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Meeting Report

Superoxide and nitric oxide – participation in cell communication

P Sarti*,[1], L Avigliano[2], A Görlach[3] and B Brüne*,[4]

[1] University of Rome, La Sapienza, The IInd Faculty of Medicine, Department of Biochemistry, Italy
[2] University of Tor Vergata, Department of Experimental Medicine and Biochemical Sciences, Italy
[3] German Heart Center Munich, Department of Experimental Pediatric Cardiology, Germany
[4] University of Kaiserslautern, Faculty of Biology, Department of Cell Biology, Germany
*Corresponding authors: B Brüne, University of Kaiserslautern, Faculty of Biology, Erwin Schrödinger Strasse 13, 67663 Kaiserslautern, Germany.
E-mail: bruene@rhrk.uni-kl.de; or P Sarti, University of Rome, La Sapienza, Piazzale Aldo Moro 5, 00185 Rome, Italy. E-mail: paolo.sarti@uniroma1.it

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Villa Vigoni Conference 'Superoxide and nitric oxide – participation in cell communication', Villa Vigoni, Loveno di Menaggio, Italy, 18–21 April 2002

The Italian-German Villa Vigoni conferences were established over 10 years ago with the intention to discuss radical-mediated reactions that affect apra- and autocrine cell communication. In the tradition of the previous meetings the 7th conference was organized at the beautiful Villa Vigoni, Loveno di Menaggio, Italy, as a joint Italian-German event with a total of 30 participants.

Life demands intra- and intercellular communication to respond and adapt to changes in the environment. Among signaling molecules, reactive oxygen (ROS) and nitrogen (RNS) species gained attention and have been the focus of this conference.

Oxygen is an ideal electron sink. By accepting four electrons and protons from the mitochondrial environment it releases water, the final product of the electron transport chain. The beauty of this system is that oxygen and water are relatively safe; only relatively, however, since the oxygen chemistry can be toxic. The dark side of the story involves single electron transfer to oxygen to produce superoxide anions. Superoxide can start reactions of chemical destruction in cells by promoting self-perpetuating chain reactions. In addition, harmful reactions may arise when hydrogen peroxide, in the presence of Fe^{2+}, generates hydroxyl radicals (Fenton reaction). However, despite their potential damaging action, ROS are used to guarantee physiological signaling and to orchestrate cell communication in vital cells. A quite similar story holds true for NO. In the last 15 years NO made its way from being considered a damaging/toxic molecule to a master regulator of cell communication. Of note, RNS include not only NO (the radical) but also those species resulting from oxidation, reduction, or adduction of NO in physiological milieus.

Although appreciating the physiological role of ROS and RNS in transmitting physiological signals a number of hot topics emerged in the center of intense discussions at the 2002 Villa Vigoni conference:

Nitric oxide: formation and actions

NO touches upon multiple aspects of intracellular signaling pathways such as gene activation, protein expression and activity regulation of enzymes. It is becoming clear that some important pathological conditions such as psoriasis are associated with too little NO formation. The molecular reason for this may be the overexpression of arginase detected in some phases of the disease. Arginase consumes arginine and thus limits substrate availability for the NO-synthases (NOSs). In turn, too little NO may promote cell proliferation instead of encountering the useful anti-proliferative impact of elevated NO concentrations generated by iNOS. In other experimental systems NO, via control of sphingomyelinase activities and formation of cGMP, attenuates ceramide formation and initiation of apoptosis. In this respect, it has been shown that the inhibition of the 'acid sphyngomyelinase' needs consideration. In addition, under different experimental conditions, characterized by laminar shear stress of the human umbilical vein endothelium, NO-elicited p21 expression may contribute to an anti-apoptotic signaling cascade. A different picture emerges when NO stimulates cyclooxygenase-2 (Cox-2) activity, most likely by enhancing the peroxide tone needed for Cox-2 activity. In this case NO production (most likely in association with elevated, i.e. higher NO concentrations), contributes to inflammation by enforcing formation of prostanoids (S Cuzzocrea, Italy). Alternative mechanisms of cell destruction under the impact of NO may arise when the formation of peroxynitrite (produced under conditions of NO and superoxide cogeneration) stimulates a secretory phospholipase A_2 activity and thus promotes arachidonate release. To intervene with pathways of $ONOO^-$ action one may consider the compartment of its generation. Thus, the efficacy of inhibitors may depend to some extent on their accessibility to those cellular compartments where these radicals are produced. Peroxynitrite is known for its potency to oxidize thiol groups and to inactivate –SH groups, especially zinc-finger

binding motifs. By using ADH (alcohol dehydrogenase) as a model system, it has been shown that superoxide will not inactivate the enzyme unless NO (generated by spermine-NONOate) is present in a 1 : 1 ratio compared to superoxide. Under these conditions inactivation of ADH is reversed by superoxide dismutase (SOD). Interestingly, however, inactivation is prevented also when the concentration of NO increases over that of O_2^-. It is proposed that NO may act as a very efficient and potent inhibitor of the $ONOO^-$ oxidative chemistry, by giving rise to a protective protein nitrosation. This may apply to conditions when an excess of NO is formed over superoxide. Therefore, one may envision that the rate of NO versus superoxide formation determines the destiny of the target proteins, whether they will be oxidized or nitrosated, with the important concept that excessive NO may prevent $ONOO^-$ chemistry (V Ullrich, Germany). Thus, the balance between NO and superoxide determines the reactivity of these partners when looking at model substrates such as ADH or endocannabinoid receptors as targets of oxidation/nitrosation. The complexity of reactions between NO and O_2^- is also reflected under cellular conditions and affects gene activation. Although some controversy still remains as to whether NO attenuates or activates transcription factors such as NF-κB, lessons from iNOS knockout mice imply an important role of NO in NF-κB activation. What is becoming clear is that besides O_2^-, NO also activates genes. There are examples where NO induces the expression of some proteins while suppressing others, which may be elegantly followed by a proteomics approach. Again, the NO over O_2^- balance will be important to fully understand the complete cell response. To fully uncover gene expression under inflammatory conditions we need to determine the time course of NO and O_2^- formation which will be mirrored by enzymes capable of generating these radicals. At the same time we need to take into consideration the expression of major antioxidative components that counteract signaling pathways evoked by either NO or O_2^-. Only the sum of radical production, radical interactions and defense mechanisms will determine a specific cell response. Therefore, it can be envisioned that the biological milieu (intracellular redox environment), which is cell type specific and which may depend on the cell phenotype, accounts for specific NO/O_2^- actions.

An additional piece of information on the pathophysiological relevance of the NO chemistry resides in the role played by NO in the control of cell respiration and ATP production. It is now agreed that NO can react very rapidly with mitochondrial complex IV in competing with oxygen. NO-binding is being characterized by a fairly low Ki, i.e. in the order of nM when oxygen tension in tissues is physiologic. Once bound to the enzyme in the active site, cell respiration is inhibited. Depending on environmental conditions, particularly on the electron flow level and fluxes through the respiratory chain, complex IV can either directly cope with NO, oxidizing it to harmless nitrite or wait for NO displacement from the active site. The latter may be a slow process and may lead to bioenergetic failure.

Oxygen control of gene expression

There is unquestionable evidence that a decrease in the oxygen content causes activation of the hypoxia inducible transcription factor HIF-1, which initiates many of the classical cell responses towards hypoxia. The oxygen/hypoxia regulated subunit HIF-1α is subjected to stability regulation and proline hydroxylation of HIF-1α allows association with the von Hippel Lindau protein (a E3-ligase) to promote 26S-proteasomal degradation. Although this scenario *per se* does not allow space for redox regulation there is experimental evidence that ROS participate in HIF-1 regulation and/or gene expression of established HIF-1 target genes. It is suggested that under normoxia the generation of ROS (O_2^-, H_2O_2, Fenton chemistry) will attenuate HIF-1α accumulation and that scavenging of ROS may simulate hypoxic conditions. However, observations from other groups rather proposed that O_2^- generation by NAD(P)H oxidase occurs in response to thrombin to stabilize HIF-1α in a p38 MAPK and Akt/PKB-dependent fashion. Apparently, HIF-1 activity and HIF-1α stabilization is under the control of O_2^- although positive and negative effects are reported. Apart from directly modulating proline hydroxylation of HIF-1α, stress-evoked signal transduction pathways may indirectly modulate established concepts of HIF-1 regulation in association with O_2^- formation. Again, a very similar situation holds true for NO. NO attenuates hypoxia-evoked HIF-1α responses but stabilizes the protein during normoxia. An explanation may be offered by different reactivities of NO due to changes in O_2^- production under normoxia *versus* hypoxia. In endothelial cells NO provokes angiogenesis which, among other control mechanisms, may be also connected with NO-induced upregulation of HIF-1α.

The angiogenetic process involves the exit of endothelial cells from quiescence to promote cell migration, to degrade the extracellular matrix and sustain cell proliferation, ultimately leading to differentiation of vascular beds into functional capillaries. Endothelial dysfunction and reduced production of NO are a predominant feature of vascular pathologies, such as atherosclerosis. Any disturbance in endothelial cell function causes the impairment of the angiogenic response during regeneration. It has been demonstrated that NO directs endothelial cells in each major step during angiogenesis. In endothelial cells NO-synthase activation provokes MAPK signaling, which in turn increases transcription and production of the growth factor (FGF-2), that in turn promotes an autocrine mechanism of cell survival. Thus, proper activation of NOS in endothelial cells can be considered a limiting step to trigger angiogenesis (M Ziche, Italy). Experimental evidence indicates further that these molecular signals attenuate an apoptotic event in the endothelium. Pathways may be activated by ACE inhibitors and drugs increasing NO production in the endothelium.

Besides paying attention to HIF-1α it became clear that ROS may stimulate expression of matrix metalloproteinases that in turn will allow blood vessel formation. Therefore, multiple targets for both NO and O_2^- need consideration during oxygen sensing, which also applies to even more complex scenarios such as angiogenesis. Again, one may envision that the formation and interaction of NO and O_2^- in terms of time and restricted compartments contributes to gene expression under hypoxia and/or normoxia.

Superoxide: formation and consequences

Superoxide, in some analogy to NO, must be considered a regulator of gene/protein expression and modulator of enzyme activity. Production of O_2^- is, among other sources, facilitated by membrane bound NAD(P)H oxidases, with the notion that ROS formation is not restricted to phagocytes. It is not surprising that individual components of the NAD(P)H oxidase are subjected to expression regulation and that NO as well as superoxide itself modulates their expression. Agonists of PPARγ suppress ROS formation in macrophages which may add to the reported anti-inflammatory actions of PPARγ-agonists. Statins reduce ROS generation in VSMC by preventing rac translocation to the membrane which in turn attenuates NAD(P)H oxidase assembly. For endothelial cells a new concept emerges when EDHF (endothelium-derived hyperpolarizing factor)/EET's (epoxyeicostrienoic acids) reduce ROS formation (F Krötz, Germany). It can be hypothesized that endothelial cell activation uses the EDHF-signal to deliver a self-terminating anti-oxidative component to limit O_2^- formation that occurs following depolarization. Besides these examples of cellular feed-back control systems the use of SOD-mimetics (Mn-type) is envisioned to attenuate acute and chronic inflammatory tissue damage. This makes perfect sense, if taking into account that ROS are implicated in the initiation of apoptosis in part by producing ceramide and/or damaging mitochondria and moreover, if considering that ROS contribute to disease states such as arteriosclerosis, myocardial infarction, and tissue remodeling.

Conclusions and perspectives

The presentations and discussions provided some detailed insights into the world of NO and O_2^- with the indication that interactions between these radicals touches on most, if not all signaling systems, thus increasing signaling complexity (Figure 1).

ROS- and RNS-signaling is important to understand cell physiology with the notion that marginal changes, i.e. flux rates of either NO or O_2^- or altered defence systems may shift vital signals used for communication into areas of pathology in close association with human diseases. We are far from understanding the world of radicals but we need to gain more information in order to predict how a cell behaves under conditions of NO/O_2^- production with the intention to intervene on demand. The most fascinating challenge will be to define the border line when radical signaling transits from physiology to pathology and how production of ROS as well as RNS are regulated to meet the requirements of patho-physiological signaling.

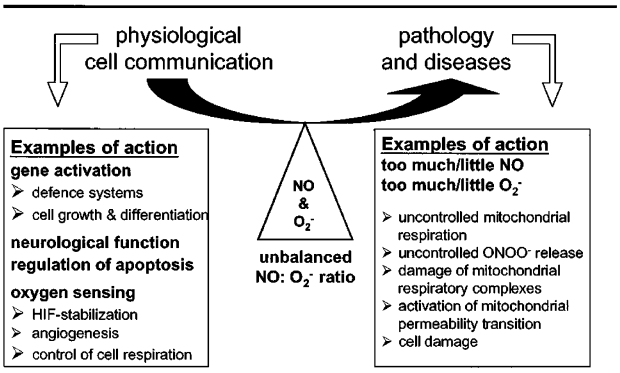

Figure 1 The role of NO and O_2^- in cell communication. Examples of radical (NO and/or O_2^-) signaling in the transition from physiology to pathology/disease states. For details see the text

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Further reading

The reader may appreciate some review articles published in CDD during the past that cover individual aspects addressed in this meeting report for further information.

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