Reactive Nitrogen and Oxygen Species: Role and Evidence of their Production in Humans

M. Lamy, M. Nys, and G. Deby-Dupont

Introduction

For a long time, it has been recognized that the effects of excessive production of reactive oxygen species leading to oxidative stress are damaging to cellular constituents with subsequent tissue necrosis. There is now evidence that reactive oxygen species can alter cell functions by acting as 'second messengers' [1] or by affecting proteins of the signal transduction pathway that leads to gene expression or to apoptosis [2–4]. But many questions remain about the role of reactive oxygen and nitrogen compounds: how and where do they act? Do they act by themselves or by way of secondary products like nitroso- or nitrated compounds, oxidized forms of thiol compounds such as GSSG (the oxidized form of gluthatione), α,β-unsaturated aldehydes or carbonylated proteins? Do they alter specific proteins of the signal transduction pathway or intervene in Ca^{2+} metabolism? Do they act at the level of the nucleus by oxidation of thiol proteins involved in the activation of gene expression? Do specific reactive oxygen species (ROS) such as nitric oxide (NO) or peroxynitrite, have specific activity on selected biomolecules? And, above all else, what can be transposed from *in vitro* studies or laboratory models to *in vivo* situations?

Redox Balance and Oxidative Stress

As early as 1973, it was determined that $\pm 2\%$ of molecular oxygen (O_2) reduced by the mitochondrion forms superoxide anion ($O_2^{\cdot -}$) and its dismutation product hydrogen peroxide (H_2O_2) [5]. This observation, ignored for a long time, is now regaining importance with the development of molecular biology studies on signal transduction and apoptosis. A clear distinction has to be made between oxidative stress and redox modifications by 'physiological doses' of reactive nitrogen and oxygen species (RNOS). Most of the studies dealing with the ROS in signal transduction pathways were done with hydrogen peroxide at doses $\geq 10^{-4}$ M, which are concentrations unlikely to be reached *in vivo* except in (or in the neighborhood) of activated phagocytes.

The redox state (E_0) of a compound is defined by its capacity to capture (oxidant compound) or to release (reducing compound) one or more electrons. A normal biological milieu is characterized by a redox equilibrium between oxidants and reductors. Despite difficulties in the precise measurement of this redox equilibrium, cytosol seems to be a reducing milieu in physiological conditions, by the presence of

J.-L. Vincent (ed.), *Yearbook of Intensive Care and Emergency Medicine 2001*
© Springer-Verlag Berlin Heidelberg 2001

glutathione (GSH), NAD/NADH and NADP/NADPH, particularly in leukocytes [6]. In physiological conditions, enzymatic and non-enzymatic antioxidants balance the production of ROS, delaying oxidation in the cellular milieu and heavy alterations of redox status. But, this balance is altered by excessive activation of NADPH-oxidase in stimulated leucocytes. *In vivo*, an excessive production of RNOS is possible in inflammatory pathologies characterized by an intense activation of leukocytes, or in unusual situations such as massive irradiation. In these situations, when the antioxidant buffering capacity is overloaded or pathologically depressed, oxidative damage is observed and in these conditions an oxidative stress is possible.

Endogenous Origins of RNOS

RNOS are small size molecules, most often highly unstable particularly when they are in a radical form. They rapidly diffuse and react with neighboring molecules in an unspecific manner. The detection and identification of RNOS remains difficult, which has often shed some doubt on their exact site of production and on the importance of their production. Nonetheless, specific enzymes are responsible for their production and for their destruction, and most of these enzymes have been identified and characterized. They have been localized in many cell types, so that RNOS production appears to be a general *in vivo* phenomenon, tightly regulated and linked to normal cell metabolism. ROS production has been particularly studied in lymphocytes, monocytes, macrophages and neturophils, these latter being the main producers of RNOS in stimulation conditions.

To summarize, it can be assumed that RNOS are produced mainly by seven pathways:

1) by NADPH-oxidase. This enzyme is a membrane-associated enzymatic complex which is responsible for the high oxygen consumption (the respiratory burst) that occurs during phagocytosis. NADPH is present in a resting form, but is quickly activated by soluble mediators and specific stimuli acting on membrane receptors. This enzyme uses NADPH as an electron donor to turn molecular oxygen to superoxide anion ($O_2^{\cdot-}$) by a monoelectronic reduction. H_2O_2 derives from superoxide anion. Hydroxyl radical ($^{\cdot}OH$) can be formed from H_2O_2 in the presence of complexed iron ions (Fenton reaction), but the *in vivo* production of $^{\cdot}OH$ by the Fenton reaction still remains unproved. H_2O_2 is also the substrate of the neutrophil enzyme myeloperoxidase (MPO), which (in presence of chloride anion) synthesizes the oxidant hypochlorous acid (HOCl). These ROS are released in the phagosomes and in the extracellular milieu, but also in the cytosol where they modify the redox status of the cell [7]
2) by the electron transport chain of mitochondria. The mitochondrial enzymes normally produce ROS as byproducts of the electron transport chain that normally transforms molecular oxygen into H_2O by a 4 electron reduction [5]. Leakages in the electron transport chain result in the monoelectronic reduction of molecular oxygen into superoxide anion, precursor of H_2O_2. Two sites responsible for electron leakage have been identified, the NADH dehydrogenase and the intersection ubiquinone-cytochrome b. Hypoxia appears to severely increase the production of RNOS in mitochondria by an enhanced activity of monoelectron-

ic reduction of oxygen and NO synthase (NOS) activity. Mitochondria are equipped with a characteristic Cu-Mn^{2+} superoxide dismutase (SOD) that neutralizes superoxide anion, preventing its reaction with other ROS, especially NO, limiting the *in situ* production of peroxynitrite. This SOD is quickly inducible to assume mitochondrial defense, especially in hyperoxic and hypoxic conditions.

3) by the metabolism of arachidonic acid. Arachidonic acid metabolism is regulated by the family of lipoxygenase and prostaglandin H (PGH) synthase enzymes. The PGH synthases function as cyclooxygenase and hydroperoxidase, going through radical steps that are responsible for secondary reactions generating superoxide anion and other ROS. Like cyclooxygenases, lipoxygenases can produce superoxide anion as side products of their enzymatic activity.
4) by cytochrome P450. The primary physiological role of the cytochrome P450 family is to detoxify lipid-soluble drugs and their metabolites. The enzymes of the cytochrome P450 family are ubiquitous. They use high-energy electrons transferred from NADPH to add hydroxyl groups to hydrophobic molecules associated with lipid bilayers. Both superoxide anion and H_2O_2 are produced during the catalytic cytochrome P450 activities. Members of this superfamily of enzymes are present in leukocytes and tissues (liver, lung) and may be physiologically important sources of ROS.
5) by xanthine-oxidase. This cytosolic enzyme converts xanthine or hypoxanthine to uric acid, and is derived from xanthine dehydrogenase by the oxidation of thiol groups or limited proteolysis that occurs during hypoxia/reoxygenation. Xanthine oxidase uses molecular oxygen as electron acceptor and generates $O_2^{\cdot-}$ and H_2O_2. Xanthine oxidase activity has been demonstrated in many tissues and cells including alveolar cells and leukocytes (especially macrophages and neutrophils). Its activity would be regulated by NO, possibly implicating the production of peroxynitrite.
6) by NOS. This enzyme (in its constitutive or inducible isoforms) generates NO which appears to possess variable pro- or anti-inflammatory cellular effects. This enzyme is expressed in many cells and its inducible isoform is expressed in macrophages, neutrophils and endothelial cells. Unregulated NO production seems to have detrimental effects, which can be largely attributed to the production of peroxynitrite (ONOO-) formed by the reaction of NO with $O_2^{\cdot-}$. $ONOO^-$ is a highly toxic compound able to modify signal transduction pathway by oxidation or nitrosylation. Other effects of NO would be the generation of iron-nitrosyl complexes with FeS-enzymes (enzymes of mitochondria), the inactivation of antioxidant enzymes such as catalase, GSH-peroxidase, and SOD. NO would also induce apoptosis by increasing the activity of capsase-3 and by induction of the permeability transition in mitochondria.
7) by several other sources such as the autoxidation of ascorbic acid, thiols, norepinephrine, and flavine coenzymes.

A Newcomer on the Set of RNOS: Peroxynitrite

In vivo Production

In vivo, $ONOO^-$ is formed from the reaction of NO with $O_2^{\dot{-}}$. The first evidence of this synthesis is based on the observation that the presence of SOD prolonged the lifetime of NO˙ by removal of $O_2^{\dot{-}}$. The constant of the reaction of NO˙ with $O_2^{\dot{-}}$ is higher than the constant of the reaction of SOD with $O_2^{\dot{-}}$ [8], indicating that the production of $ONOO^-$ *in vivo* is highly probable despite the presence of SOD, when NO and $O_2^{\dot{-}}$ are formed simultaneously, and particularly when NO is produced in large quantities.

NOS is present in many cells that also possess the enzymatic equipment responsible for $O_2^{\dot{-}}$ synthesis. The existence of $ONOO^-$ in biological systems was demonstrated for the first time by Beckman et al [9]. Since then, the production of $ONOO^-$ has been identified in tissues, in cells, and even out of the cells. In macrophages and neutrophils, $ONOO^-$ would be responsible for the major part of the luminol-dependent chemiluminescence observed when these cells are stimulated [10]. $ONOO^-$ produced by the phagocytes appears as a major cytotoxic agent released during sepsis and used for the host defense against invading microorganisms, but also produced in excess in inflammation and in ischemia/reperfusion.

Concentrations of NO in the endothelium have been estimated to be between 100 to 400 nM, but when endothelial cells are stimulated (by endotoxins for example) or in pathological conditions of ischemia-reperfusion, concentrations of 0.5 µm NO have been measured on the surface of the endothelial cells [11]. By luminol-dependent chemiluminescence, an immediate oxidant production by stimulated aortic endothelial cells was observed that was efficiently inhibited by the NOS inhibitor, nitro-L-arginine methyl ester, and by SOD, implying dependence on the presence of both NO˙ and $O_2^{\dot{-}}$, and thus of $ONOO^-$, for oxidant production. Vascular endothelium would also generate peroxynitrite in response to carbon monoxide [12], which is a byproduct of endothelial heme oxygenase activity, an enzyme induced by the exposure of the endothelial cells to free hemoglobin.

It also appears that NO is produced inside the mitochondria where it plays a role in the regulation of the respiration by its binding to cytochrome c oxidase [13]. As mitochondria normally form $O_2^{\dot{-}}$, albeit at a low level, the production of $ONOO^-$ is possible, a production that could be enhanced during and after ischemia when the damaged mitochondrial respiratory chain uncompletely reduces O_2 and releases $O_2^{\dot{-}}$.

It is likely that, in stimulating and pathological conditions, $ONOO^-$ could be formed in most of the cells which contain inducible or constitutive NOS and enzymes synthesizing $O_2^{\dot{-}}$ such as NADPH oxidase and xanthine oxidase, and that $ONOO^-$ is particularly formed in mitochondria submitted to pathological conditions such as ischemia reperfusion. Endotoxins were reported to trigger the formation of $ONOO^-$ in the lungs [14], data from which it could be extrapolated that the production of $ONOO^-$ is highly probable in sepsis.

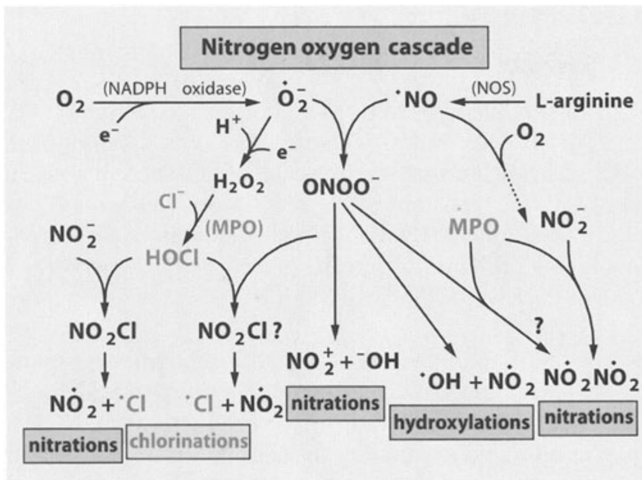

Fig. 1. Schematic overview of the mechanisms of production of peroxynitrite ($ONOO^-$) and its reaction with other reactive oxygen species.
NOS: NO synthase; *MPO:* neutrophil myeloperoxidase; $O_2^{\dot{-}}$*:* superoxide anion; $^{\cdot}NO_2$*:* nitryl radical; NO_2^-*:* nitrite ion; NO_2^+*:* nitronium ion; NO_2Cl*:* nitryl chloride; $^{\cdot}Cl$*:* chlorine radical; *HOCl:* hypochlorous acid; $^{\cdot}OH$*:* hydroxyl radical; ^-OH*:* hydroxyl anion

Interactions of Peroxynitrite with Myeloperoxidase in Phagocytes

In neutrophils, a net of interactions is possible between the ROS and the RNOS (Fig. 1). The enzymatic equipment of neutrophils allows them to synthesize $NO^{\cdot}$ by their NOS, $O_2^{\dot{-}}$ by their NADPH-oxidase and HOCl by their myeloperoxidase using H_2O_2 derived from the important flux of $O_2^{\dot{-}}$ produced by the stimulated neutrophils. They also produced $ONOO^-$ from the diffusion-controlled reaction between NO and $O_2^{\dot{-}}$, and this $ONOO^-$ can be reduced to NO_2^-. HOCl may react with this latter molecule to form nitryl chloride (NO_2Cl) which can undergo heterolysis to nitryl cation (NO_2^+) responsible for nitration. There is also a possibility of $^{\cdot}NO_2$ production (and thus of nitration) by the activity of myeloperoxidase using NO_2^- or reacting directly with peroxynitrite [15, 16]. In 1996, a nitration of bacteria ingested by cytokine-stimulated neutrophils was observed [17], confirming that these cells produce $ONOO^-$ as an oxidant in host defense. However, in situations of excessive activation and degranulation, $ONOO^-$ and myeloperoxidase can be released out of the cells, damaging the neighboring cells and tissues. It was demonstrated that human phagocytes and lymphocytes produce $ONOO^-$ when they are challenged with lipopolysaccharide [18].

Role of RNOS

ROS are powerful antimicrobial agents. Their production by stimulated phagocytes allows the destruction of microoorganisms into the phagolysosome, but ROS are also damaging agents for the neighboring cells and tissues when they are produced

in excess [19]. They are especially damaging for the unsaturated lipids of membranes, of which the permeability is altered by lipoperoxidation. Lipoperoxidation produces stable lipid-derived aldehydes (such as hydroxynonenal) able to diffuse and react with amino acids and that seem be responsible for immunosuppression. Nucleic acids are altered by ROS (reaction with thymine, guanine and deoxyribose), leading to DNA breaks and mutations. ROS are also responsible for depolymerization of hyaluronic acid, for protein alterations (tryptophan, histidine and cysteine are frequent targets) and for zymogen activation. $HO^{\cdot}$ and lipid radicals are highly reactive by their radical nature, but HOCl and to a lesser extent H_2O_2 are also dangerous oxidant species by their longer lifetime, what allows them to reach cellular parts distant from their site of production.

The small quantities of NO biosynthesized by the constitutive isoforms of NOS have important physiological roles in cardiovascular, nervous, immune, and inflammatory systems. There is little or no evidence of cytotoxic effects of physiological amounts of NO, but in special cases, neuronal NOS can generate sufficient amounts of NO to damage target cells in the central nervous system. Inducible NOS can generate large quantities of NO leading to profound and life-threatening hypotension, as observed in sepsis, or associated with widespread cytotoxicity (cytostatic or cytocidal actions) not only for invading microorganisms [20], but also for host cells. The mechanisms of this cytotoxicity remain a hotly debated topic. NO may directly interact with protein-bound iron and iron-sulfur causing their destruction or inactivation. It also reacts with oxygen to form N_2O_3 and to release NO_2^-, an oxidant species that can be used by myeloperoxidase or react with HOCl to produce nitrating (and chlorinating) species. But the most popular mechanism for NO cytotoxicity implicates $ONOO^-$, derived from NO and $O_2^{\cdot-}$.

Despite its instability, the lifetime of $ONOO^-$ seems sufficient to permit reaction at long distance, crossing through the cell membranes and reaching the DNA [21]. With a model of microbeads covered with macrophages, it has been demonstrated that NO rapidly reacts with oxygen to form N_2O_3 and to release NO_2^-, and with $O_2^{\cdot-}$ to form $ONOO^-$, which could reach distance of ± 30 µm before being decomposed [22]. Albeit short, this distance allows $ONOO^-$ to be cytotoxic for the producer cells themselves and for the neighboring cells. Numerous *in vivo* reactions of $ONOO^-$ have been reported, with enzymes and biomolecules, but the exact mechanisms of these reactions, just as their consequences, most often remain uncompletely understood. $ONOO^-$ can react with SOD yielding a stable yellow protein adduct (identified as nitrotyrosine) and releasing copper [23].

As $ONOO^-$ attacks heme molecules, its reaction with the heme enzymes is thus expected. Recent data confirmed that $ONOO^-$ reacts with the enzymes of the mitochondrial respiratory chain, with cyclo-oxygenase, with peroxidases and with NOS. Peroxynitrite also oxidizes tetrahydrobiopterin, a cofactor of NOS [24]; it thus plays a role in the regulation of $NO^{\cdot}$ production. The reaction of $ONOO^-$ with amino acids and proteins generates spontaneous chemiluminescence, that appears to be due especially to the attack of tryptophan residues [25]. We demonstrated that the reaction of $ONOO^-$ with tryptophan was accompanied by a light emission and by the generation of free radical species [26]. We also demonstrated that $ONOO^-$ reacted in a similar manner (with light emission and generation of free radical species) with other heterocyclic molecules such as histamine and serotonin, a tryptophan-re-

lated compound. Tyrosine, free or bound in proteins, appears to be a main target for ONOO-, by a reaction yielding nitrotyrosine and catalyzed by SOD [27]. Direct evidence of radical reaction of $ONOO^-$ with tyrosine in plasma was furnished by electron spin resonance (ESR) detection of peroxynitrite-induced-tyrosine centered protein radicals [28]. However, the reality of tyrosine nitration at physiological pH remains debated [29].

Many other compounds are targets for $ONOO^-$: glycosaminoglycans and particularly chondroitin sulfates which are essential constituents of basement membranes [30], pterines and heme molecules such as cytochrome P450 [31]. With thiol compounds, $ONOO^-$ generates oxidation products, with intermediate thiyl radicals that have been detected by ESR [32]. Base modifications and strand breakages of nucleic acids by $ONOO^-$ have also been described [33]. By still unclear mechanisms, $ONOO^-$ seems to attack double bonds in lipids and is implicated in the peroxidation of lipoproteins (at the level of lipids and apoproteins) [34]. Until now the products of lipid oxidation by $ONOO^-$ are not exactly identified, and their potential nocious effects remain unknown [35].

Except for the normal phagocytosis functions of monocytes/macrophages and neutrophils, the *in vivo* formation of $ONOO^-$/ONOOH is thus undesirable. The normal cellular concentration of SOD seems sufficient to assume the complete disparition of $O_2^{\cdot-}$ on condition that NO is produced at nanomolar levels, in the concentration range needed for signaling functions. But, the NO concentrations produced by stimulated phagocytes are much higher, reaching the µM range, so that the *in situ* generation of $ONOO^-$/ONOOH is certain.

RNOS in Signal Transduction and Apoptosis

Signal transduction is a network of phenomena resulting in the activation of gene expression when cytosolic proteins known as nuclear transduction factors have entered into the nucleus and have bound to specific DNA loci. Nuclear factors induce the expression of cell proteins that intervene in host defense as in immunity reaction and inflammation, but also to the triggering of programmed cell death known as apoptosis (often presented as cell suicide) [36]. The mechanisms by which the cell machinery is oriented to the expression of selected genes or apoptosis remain largely unexplained. Signal transduction and apoptosis are processes of homeostasis. To work correctly, they need to be tightly regulated. Alterations of the redox equilibrium in the cell medium are directly implicated in the mechanisms of signal transduction and the thiol (-SH) functions appear to play a main role. They are present in a lot of compounds that intervene in the signal transduction pathway: cell receptors, caspases, nuclear factors and even nuclear proteins that regulate the binding of nuclear factors to their corresponding DNA elements. These -SH functions are easily oxidable and are ideal targets for the RNOS.

Role of ROS

Evidence has accumulated that ROS can intervene as intracellular messengers in the regulation of signal transduction and apoptosis [1–4]. For this function, ROS have to fulfill special conditions: small size, easy diffusion capacity, ubiquity, rapid synthesis and destruction. ROS fulfill these conditions, but they are also highly toxic by their oxidative capacity, so that only low concentrations of ROS could be considered as physiological cellular messengers. A normal role of ROS would be the modification of the redox balance by the consumption of reductor molecules (GSH, NADH, NADPH) or the oxidative modification of protein structures. The oxidative alteration of proteins could allow them to interact with receptors, to modify essential enzymatic activities or to bind to specific targets on DNA and trigger the signal transduction and gene expression [37]. In leukocytes, alterations of the redox state have been demonstrated to modify signal transduction.

In 1991, Bauerle and coworkers underlined the role of oxidant compounds (H_2O_2 or ionization radiation) on the activation of the nuclear transduction factors of the nuclear factor-kappa B (NF-κB)/Rel family [1]. Since these pioneer observations, the effects of ROS have been particularly studied on the member of this family commonly referred to as NF-κB, which is the heterodimer p50: p65 (Rel A) [38]. NF-κB is a unique factor by its rapidity of activation and its mechanism of regulation. It plays a major role in situations where a rapid gene induction is necessary for cell life (immunity response and inflammation) [39]. It is present in all cell types where it is present in an inactive form in the cytosol, bound to its specific inhibitor, the IκB [38]. NF-κB induces a large number of genes, especially the genes implicated in the immune and inflammatory responses. In most cell lines, it protects against apoptosis. NF-κB is activated by a host of stimuli (pro-inflammatory cytokines, bacteria, lipopolysaccharide [LPS], viruses…). Its activation starts with the phosphorylation of IκB followed by ubiquitination and leading to rapid degradation of IκB by the proteasome. NF-κB is released and quickly translocated into the nucleus. Regulation of NF-κB activation is controlled mainly by the level of IκB phosphorylation and ubiquitination, but also by the activity of the proteasome [38, 40].

Other nuclear transcription factors and several cascades of protein kinases are sensitive to ROS, and the common target for ROS appears to be a regulation domain which is activated by phosphorylation. In leukocytes, physiological concentrations of H_2O_2 are produced in response to cytokines, and H_2O_2 thus acts as second messenger [41]. The mechanisms of this activation by ROS remain unexplained. ROS would intervene by oxidation of thiol functions and glutathione would be a main target for this oxidation. The redox-sensitive transcription factors hypoxia inducible factor-1a (HIF-1a) and NF-κB are tightly regulated by the GSH-GSSG balance in lungs [42]. H_2O_2 would act by favoring the phosphorylation of tyrosine kinases or by oxidation (at the level of a cystein residue) of the protein tyrosine phosphatases (PTPs) which regulate signal transduction by dephosphorylation of the protein kinases [39]. Products of lipid oxidation (oxidized low density lipoprotein [LDL], oxidized forms of cholesterol and lipid aldehydes like 4-hydroxynonenal) also act on the regulation of gene expression for adhesion molecules, heat shock proteins, cytokines, growth factors, etc. [43]. Whatever their mechanism of action, the role of ROS is confirmed by the use of antioxidant compounds (N-acetyl-cysteine) and enzymes

(catalase), which inhibit signal transduction, mainly by inhibition of tyrosine phosphorylation [44]. But many problems remain unsolved, and the role of ROS in transduction signal activation is still contested. A recent study with endothelial cells demonstrated that H_2O_2 induced the DNA binding activity of NF-κB; however, this binding was transcriptionally 'silent' as it could not activate intercellular adhesion molecule (ICAM-1) expression [45]. Problems also remain concerning the role of ROS/RNOS in apoptosis. After an oxidative stress induced by H_2O_2, the cell can be destroyed by apoptosis or necrosis, and this orientation would depend on the concentration of H_2O_2: at doses ≥ 1 mM (oxidative stress), the cells are moving toward necrosis, and at micromolar concentrations, the cells move toward apoptosis. This difference of evolution would be dependent on the ATP level [46].

Peroxynitrite: a Key Point in Signal Transduction Alterations?

In the last years, an increasing number of studies have implicated $ONOO^-$ in the regulation of signal transduction, by damaging receptors or by intervening at other intracellular sites of the signal transduction [47, 48]. By nitration of essential tyrosine residues, $ONOO^-$ can induce the dysfunction of enzymes implicated in signal transduction and apoptosis, such as tyrosine kinases (Fig. 2). Nitrated proteins would thus be not only the inactive 'footprint' of $ONOO^-$ but also active contributors in disease processes. Tyrosine kinases must be phosphorylated on their active tyrosine site to participate to the signal transduction, and they must further be dephosphorylated to stop the signal. The enzymes that are implicated in the dephosphorylation are also tyrosine-bearing enzymes. Nitration of the tyrosine residues may alter the enzyme functions by hindering the phosphorylation of tyrosine kinases and by inactivating the dephosphorylating enzymes. Peroxynitrite may thus act

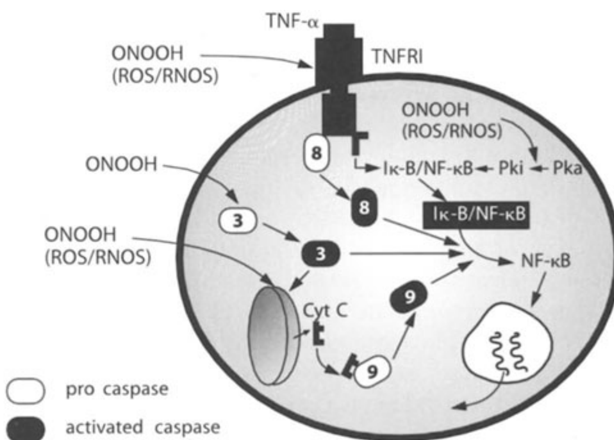

Fig. 2. Simplified view of signal transduction processes, and possible points of intervention of peroxynitrite (and reactive nitrogen-oxygen species: ROS/RNOS).
Pk: protein kinase (*i:* inactive; *a:* active); *TNFR:* tumor necrosis factor receptor; *NF-κB:* nuclear factor κB; *IκB:* inhibitor of NF-κB; *cyt c:* cytochrome C; *N:* nucleus

as a deregulating agent in apoptosis, blocking or increasing the transduction of the signal, according to the place where it is produced and the type of enzymes it attacks [49, 50]. Peroxynitrite also intervenes at other sites in the apoptosis pathway. It was recently reported that $ONOO^-$ was an activator of procaspases into caspases, the cysteine aspartases which are excutioneers of apoptosis triggered by activation of cell surface receptors. $ONOO^-$ was demonstrated to activate procaspase 3 and to act on mitochondria; from mitochondria, cytochrome c is released which activates procaspases 3 and 9 [51, 52]. Peroxynitrite could also affect transcription factors by altering their cysteine residues and by oxidation of their redox sensitive iron-sulfur clusters and zinc-finger moieties, destroying the ability of proteins to bind to DNA or to recognize the correct DNA sequences [47].

Evidence for Reactive Oxygen and Nitrogen Species in Humans

In human pathologies characterized by an acute inflammatory reaction such as sepsis and acute lung injury (ALI), to demonstrate a production of ROS is not an easy task and direct demonstrations are not easy to obtain. But, indirect proof is accumulating that ROS and RNOS are produced in blood and in lungs, mainly by the activity of leukocytes. Proof of an oxidative activity *in vivo* has been obtained by measuring the alterations of antioxidant status of blood (decrease in vitamin E concentrations, decrease in total antioxidant potential of plasma, fall in the activities of antioxidant enzymes) [53–54]. Broncho-alveolar fluids that are obtained from lung lavage during ALI and/or sepsis have been extensively studied [19, 55]. Oxidized anti-proteinases were found as early as 1986 [19], and granulocytic enzymes were measured as markers of phagocyte activation [56]. These studies accumulated data proving that an oxidative activity, largely linked to phagocyte activation, occurred in the lung during acute inflammation pathologies (Fig. 3).

Protein Nitrations: a Marker of *in vivo* Oxidative Stress?

For a few years, new methods have been proposed to identify the consequences of oxidative stress in biological fluids. These new techniques propose to measure 'nitrated proteins' as a marker of *in vivo* production of peroxynitrite, and these nitrated proteins are mainly considered to be 3-nitrotyrosine-associated proteins [57, 58]. They could be measured by immunological techniques (immunohistochemistry or ELISA with mono- or polyclonal antibodies, and Western blot analysis) or by quantitative analytical methods such as high performance liquid chromatography (HPLC) or gas liquid chromatography coupled to mass spectometry (GCMS).

Many results have accumulated in humans reporting the presence of 3-nitrotyrosine, free or bound to proteins, in various pathologies, often associated with acute inflammation and septic diseases characterized by neutrophil activation and infiltration (Table 1). Nitrotyrosine was detected in extra- and intracellular proteins in human whole blood containing PMA-activated neutrophils [59]. Extensive nitration of protein tyrosines in human atherosclerosis was detected by immunohistochemistry [60]. In Western blots of crude homogenates of atherosclerotic aorta, the antiser-

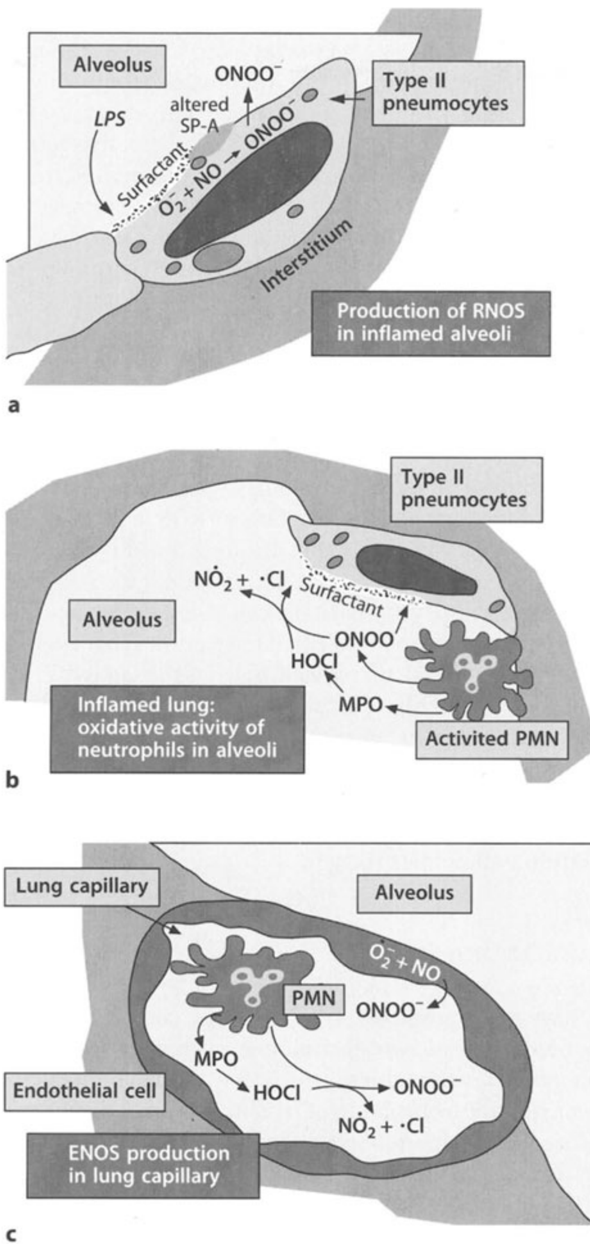

Fig. 3. Main points of peroxynitrite production in the lungs during inflammatory processes (cell activation by endotoxin, by cytokines...), highlighting the role of mitochondria in hypoxia consecutive to respiratory distress. NO• is produced by activated lung cells and by stimulated phagocytes. Superoxide anion ($O_2^{\dot{-}}$) is produced by xanthine oxidase in hypoxic cells and by stimulated phagocytes

um to nitrotyrosine reacted with a wide range of protein bands (approximately 180 to 30 kd) [61].

In the respiratory tract, the reality of local synthesis of NO by constitutive and inducible NOS is no longer contested. NOS is present in airway and alveolar epithelial cells, alveolar macrophages, neutrophils, mast cells, vascular endothelial and smooth muscle cells. Most of these cells are also equipped with NADPH oxidase responsible

Table 1. Summary of the studies reporting the detection of nitrated proteins in humans

Pathology and/or site of detection	Type of study	Reference [number]
Atherosclerosis (coronary arteries)	human	Beckman JS, et al 1994 [60]
Acute lung injury	human	Haddad IY, et al 1994 [64]
Rheumarthritis (synovial fluid,blood)	human	Kaur H & Halliwell B 1994 [69]
Acute lung injury	human	Kooy NW, et al 1995 [66]
Myocardial inflammation	human	Kooy NW, et al 1997 [71]
Atherosclerosis (intima)	human	Leeuwenburgh C, et al 1997 [72]
Obliterative bronchiolitis	human	McDermott CD, et al 1997 [67]
Cystic fibrosis	human	van der Vliet A, et al 1997 [73]
Idiopathic pulmonary fibrosis	human	Saleh D, et al 1997 [74]
Bronchopulmonary dysplasia	human (infants)	Banks BA, et al 1998 [75]
Perennial nasal allergy	human	Sato MN, et al 1998 [76]
Asthma	human	Saleh D, et al 1998 [77]
ARDS (with NO administration)	human	Lamb NJ, et al 1999 [78]
Lung allotransplantation	human	Andrade, et al 2000 [68]
Bronchopneumonia/ARDS	human	Mathy-Hartert, et al 2000 [56]

for $O_2^{\cdot-}$ production, and with xanthine dehydrogenase, a ubiquitous cytosolic enzyme of which the activity of dehydrogenase can be shifted to an activity of oxidase generating $O_2^{\cdot-}$ in pathological circumstances such as ischemic situations. All the conditions are thus met to permit the simultaneous production of NO and $O_2^{\cdot-}$, particularly in cases of infection and inflammation. In these pathological conditions, the production of NO is increased as a host defense against pathogens [20, 62] and the respiratory burst of phagocytes releases oxidant species. The probability is thus high of an *in situ* production of $ONOO^-$ that will be responsible for oxidative alterations. Nitration is one of these oxidative alterations. As neutrophil infiltration and activation is a common event in lung inflammation, nitration could be further enhanced by myeloperoxidase released by neutrophils. Quantitation of nitrotyrosine levels was performed with polyclonal antibodies in lung sections from patients with acute lung injury and hyperoxic injury, using quantitative immunofluorescence [14, 63–66]. Nitrotyrosine has been found in inflammatory cells, airway epithelium and vascular epithelium of human lung allotransplants [67]. Nitrotyrosine and other NO-derived products (nitrites and nitrates) were found in epithelial lining fluid of lung allotransplants, in which chlorotyrosine was also present indicating an interaction with neutrophil myoloperoxidase [68]. Nitrated proteins were also detected in other respiratory pathologies such as asbestosis, idiopathic pulmonary fibrosis, asthma, etc. (Table 1) [14, 60, 66, 69–78].

In the lung, $ONOO^-$ is reported to be responsible for the alterations of surfactant of type II alveolar cells [79]. A recent study demonstrated an increase of nitrated proteins in bronchoalveolar lavage fluid (BAL) of acute respiratory distress syndrome (ARDS) patients treated with inhaled $NO^{\cdot}$, as a consequence of $ONOO^-$ formation or myeloperoxidase activity [78]. These observations raise the question of a possible noxious role of NO administration in ARDS patients [50]. We found elevat-

ed concentrations of nitrated proteins in BAL fluids of patients with ventilator-associated bronchopneumonia and/or ARDS [56]. These nitrated protein concentrations correlated to the number of neutrophils and the presence of active neutrophil enzymes (myelopeoxidase and elastase). It is plausible that these nitrated proteins are the footprint of ROS and more specifically the markers of peroxynitrite production as the result of *in situ* NO and superoxide anion production by the activites of NOS and NADPH-oxidase. These nitrated proteins could also originate from the interactions of nitrites (derived from NO) with the activity of myeloperoxidase and its by-product HOCl.

There is thus considerable evidence of peroxynitrite-induced nitrations and alterations *in vivo*, but are nitrated proteins simply markers of oxidative processes that previously occurred or can these altered proteins be cytotoxic for the neighboring cells and tissues, particularly in the lungs? This question is far from being solved, but it is now clear that tyrosine nitration can perturb the signal transduction in the cells, that nitration of guanine may result in DNA mutations, and that nitration of enzymes leads to dysfunctions. In the lung, nitration of surfactant protein A causes a loss of the ability of this protein to aggregate lipids or to bind to mannose receptors, which could compromise the antibacterial functions of surfactant protein A. In our studies on nitrated proteins in BAL fluids, we also found that these BAL fluids were cytotoxic for healthy alveolar cells (type II alveolar cells, A549) in culture in correlation with the concentrations of myeloperoxidase, that we explained by an interaction of myeloperoxidase activity with H_2O_2 or NO-derived products released by living alveolar cells.

However, more *in vivo* studies identifying the target proteins for nitration are needed to assure that nitrated proteins are more than biomarkers of oxidative activity. The recent description of a 'nitrotyrosine dinitrase' activity in rat tissue homogenates suggest that regulation or defense mechanisms against tyrosine nitration exist *in vivo* [80]. It is thus rather plausible that excessive nitrations would have toxic consequences.

The Effects of Oxidative Stress on Signal Transduction *in vivo*

The *in vivo* effects of oxidative stress on signal transduction are less documented. Data obtained with cell cultures demonstrated the role of ROS in the signal transduction pathways activated by the proinflammatory cytokines IL-1β and TNF-α. These two cytokines regulate the redox-sensitive transcription factor NF-κB, which participates in a variety of immune, inflammatory, and acute-phase responses. As this nuclear factor is more and more recognized as an anti-apoptotic factor, sustained NF-κB activation in the lung of ARDS patients may increase the survival of proinflammatory neutrophils, perpetuating the process of lung inflammation. In the lungs of patients with ARDS, increased numbers of neutrophils activated to produce ROS and inflammatory cytokines are present, and have a decreased rate of apoptosis [81, 82]. In sepsis, an increased activation of NF-κB in peripheral blood monocytes correlated with poor outcome [83]. Alveolar macrophages from patients with ARDS showed increased NF-κB activation compared with those of ventilated patients without ARDS, in relation to the acute inflammation response in the lung [84].

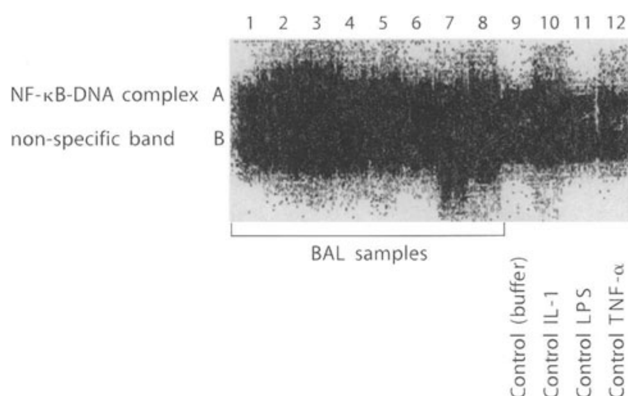

Fig. 4. Detection of NF-κB activation in alveolar cell in culture incubated with bronchoalveolar lavage fluids of intensive care patients (bronchopneumonia and/or ARDS). Comparison with NF-κB activation obtained with lipopolysaccharide (LPS), interleukin (IL)-1β and tumor necrosis factor (TNF)-α (well-known activators of this transduction factor)

Recently, we observed that BAL fluids of patients with ventilator-associated bronchopneumonia (VAP) and/or ARDS increased the activity of NF-κB in normal alveolar cells, and that this increase of NF-κB activity was significantly different in VAP and ARDS groups compared to a group of ventilated patients without these pathologies (Fig. 4). As expected, this activation was correlated with the BAL concentrations of IL-1, but also with the concentrations of IL-8, active myeloperoxidase and the number of neutrophils in BAL. For a better understanding of these observations, we tested the effects of specific ROS on the activation of NF-κB in alveolar cells by using isolated myeloperoxidase and the NO-derived species nitrites and peroxynitrite. Concentrations of nitrites and peroxynitrite ranging from 10^{-7} to 10^{-5} M were found to activate NF-κB to the same level as 100 U TNF-α, 10^{-5} M H_2O_2, and 10^{-5} M HOCl, compounds which were previously reported as NF-κB activators. We also found that 5 μg of human myeloperoxidase was as active as TNF-α, and attributed this activity to the *in situ* reaction of myeloperoxidase with H_2O_2 or NO-derived species that were produced by the alveolar cells [15, 16, 58].

Conclusion

The importance of RNOS is no longer contested in normal and pathological pathways, and NO-derived species, especially peroxynitrite, are considered as responsible for previously reported toxic effects of NO. Some trends are also clear in the subject of ROS and oxidative stress on signal transduction and apoptosis. But the fields of RNOS research changes rapidly and most of our knowledge is derived from laboratory studies on animal models or isolated cells. These studies, albeit necessary and remarkable, can bring simplified concepts, giving the idea of a clear sequence of events that starts with stimuli and ends with oxidative stress leading to cell damage or gene activation. However, *in vivo* studies do not fit these simple schemes for the

role of oxidative stress and ROS in humans. In inflammatory situations, an oxidative stress is expected arising from an excessive stimulation of neutrophils of which the apoptosis appears to be delayed. Interventions that inhibit RNOS production and signal transduction alterations could have a beneficial anti-inflammatory response *in vivo*, but could also create immunosuppression. To secure anti-RNOS therapies, the role of specific RNOS remains to be more clearly delineated, especially by studies using 'physiological' concentrations of ROS.

References

1. Schreck R, Baeuerle PA (1991) A role for oxygen radicals as second messengers. Trends Cell Biol 1:2–3
2. Chandra J, Samali A, Orrenius S (2000) Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 29:323–333
3. Lo Y, Wong J, Cruz T (1996) Reactive oxygen species mediate cytokine activation of c-Jun NH_2-terminal kinases. J Biol Chem 271:15703–15707
4. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA (2000) Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med 28:1456–1462
5. Boveris A, Chance A (1973) The mitochondrial generation of hydrogen peroxide. Biochem J 134:707–716
6. Fialkow L, Downey GP (1997). Reactive oxygen intermediates as signaling molecules regulating leukocyte activation. In: Forman HJ, Cadenas E (eds) Oxidative Stress and Signal Transduction. Chapman & Hall, New York, pp 200–235
7. Liang B, Petty HR (1992) Imaging neutrophil activation: analysis of the translocation and utilization of NAD(P)H-associated autofluorescence during antibody-dependent target oxidation. J Cell Physiol 152:145–156
8. Kissner R, Nauser T, Bugnon P, Lye PG, Koppenol WH (1997) Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. Chem Res Toxicol 10:1285–1292
9. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87:1620–1624
10. Carreras MC, Pargament GA, Catz SD, Poderoso JJ, Boveris A (1994) Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. FEBS Lett 341:65–68
11. Malinski T, Taha Z (1992) Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 358:676–678
12. Thom SR, Xu YA, Ischiropoulos H (1997) Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure. Chem Res Toxicol 10:1023–1031
13. Ghafourifar P, Richter C (1997) Nitric oxide synthase activity in mitochondria. FEBS Lett 418: 291–296
14. Wizeman TM, Gardner CR, Laskin JD, et al (1994) Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. J Leukoc Biol 56:759–768
15. Eiserich JP, Hristova M, Cross CE, et al (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 391:393–397
16. Sampson JB, Ye Y, Rosen H, Beckmann JS (1998) Myeloperoxidase and horseradish peroxidase catalyze tyrosin nitration in proteins from nitrite and hydrogen peroxide. Arch Biochem Biophys 356:207–213
17. Evans TJ, Buttery LD, Carpenter A, Springall DR, Polak M, Cohen J (1996) Cytokine-treated human neutrophils contain inducible intric oxide synthase that produces nitration of ingested bacteria. Proc Natl Acad Sci USA 93:9553–9558
18. Gagnon C, Leblond FA, Filep JG (1998) Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide. FEBS Lett 431:107–110
19. Cochrane CG (1991) Cellular injury by oxidants. Am J Med 91 (Suppl 3C):23S–30S

20. Fang FC (1997) Mechanisms of nitric oxide-related antimicrobial activity. J Clin Invest 100: S43–S50
21. Beckman JS, Chen J, Ischiropoulos H, Crow JP (1994) Oxidative chemistry of peroxynitrite. Methods Enzymol 233:229–240
22. Chen B, Keshive M, Deen WM (1998) Diffusion and reaction of nitric oxide in suspension cell cultures. Biophys J 75:745–754
23. MacMillan-Crow LA, Crow JP, Thompson JA (1998) Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. Biochemistry 37:1613–1622
24. Milstien S, Katusic Z (1999) Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. Biochem Biophys Res Commun 263:681–684
25. Pollet E, Martinez JA, Metha B, Watts BPJr, Turrens JF (1998) Role of tryptophan oxidation in peroxynitrite-dependent protein chemiluminescence. Arch Biochem Biophys 349:74–80
26. Mouithys-Mickalad A, Kohnen S, Deby C, Noels AF, Lamy M, Deby-Dupont G (1999) Peroxynitrite reacts with biological nitrogen-containing cyclic molecules by a radical pathway, as demonstrated by ultraweak luminescence coupled with ESR technique. Biochem Biophys Res Commun 259:460–464
27. Ischiropoulos H (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys 356:1–11
28. Pietraforte D, Minetti M (1997) Direct ESR detection of peroxynitrite-induced tyrosine centered protein radicals in human blood plasma. Biochem J 325:675–684
29. Pfeiffer S, Mayer B (1998) Lack of tyrosine nitration by peroxynitrite generated at physiological pH. J Biol Chem 273:27280–27285
30. Hassan MS, Mileva MM, Dweek HS, Rosenfeld L (1998) Nitric oxide products degrade chondroitin sulfates. Nitric Oxide 2:360–363
31. Roberts ES, Lin Hl, Crowley JR, Vuletich JL, Osawa Y, Hollenberg PF (1998) Peroxynitrite-mediated nitration of tyrosine and inactivation of the catalytic activity of cytochrome P450 2B1. Chem Res Toxicol 11:1067–1074
32. Vásquez-Vivar J, Santos AM, Junqueira VBC, Augusto O (1996) Peroxynitrite- mediated formation of free radicals in human plasma: EPR detection of ascorbyl, albumin-thiyl and uric acid-derived free radicals. Biochem J 314:869–876
33. Burney S, Caulfield JL, Niles JC, Wishnok JS, Tannenbaum SR (1999) The chemistry of DNA damage from nitric oxide and peroxynitrite. Mutat Res 424:37–49
34. Graham A, Hogg N, Kalyanaraman B, O'Leary V, Darley-Usmar V, Moncada S (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. FEBS Lett 330:181–185
35. O'Donnell VB, Eiserich JP, Bloodsworth A, et al (1999) Nitration of unsaturated fatty acids by nitric oxide-derived reactive species. Methods Enzymol 301:454–470
36. Kidd VJ (1998) Proteolytic Activities that mediate apoptosis. Annu Rev Physiol 60:533–573
37. Storz G, Tartaglia LA, Ames BN (1990) Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science 248:189–194
38. Ghosh S, May MJ, Kopp EB (1998) NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. Annu Rev Immunol 16:225–260
39. Abraham E (2000) NF-κB activation. Crit Care Med 28 (Suppl):N100–N104
40. Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiqitination: the control of NF-κB activity. Annu Rev Immunol 18:621–663
41. Schieven GL (1997) Tyrosine phosphorylation in oxidatve stress. In: Forman HJ, Cadenas E (eds) Oxidative Stress and Signal Transduction. Chapman & Hall, New York, pp 181–199
42. Haddad JJE, Olver RE, Land SC (2000) Antioxidant/pro-oxidant equilibrium regulates HIF-1α and NF-κB redox sensitivity. Evidence for inhibition by glutathione oxidation in alveolar epithelial cells. J Biol Chem 275:21130–21139
43. Leonarduzzi G, Arkan MC, Basaga H, Chiarpotto E, Sevanian A, Poli G (2000) Lipid oxidation products in cell signaling. Free Radic Biol Med 28:1370–1378
44. Lee SR, Kwon KS, Kim SR, Rhee SG (1998) Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J Biol Chem 273:15366–15372
45. True AL, Rahman A, Malik AB (2000). Activation of NF-κB induced by H_2O_2 and TNF-α and its effects on ICAM-1 expression in endothelial cells Am J Physiol 279:L302–L311

46. Lelli JL Jr, Becks LL, Dabrowska MI, Hinshaw DB (1998) ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. Free Radic Biol Med 25:694–702
47. Spear N, Estévez AG, Radi R, Beckman JS (1997) Peroxynitrite and cell signaling. In: Forman HJ, Cadenas E (eds) Oxidative Stress and Signal Transduction. Chapman & Hall, New York, pp 32–51
48. Mallozzi C, Di Stasi AM, Minetti M (1997) Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3. FASEB J 11:1281–1290
49. Brito C, Naviliat M, Tiscornia AC, et al (1999) Peroxynitrite inhibits T lymphocyte activation and proliferation by promoting impairment of tyrosine phosphorylation and peroxynitrite-driven apopototic death. J Immunol 162:3356–3366
50. Baylock MG, Cuthbertson BH, Galley HF, Ferguson NR, Webster NR (1998) The effect of nitric oxide and peroxynitrite on apoptosis in human polymorphonuclear leukocytes. Free Radic Biol Med 25:748–752
51. Ischiropoulos H (1998) Living and dying with reactive species. Focus on "peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease". Am J Physiol 274: C853–C854
52. Lin KT, Xue JY, Lin MC, Spokas EG, Sun FF, Wong PYK (1998) Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. Am J Physiol 274:C855–C860
53. Goode HF, Webster NR (1993) Free radicals and antioxidants in sepsis. Crit Care Med 21: 1770–1776
54. Leff JA, Parsons PE, Day CE, et al (1993) Serum antioxidants as predictors of adult respiratory distress syndrome in patients with sepsis. Lancet 341:777–780
55. Pacht ER, Timmerman AP, Lykens MG, Merola AJ (1991) Deficiency of alveolar fluid glutathione in patients with sepsis and the adult respiratory distress syndrome. Chest 100:1397–1403
56. Mathy-Hartert M, Damas P, Nys M, et al (2000) Nitrated proteins in bronchoalveolar lavage fluid of patients at risk of ventilator-associatedbronchopneumonia. Eur Respir J 16:296–301
57. Herce-Pagliai C, Kotecha S, Shuker DE (1998) Analytical methods for 3-nitrotyrosine as a marker of exposure to reactive nitrogen species: a review. Nitric Oxide 2:324–336
58. van der Vliet A, Eiserich JP, Shigenaga MK, Cross CE (1999) Reactive nitrogen species and tyrosine nitration in the respiratory tract. Epiphenomena or a pathophysiological mechanism of disease? Am J Respir Crit Care Med 160:1–9
59. Salman-Tabchem S, Guerin MC, Torreilles J (1995) Nitration of tyrosyl-residues from extra- and intracellular proteins in human whole blood. Free Rad Biol Med 19:695–698
60. Beckmann JS, Ye YZ, Anderson PG, et al (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol Chem Hoppe Seyler 375:81–88
61. Buttery LD, Springall DR, Chester AH, et al (1996) Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab Invest 75:77–85
62. Gaston B, Drazen JM, Loscalzo J, Stamler JS (1994) The biology of nitrogen oxides in the airways. Am J Respir Crit Care Med 149:538–551
63. Salvemini D, Wang ZQ, Bourdon DM, Stern MK, Currie MG, Manning PT (1996) Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. Eur J Pharmacol 303: 217–220
64. Haddad IY, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S (1994) Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. J Clin Invest 94:2407–2413
65. Royall JA, Kooy NW, Beckman JS (1995) Nitric oxide-related oxidants in acute lung injury. New Horiz 3:113–122
66. Kooy NW, Royall JA, Ye YZ, Kelly DR, Beckman JS (1995) Evidence for in vivo peroxynitrite production in human acute lung injury. Am J Respir Crit Care Med 151:1250–1254
67. McDermott CD, Gavita SM, Shennib H, Giaid A (1997) Immunohistochemical localization of nitric oxide synthase and the oxidant peroxynitrite in lung transplant recipients with obliterative bronchiolitis. Transplantation 64:270–274
68. Andrade JA, Crow JP, Viera L, et al (2000) Protein nitration, metabolites of reactive nitrogen species and inflammation in lung allografts. Am J Respir Crit Care Med 161:2035–2042
69. Kaur H, Halliwell B (1994) Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Lett 350:9–12

70. Akaike T, Noguchi Y, Ijiri S, et al (1996) Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals Proc Natl Acad Sci USA 93:2448–2453
71. Kooy NW, Lewis SJ, Royall JA, Ye YZ, Kelly DR, Beckman JS (1997) Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite. Crit Care Med 25:812–819
72. Leeuwenburgh C, Hardy MM, Hazen SL, et al (1997) Reactive nitrogen intermediates intermediates promote low density lipoprotein oxidation in human atherosclerotic intima J Biol Chem 272:1433–1436
73. van der Vliet A, Eiserich JP, Marelich GP, Halliwell B, Cross CE (1997) Oxidative stress in cystic fibrosis: does it occur and does it matter? Arch Pharmacol 38:491–413
74. Saleh D, Barnes PJ, Giaid A (1997) Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 155: 1763–1769
75. Banks BA, Ischiropoulos H, Mc Clelland M, Ballard RA (1998) Plasma 3-nitrotyrosine is elevated in premature infants who develop bronchopulmonary dysplasia. Pediatrics 101:870–874
76. Sato M, Fukuyama N, Sakai M, Nakazawa H (1998) Increased nitric oxide in nasal lavage fluid and nitrotyrosine formation in nasal mucosa – indices for severe perennial nasal allergy. Clin Exp Allergy 28:597–605
77. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A (1998) Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. FASEB J 12:929–937
78. Lamb NJ, Quinlan GJ, Westerman ST, Gutteridge JMC, Evans TW (1999) Nitration of proteins in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome receiving inhaled nitric oxide. Am J Respir Crit Care Med 160:1031–1034
79. Haddad IY, Ischiropoulos H, Holm BA, Beckman JS, Baker JR, Matalon S (1993) Mechanisms of peroxynitrite-induced injury to pulmonary surfactants. Am J Physiol 9:L555–L564
80. Kamisaki Y, Wada K, Bian K, et al (1998) An activity in rat tissues that modifies nitrotyrosine-containing proteins. Proc Natl Acad Sci USA 95:11 584–11 589
81. Matute-Bello G, Liles WC, Radella F, et al (1997) Neutrophil apoptosis in the acute respiratory distress syndrome. Am J Respir Crit Care Med 156:1969–1977
82. Parsey MV, Kaneko D, Shenkar R, et al (1999) Neutrophil apoptosis in the lung after hemorrhage or endotoxemia: apoptosis and migration are independent of IL-1β. Clin Immunol 91:219–225
83. Bohrer H, Qiu F, Zimmerman T, et al (1997) Role of NF-κB in the mortality of sepsis. J Clin Invest 156:1969–1977
84. Schwartz MF, Moore EE, Moore FA, et al (1996) NF-κB is activated in alveolar macrophages from patients with acute respiratory distress syndrome. Crit Care Med 24:1285–1292