidative signal [60].

A more targeted approach should address the enzymatic sources of $^{\bullet}O_2^-$. NADPH oxidases are complex in nature [69] and still no specific inhibitors with adequate affinities for these enzymes exist. Gliotoxin, a bacterial secondary metabolite, shows specificity but has a low affinity [70]. NOX2 (gp91phox) is the main oxidase of polymorphonuclear neutrophils (PMN) involved in bacterial killing and hence this enzyme should only be a pharmacological target when PMN attack the body’s own tissue. Prolonged treatment with angiotensin II induces the endothelial NADPH oxidase isozymes NOX1 and NOX4 [69]. Thus, these oxidases could be targets for treatment of hypertension.

If xanthine oxidase is involved in $^{\bullet}O_2^-$ production, the use of allopurinol or related quasi-substrate inhibitors could be beneficial. Indeed, positive effects of allopurinol have been reported [71–73]. However, it should be kept in mind that the conversion of xanthine dehydrogenase to the oxidase form requires either oxidation of SH-groups or proteolysis [74,75] and that enhanced purine nucleotide degradation may be necessary to provide sufficient substrate for the oxidase form. Therefore, better targets may exist upstream to the action of xanthine oxidase. Such may be the conditions for mitochondrial dysfunction linked to superoxide instead of water formation accompanied by ADP degradation instead of ATP synthesis. What was believed to be a simple autoxidation of respiratory chain components has since emerged as a sophisticatedly regulated process with several control points for the prevention of superoxide overproduction, which is efficiently handled by dismutation of $^{\bullet}O_2^-$ catalyzed by Mn-SOD and the glutathione-dependent reduction of H_2O_2 to water.

There are strong indications that many of the chronic diseased states are accompanied by a gradual increase in mitochondrial superoxide formation. Initial events of cell activation, such as the influx of extracellular Ca^{2+} or NADPH oxidase activation, can lead to opening of the PTP, followed by increased $^{\bullet}O_2^-$ production, which can be prevented by cyclosporine A or D. The influence of Bcl-2 and of Bax proteins on the PTP are not yet fully understood but are strongly antioxidative or oxidative, respectively [50]. Compounds like carvedilol are not only β-receptor blockers but also have antioxidative properties [76], which may relate to a putative NADH oxidase inhibition in the mitochondrial system.

A new approach was taken as soon as peroxynitrite was recognized as a powerful cellular oxidant associated with oxidative stress. Its physiological functions are probably performed at levels between 50 and 500 nM with levels in pathophysiological situations reaching approximately

5 μM as calculated from bolus administration. Such levels can, however, only be reached if the cellular antioxidant system is already compromised by oxidative stress. It is interesting to note that uric acid joins the antioxidant system for $ONOO^-$ and this has initiated the search for even more effective trapping agents for peroxynitrite. For many compounds, however, the reaction with $ONOO^-$ leads to the production of radical intermediates, which may show pro-oxidant properties and start radical chain reactions. Ideally, a peroxynitrite eliminating agent should catalyze just the isomerization to nitrate and indeed some of the Mn-porphyrin drugs act via this pathway [77,78]. Peroxynitrite formation can also be prevented by inhibition of nitric oxide synthases, preferentially NOS-2, but this will only be feasible in the very destructive phase of $ONOO^-$ action and would indeed be contraindicated in the earlier phase when $^{\bullet}NO$ acts as a trapping agent for $^{\bullet}O_2^-$, leading to endothelial activation as a first step in the innate immune response.

Very little is known about the complex situation when $^{\bullet}O_2^-$ formation by mitochondria, xanthine oxidase or activated leukocytes exceeds $^{\bullet}NO$ synthesis. This is the commencement of free radical chemistry and hence, the situation in which antioxidants could be employed. However, the physiological antioxidants like vitamin C or E but also catechol-based plant constituents have pharmacokinetic problems, or in higher concentrations, may even become pro-oxidants. Therefore, it seems more promising to reinforce the cellular reducing systems mainly based on thiol biochemistry such as GSH reductase, thioredoxin or glutaredoxin reductases. This is still out of pharmaceutical reach but the excessive supply of *N*-acetylcysteine as a cell-permeable source for thiols represents the first step in this direction. There is no selectivity in such treatment when given systemically, but assuming that an excess of cellular cysteine would not be harmful, an overstimulated immune system as in AIDS could indeed be protected by *N*-acetylcysteine [79].

The final goal should be the achievement of selectivity in the stimulation of reduction or the inhibition of oxidation. This could be achieved by using selective uptake mechanisms for drugs as has been proposed for positively charged compounds which accumulate in mitochondria, such as positively-charged Mn-porphyrins as catalysts for hydrogen peroxide, peroxynitrite or superoxide degradation [67,68].

Great potential exists in the treatment of diseases caused by microorganisms with specialized reducing or oxidizing pathways as found with *Plasmodium falciparum* which can be fought quite simply by applying redox cyclers [80]. For plants this principle is already in use for most of herbicides currently on the market.

While approaching a pharmacological control of cellular redox processes it is essential to consider the interactions of the $^{\bullet}O_2^-$ and $^{\bullet}NO$ synthesizing systems as a physiological signaling network. The main intermediates, $^{\bullet}O_2^-$, H_2O_2, $^{\bullet}NO$, NO^+ and $ONOO^-/^{\bullet}NO$, are strictly controlled for the purpose of redox regulation. Pathophysiological conditions arise by overpowering the cellular antioxidant systems leading to a vicious circle with $^{\bullet}OH$ and $^{\bullet}NO_2$ radicals as radical chain initiators. This situation would require pharmacological interference and so far, antioxidants here could find an application.

For differentiation of redox pathways in different cells of the human body, one may have to rely on isoenzyme patterns as are evolving for nitric oxide synthases or NADPH oxidases. Bioinformatics may turn out to be helpful [81,82] in dissecting redox pathways and in searching for appropriate targets in isoenzymes which allow attacking one but not the other. However, when looking at the complex network of redox signaling it can be foreseen that influencing the redox state by drugs will have to be accompanied by additional interventions in the network.

6. Conclusions and outlook

With the regulation of vessel function as a paradigm some interesting principles of redox regulation have been established. It becomes obvious that redox signaling has a similar potential than Ca^{2+} signaling or phosphorylation/dephosphorylation but due to the complex chemistry involved, many details are still lacking. There is no doubt that redox regulation is part of the cellular signaling network and that connections to the other regulatory mechanisms exist. For a pharmacologist this alone makes it worthwhile to become acquainted with the chemistry of redox regulation, even if the practical applications for drug development are only now under consideration.

The essentials of redox regulation became apparent from PGI_2 synthase nitration which could be completely inhibited by SOD. Combination of $^{\bullet}O_2^-$ with $^{\bullet}NO$ to form peroxynitrite proved to be in the core of redox regulation and by the further reaction of $^{\bullet}NO$ and peroxynitrite with $^{\bullet}O_2^-$, an entire system of posttranslational protein modifications could be derived [83]. Here we propose a sequential model of $^{\bullet}O_2^-$ generation, which finds support in own observations and those previously reported in the literature. Cellular activation begins with NADPH oxidase and further stimulation involves the mitochondria, xanthine oxidase and finally the conversion of NO synthases into oxidases, which marks the situation of oxidative stress (Fig. 3). The borderline between redox regulation and oxidative stress may vary but for practical purposes, it may be convenient to consider reversible oxidations/reductions as redox signaling if they are linked to regulatory processes, and to call it oxidative stress if irreversible oxidations can be observed. In the case of PGI_2 synthase nitration it is still open whether a denitration exists. A more rapid proteasomal degradation followed by new protein synthesis would be an alternative but this would not fulfill the exact definition of chemical reversibility. There is no doubt that repair of oxidative stress-induced damage is an

important process as a phase III which has not been considered in this review, but in which redox regulation will certainly also be involved.

As for the problem of denitration, many more questions remain to be answered. The reduction of disulfides or methionine sulfoxides is clearly established but the necessary network of reductases and electron transfer remains to be elucidated. A sufficient supply of NADPH is a prerequisite and this is corroborated by the observations that NADPH sources may be switched off at the beginning of $^{\bullet}O_2^-$ release to avoid futile cycles. This important aspect of redox regulation needs investigation and may be important for therapeutic interventions. It is already evident that enhancing the reductive power of cells may be as important as preventing the oxidative activation when attempting to influence the development of oxidative stress at the reversible phase of redox regulation. Observations in clinical practice confirm that a supply of antioxidants will assist the reductive phase in a preventive way but certainly is of little effect when the damage by oxidative stress has already been established.

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