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BREAKTHROUGHS AND VIEWS

Superoxide as a Messenger of Endothelial Function

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From time to time we have to revise traditional thinking and to include new aspects which previously had been incompatible with the pictures we were brought up with. In cell regulation one of such facts was the role of nitric oxide (NO) as a messenger. Hardly anybody could have guessed that such a small molecule being a gas, and even more unexpected, as a radical, would be identified as the "endothelium-derived relaxing factor" (EDRF) [1–3]. Radicals we had learned are the foes of cellular structures and dynamics and have to be controlled by antioxidants; otherwise "oxidative stress" develops and pathophysiology starts. In the case of NO we had to revive our chemical knowledge of NO reacting with dioxygen with third order kinetics being responsible for the fact that at low NO concentrations and physiological O_2 levels, NO can be a rather stable molecule. Through its unpaired electron NO can develop strong and rapid interactions with transition metal ions, e.g., with ferrous hemoproteins, which provides the basis for its important function as an activator of guanylyl cyclase [4, 5].

This review brings into focus the superoxide anion ($\cdot O_2^-$), as another low molecular weight radical which also had been banned from physiological functions and as a "reactive oxygen species" (ROS) has been put together with OH-radicals, H_2O_2, alkoxy- and peroxy-radicals into the club of nasty, life-threatening and destructive oxygen-derived toxicants [6–8]. Such a role seemed to be supported by its formation in leukocytes and macrophages, but without considering that not O_2^- but hydrogen peroxide, hypochlorite or peroxynitrite are the ultimate oxidants in such defense processes. Even the long known thermodynamics of superoxide and its use as a reductant for cytochrome c could not change its image as a radical of oxidative power [9, 10]. One reaction, however, has been supportive for a role as a dangerous molecule in the cell: its reduction of protein-bound Fe^{3+} which after uptake of an electron is released as free ferrous iron into the cytoplasm and then can enter the Haber–Weiss cycle to generate OH radicals from hydrogen peroxide [11, 12]. This reaction leads to cellular toxicity and thus explains the existence of superoxide dismutases (Cu, Zn-SOD, Mn-SOD) as cellular antioxidants. Otherwise no function for this ubiquitous group of enzymes seemed to exist although SOD mutations had shown a link to the development of degenerative diseases [13, 14]. Based on current results we here present evidence that SOD's are essential for providing a low basal superoxide level in order to allow an additional and targeted formation of superoxide to act as a messenger system.

ENZYMATIC SOURCES OF SUPEROXIDE

There are several enzymes known to generate cellular superoxide in a controlled way. One of the probably important ones is a membrane-located NADPH-oxidase also present in the endothelium [15, 16]. This system has close similarity to, but is not identical with, the corresponding enzyme complex in phagocytes but its activity outside phagocytes is found quite low. It was through the detection of expression of the gp91-phox, p22-phox, p67-phox and p47-phox and rac components of the phagocyte NADPH-oxidase that $\cdot O_2^-$ generation by endothelial cells is now recognized as a separate activity and not a consequence of autooxidation [17, 18]. Of pathophysiological importance became this system by the finding that prolonged angiotensin II exposure of endothelial cells *in vitro* and *in vivo* could increase the mRNA of the gp91 phox component and also $\cdot O_2^-$ formation triggered by this agonist of endothelium-mediated vasoactivity clearly was enhanced [19, 20]. It is likely, but not directly proven, that endothelial $\cdot O_2^-$ formation by the NADPH oxidase counteracts the NO-mediated vessel relaxation [19, 21]. Previous studies with knock-out mice for p47 phox

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lead to the assumption, that there must exist more than only one type of NADPH-oxidase in non-phagocytic cells [20]. Recently there have been discovered three new members of the NADPH-oxidase family, named Mox1,2 [22], ThOX1,2 [23] and Renox [24]. The function of these enzymes seems to be linked to growth control, since Mox1,2 overexpression or stimulation by growth factors like platelet-derived growth factor leads to increased superoxide formation and cell proliferation [22]. Although ThOX1,2 is an NADPH-oxidase, it generates hydrogen peroxide instead of superoxide. Another very interesting finding is a splice variant of the Mox1 NADPH-oxidase (NOH-1; NADPH-oxidase homology 1), which surprisingly has the function of a voltage-gated proton channel. NOH-1 plays an important role in cellular defense against acidic stress [25].

A second and well known $\cdot O_2^-$ generating system consists in xanthine/xanthine oxidase, which is found membrane-associated on endothelial and other cells and has been made responsible for vascular oxidative reactions leading to endothelial dysfunction [26–28]. The problem here arises from the fact that $\cdot O_2^-$ formation by this enzyme requires a conversion of xanthine dehydrogenase to its oxidase form by either thiol oxidation or proteolytic cleavage [29] and also that xanthine or hypoxanthine as substrates may only become available in sufficient amounts after ATP or GTP degradation, i.e., under conditions of mitochondrial damage and dysfunction. Therefore it is likely that $\cdot O_2^-$ formation by xanthine oxidase occurs as a rather late event in vascular damage, but then it will become effective in the trapping of NO as will be shown in the next section.

There are two more sources of $\cdot O_2^-$ that should be mentioned although the conditions for their activation are even less clearly defined. One is an increased mitochondrial $\cdot O_2^-$ production, which should not simply be termed "autooxidation" since it may comprise a complicated process involving a rise in mitochondrial Ca-levels, permeability transition, a loss of the membrane potential [30] and eventually a specific one-electron-reduction of dioxygen by the ubiquinone system of the respiratory chain [31, 32].

The fourth source of $\cdot O_2^-$ could be NOS-3 itself which like most cytochrome P450 dependent monooxygenases has an oxidase activity leading to the release of $\cdot O_2^-$. Loss or deficiency of the tetrahydrobiopterin (BH_4) cofactor or lack of the substrate L-arginine can provoke the oxidase function of NOS and liberate $\cdot O_2^-$ instead of NO [33–36]. In the resulting property of NOS as an oxidase it is immediately apparent that the enzyme itself could antagonize NO synthesis by trapping NO partially or completely dependent on the degree of conversion to the oxidase form.

In summary at least four $\cdot O_2^-$ generating systems can be defined which alone or in combination can be activated to provide $\cdot O_2^-$ in a defined way. Since NO is synthesized by many events, e.g., by shear stress and several agonists [37], the simultaneous generation of $\cdot O_2^-$ will lead to a fast reaction between the two radicals causing an immediate decrease in the free NO-levels which will result in vasoconstriction and loss of other NO-dependent functions.

SUPEROXIDE AS AN NO SCAVENGER

This new aspect came up after the significance of NO was recognized and its reaction with $\cdot O_2^-$ was found to be extremely fast ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) [7]. Already a rough calculation revealed that the existence of NO and $\cdot O_2^-$ is mutually exclusive, i.e., an excess of $\cdot O_2^-$ eliminates the messenger functions of NO, but vice versa NO would counteract possible actions of superoxide and hence could be considered an antioxidant. Indeed, some reports suggest antioxidant functions of NO [7, 37–39], but the mechanisms behind are chemically and physiologically complex and not subject of this review.

Scavenging of NO as an important messenger makes $\cdot O_2^-$ an antagonist to NO and already simply by this property $\cdot O_2^-$ becomes a messenger molecule itself [40–44]. This can be best demonstrated in the vascular system where NO release by the endothelial NO-synthase (NOS-3) controls vascular tone by activating guanylyl cyclase of smooth muscle to cause relaxation of the vessels by a cGMP-dependent phosphorylation cascade [5, 45–47]. Superoxide by counteracting this NO action indirectly develops vasoconstricting properties which has been repeatedly postulated and experimentally shown [42, 43, 48]. The question arises, however, whether trapping of NO is the basis of a physiological regulatory process or rather exists as a side reaction unavoidably associated with the ubiquitous occurrence of $\cdot O_2^-$ derived from unspecific autooxidations of many reducing components of the cell. The existence of the previously listed defined sources for $\cdot O_2^-$ favors the hypothesis of a superoxide driven redox regulation.

PEROXYNITRITE AS A SUPEROXIDE-DERIVED EFFECTOR

Since $\cdot O_2^-$ appears too weak as a direct oxidant a derived peroxide would be a better candidate for mediating oxidative effects on regulatory biomolecules. The reaction product of NO and $\cdot O_2^-$ is the unstable molecule peroxynitrite ($^-$O—O—N═O) [7]. Its chemical reactivity is primarily due to its easy protonation ($pK = 6.8$) and a facilitated O—O bond cleavage in the trans-configuration of its acid form [7].

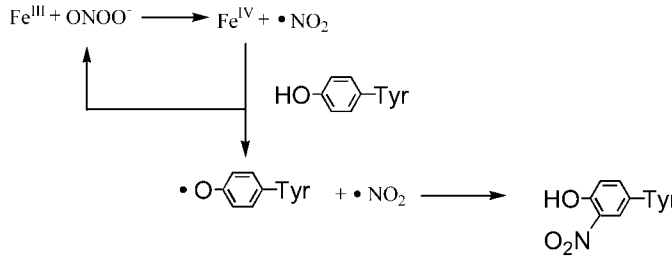
$$O{=}N{-}OO^- \xrightarrow[pK\ 6.8]{} O{=}N{-}O{-}O{-}H \rightarrow \cdot NO_2 + \cdot OH$$

Its tendency to concomitantly generate the $\cdot NO_2$ and the OH-radical has led to the opinion that peroxynitrite is a highly reactive and hence destructive molecule, causing oxidations of almost all organic molecules and nitrations as well as hydroxylations of phenolic compounds [49, 50]. We found that such statements may certainly apply to chemical systems using quite high concentrations (0.1–1 mM) of peroxynitrite. Under physiological conditions, however, the peroxynitrite anion but even better its acid form can be efficiently trapped by cellular antioxidants [51, 52]. Thus the effective tissue concentrations of peroxynitrite arising from the reaction of NO and $\cdot O_2^-$ may reach levels that never can cause oxidations of macromolecules by the mechanisms described above. However, catalysis by metal centers can replace the protonation with the consequence of higher valence states of transition metals to be formed which then cause oxidations and nitrations already at low peroxynitrite concentrations but by different mechanisms [53, 54].

Indeed, by the use of nitrotyrosine-specific antibodies MacMillan–Crow and associates [55, 56] could trace nitrotyrosine-containing proteins among which Mn-SOD could be identified [56]. In our laboratory the nitration and inhibition of prostacyclin (PGI_2) synthase had been reported and has been identified as a peroxynitrite-mediated reaction that is catalyzed by the heme-thiolate active site of this P450 enzyme at very low concentrations of peroxynitrite [57, 58]. Model investigations with cytochrome P450 enzymes allow to conclude on the mechanism shown in Scheme 1.

Since PGI_2 next to NO is an important mediator of vasorelaxation [59], this finding suggested that $\cdot O_2^-$ via formation of peroxynitrite was able not only to scavenge NO but secondarily also to inhibit PGI_2 synthase [60].

Further experiments with coronary arteries confirmed this finding and revealed that PGI_2 synthase in endothelial cells and even whole arteries was the only protein nitrated by peroxynitrite bolus concentrations up to 10 μM [60]. In this model the physiological consequences of PGI_2 synthase inhibition could directly be demonstrated: the PGI_2 dependent vasorelaxation after an angiotensin II mediated contraction was abolished by peroxynitrite and even a secondary vasoconstriction was the consequence. Using pharmacological tools this vasospasms originated from an action of the unmetabolized substrate PGH_2 at the thromboxane A_2 receptor which has the unique property of being activated by thromboxane A_2 as well as PGH_2 [61]. Thus, the previously unexplained dual specificity of the thromboxane A_2/PGH_2 receptor gains physiological relevance for potentiating the superoxide antagonism for



SCHEME 1

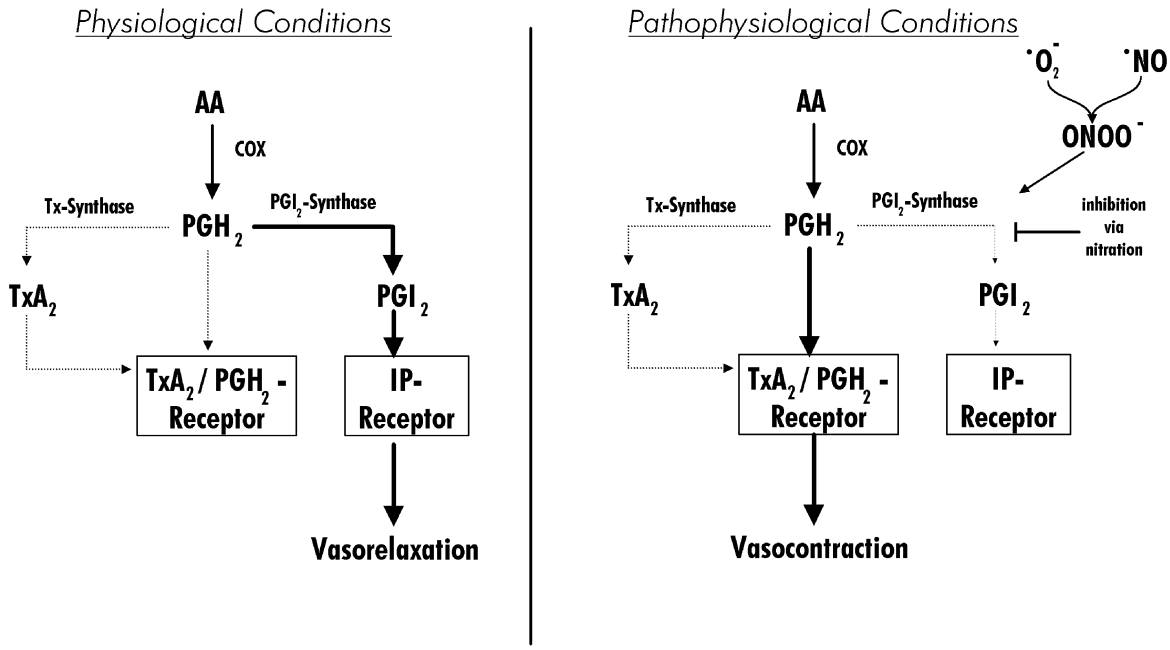
NO and PGI_2.The events observed in the coronary artery model can be summarized in Scheme 2.

In this scenario $\cdot O_2^-$ is considered a new messenger causing a double antagonistic action to NO. What, however, is the evidence that peroxynitrite indeed can be formed in endothelial or other NO producing cells and that sufficient amounts can be built up to perform the action that is attributed to peroxynitrite? Our laboratory has presented evidence for a nitration of PGI_2 synthase and consequently vasodysfunction under such conditions [62] and atherosclerotic tissue showed the same effects [63].

It often had been suggested that endotoxin (LPS) exposure to endothelial cells causes oxidative changes that are responsible for subsequent endothelial dysfunction [64]. This situation could be mimicked in the coronary artery model. LPS exposure of the segments leads to a vasospasm after a preincubation time of at least 30 min and was accompanied by a decrease in PGI_2 formation and enzyme nitration. Most importantly, these effects were abolished when polyethyleneglycolated Cu,Zn-SOD was coincubated during angiotensin II stimulation. Since inhibition of NOS was preventing the LPS-effects as well, it can be safely concluded that $\cdot O_2^-$ together with NO was responsible for the nitration and inhibition of PGI_2 synthase. With regard to the source of $\cdot O_2^-$ the results are still preliminary but inhibition of both the nitration and the vasospasm by allopurinol suggest xanthine oxidase as a major contributor. However, it should be recalled that the conversion of xanthine dehydrogenase to its oxidase form requires an additional oxidative or proteolytic step upstream of xanthine oxidase action and hence the underlying mechanism of LPS-mediated peroxynitrite formation must be more complex [*FASEB J.*, submitted].

PATHOPHYSIOLOGICAL IMPLICATIONS

The action of $\cdot O_2^-$ via peroxynitrite as an antagonist of NO and PGI_2, together with the concomitant activation of the TxA_2/PGH_2 receptor, represents a missing mechanistic link for many related findings on oxidative endothelial dysfunction [62, 63]. Based on such sophis-



SCHEME 2

ticated interactions the release of superoxide may, however, not just cause vasospasm as a pathological event but may serve a physiological regulatory function in host defense. Eliminating NO as well as PGI_2 as the main endothelial messengers will abolish the vasorelaxing properties of the endothelium. Together with stimulation of the TxA_2/PGH_2 receptor by the unmetabolized PGH_2 the endothelium will express adhesion molecules [65] followed by leukocyte attachment and by contraction of the endothelial cells the endothelial barrier will become leaky and penetrable for blood monocytes [66, 67]. This would be an important first step in tissue defense or the wound healing process.

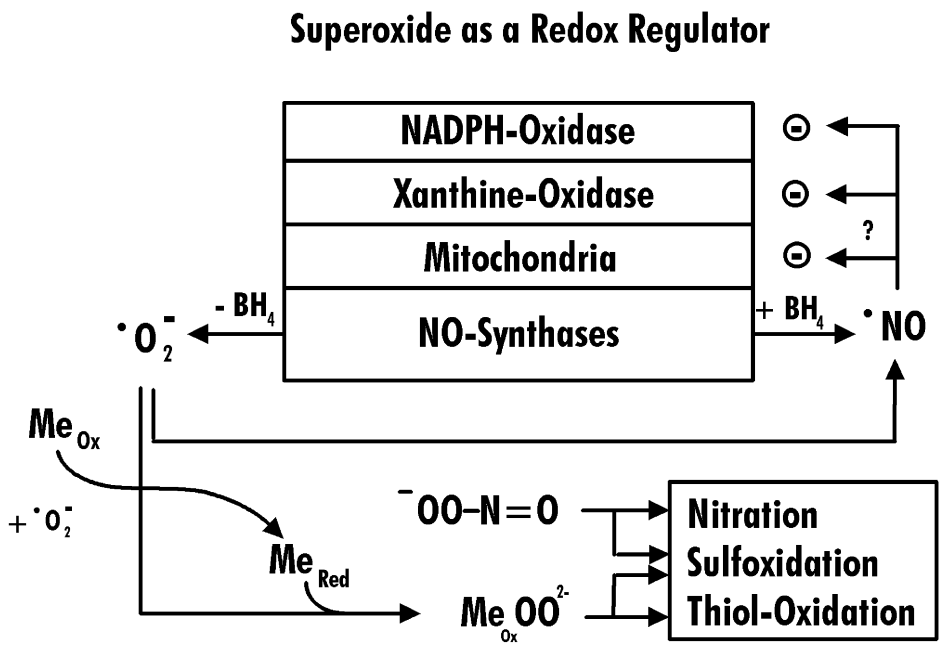
If, on the other hand, $\cdot O_2^-$ production becomes excessive, like in ischemia/reperfusion, the tissue damage by infiltrating leukocytes may become destructive and directed against tissue cells. Even the aging process studied in rats was accompanied by an increased superoxide production as measured by lucigenin chemiluminescence, lower NO levels and interestingly a nitration of mitochondrial Mn-SOD [van der Loo *et al.*, submitted]. Therefore, many events causing prolonged stimulation of the endothelium may also involve an increased $\cdot O_2^-$ production with a consequent drop of NO and PGI_2 levels leading to endothelial dysfunction or vascular diseases.

SUPEROXIDE AS A MESSENGER IN THE ABSENCE OF NO

The unique consequences of the superoxide reaction with NO leads to the question whether $\cdot O_2^-$ can perform redox regulations without NO. At unphysiological low pH values the protonated $\cdot O_2H$ radical is a more reactive species but at physiological pH its concentration is too low ($pK = 4.8$) and its radical mechanism not suited to cause sulfoxidations of methionine, sulfenic acid formation or the oxidation of vicinal dithiols to disulfides. However, taking into account the reducing capacity of O_2^- it has been confirmed that ferritin-bound ferric ions can be reduced and released [11] and also Cu^I or Mn^{II} levels may increase as a consequence of superoxide formation. These metals tend to associate with protein structures and then would be able to react with O_2^- by formation of peroxo- or oxo species; e.g.

$$Fe^{3+} + O_2^- \xrightarrow[-O_2]{} Fe^{2+} \xrightarrow{+\cdot O_2^-} Fe^{III}OO^{2-} \text{ or } Fe^{V}{=}O$$

Such species are more reactive than H_2O_2 and can act locally and hence selectively at metal-binding domains. There are indications that guanylyl cyclase can be inhibited by O_2^- even in the absence of NO [68, 69] and also for calcineurin a superoxide-dependent inhibition has been reported [70]. Both enzymes have multiple metal binding sites and contain vicinal dithiols as redox regulatable sites. The zinc finger regions of PKC also have been reported to be sensitive to superoxide [71]. Elevated Fe^{II} and Cu^I levels occur in neurodegenerative diseases and therefore may transmit the action of O_2^- by the above mentioned mechanism [72, 73]. Since NO is abundant in brain both mechanisms of



SCHEME 3

superoxide activation, the formation of peroxynitrite and the activation by reduced transition metals, may occur simultaneously.

SUMMARY AND OUTLOOK

There is no doubt that redoxregulation can involve superoxide as a mediator and that its release by the four oxidase systems mentioned must be carefully controlled. Superoxide dismutases guarantee a low basal level as a prerequisite for NO to exert its messenger functions. NO not only scavenges O_2^- and therefore behaves as an antagonist but also inhibits the activity of xanthine oxidase [74] and NADPH-oxidase [75] and even may limit electron transport in mitochondria [76]. Vice versa O_2^- will counteract the physiological functions of NO by various mechanisms and therefore will lead to cell activation, growth and proliferation. A simplified Scheme 3 summarizes the present results and the working hypothesis of O_2^- action.

Thus, our picture of superoxide as an autooxidation-derived reactive oxygen species leading to oxidative stress needs refinement and addition of its newly established regulatory properties. It can be expected that such new vistas will also find pharmacological applications by using SOD overexpression [77], chemical O_2^- or peroxynitrite quenchers or selective inhibitors for the various oxidases involved.

It was the aim of this review to bring into focus the possible messenger functions of superoxide and to shed light on the chemistry involved. We therefore did not consider in detail the many reports and findings on the involvement of reactive oxygen species in endothelial function, since there already exist excellent and recent reviews on this medically important area [78–81].

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