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Radical Generation and Alterations of Erythrocyte Integrity as Bioindicators of Diagnostic or Prognostic Value in COPD?

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) has recently been viewed as an inflammation-dependent systemic disease. Oxidative modifications in the pulmonary microenvironment can result in a number of functional changes in pulmonary tissue as well as in the blood. Studies have been carried out to detect whether oxidatively modified molecules or cells could be considered possible markers of the disease. We hypothesize here that new insights into COPD could come from enzymes involved in deliberate radical generation (*i.e.*, Nox and NOS family enzymes) as well as from alterations of erythrocyte integrity and function, which could become bioindicators of diagnostic or prognostic value in the near future. *Antioxid. Redox Signal.* 10, 829–836.

CHRONIC INFLAMMATION represents a key feature in the pathogenesis of chronic obstructive pulmonary disease (COPD). Oxidative stress associated with chronic inflammation has important consequences for several elements of lung physiology (34). Hence, COPD, previously considered chiefly a respiratory disease, has more recently been viewed as an oxidative stress–associated disease and, more important, as a systemic disease (5). In this context, several studies have been carried out to detect possible bioindicators in the blood of patients with COPD (32, 55). Accordingly, with the idea that enzymes involved in deliberate generation of free radicals [*i.e.*, NADPH oxidases (Nox) and nitric oxide synthases (NOS)] could be pivotal in the pathogenesis of the disease but could also represent important progression markers, we suggest here that these enzymes or their regulation are upstream to systemic oxidant/antioxidant status. The radicals produced by these enzymes are upstream of (a) all reactive oxygen and nitrogen species (ROS/RNS) generated in tissues, (b) the complex antioxidant machinery including the pivotal role played by GSH-linked antioxidant enzymes, and (c) several inflammatory signaling pathways, such as peroxidases, cyclooxygenases, proteases, cytokines. Obviously, all these targets are also potential biomarkers of COPD (for a review, see ref. 4), although a complete description of all these pathways exceeds the space limitations of this review.

In particular, we hypothesize that the appearance in the peripheral blood of COPD patients of molecules or cells oxidatively modified by Nox and NOS enzymes could provide fruitful information of diagnostic or prognostic value.

THE REDOX STATE IN COPD

A number of oxidatively modified molecules have been successfully identified in the breath condensate as well as in the blood of COPD patients (6, 8, 34, 40, 44), and therapeutic administration of antioxidants has been introduced into clinical practice (49). Several groups reported that oxidative stress is considerably increased in patients with COPD, in particular during exacerbation. Protein carbonylation, which is a nonspecific marker found in most of oxidative stress–associated diseases, was detected, for example, both in the femoral quadriceps (7, 50) and in peripheral erythrocytes and platelets (13) of COPD patients. Knockout mice studies revealed that emphysema (24), a common complication of both smokers and nonsmokers with COPD, may be linked to ROS generation, not only through the

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Toll-like receptors (see later), but also to the antioxidant machinery through the Nrf2 transcription factor (24). This leucine zipper–type transcription factor binds to the antioxidant response elements and induces phase 2 enzymes and antioxidant genes. Without a further lengthy description of all redox changes found in COPD, which is outside the scope of this report, in Table 1 we recapitulate some of the biologic targets described as oxidatively modified.

Although well established, this scenario provided few insights into the field in terms of diagnostic value. It has been suggested that some aspects of the oxidative alterations occurring in COPD could determine peculiar changes in blood. Blood can be considered a source of as well as a target for ROS/RNS (39) and can provide innovative bioindicators and pathogenic determinants in this pathology.

SMOKING AND COPD

Smoke has an important pulmonary and extrapulmonary toxicity. An overwhelming number of studies demonstrated the relation of smoking not only to lung cancer but also to numerous widespread systemic diseases such as atherosclerosis and COPD. A large proportion of COPD patients are smokers or ex-smokers. The systemic effects of smoke, as to COPD, are due to or coupled with oxidative stress and inflammatory mediators. Cigarette smoke contains huge amounts of oxidant molecules, potentially causing an oxidative burden to the respiratory apparatus (48). Smoke-induced oxidative stress can be evaluated by measuring ROS/RNS production by peripheral blood cells or evaluating oxidized target molecules (*e.g.*, lipid peroxidation or oxidized proteins). For example, peroxidation of polyunsaturated fatty acids of cell membranes and the formation of 3-nitrotyrosine, mediated by nitric oxide and peroxynitrite, has been reported to be significantly increased in the plasma and platelets from blood of smokers (45, 47). Although numerous biomarkers of oxidative damage have been identified (33, 37, 62), overlapping largely with those of COPD (see a comparison in Table 1), it is not established whether oxidative-stress markers could become bioindicators of diagnostic or prognostic value for the development of systemic diseases in smokers. This aspect is particularly relevant in this context because not all smokers acquire one or more of systemic diseases, clearly suggesting the existence of other mechanisms influencing disease development, and, beyond doubt, genetic susceptibility is one of them (for a recent review, see ref. 68).

ROLE OF NOX FAMILY NADPH OXIDASES

Leukocytes infiltrating the lung have long been considered sources of excess ROS/RNS production associated with COPD, because these cells are detected in higher numbers in this and other inflammatory diseases of the lung (*i.e.*, cystic fibrosis and asthma) (6). Activated neutrophils and macrophages are rich sources of superoxide ($O_2^{\cdot -}$) and ROS produced by the phagocytic NADPH oxidase system.

However, growing evidence indicates that other cells of the lung and vascular system are also capable of deliberate ROS release under proinflammatory conditions (31). Several of these novel ROS sources were identified as NADPH oxidases (Nox family) homologous to the phagocytic enzyme (28). The Nox family in humans encompasses seven members: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 ($gp91_{phox}$, the core of the phagocytic oxidase, is designated Nox2). Proposed functions of these novel oxidases include roles in extracellular matrix modifications, hormone biosynthesis, fertilization, and re-

Table 1. Markers of Oxidative Stress in Patients with COPD and Comparison with Non-COPD Smokers

Parameter	*COPD*	*Smokers*	*References*
F2-isoprostanes, Ethane, plasma malonyldialdehyde	Increased	Increased	14, 25, 40, 43, 44
Plasma antioxidant power	Decreased	Decreased	17, 43, 50
Plasma total thiols	Decreased, increased	Variable	43, 50, 55, 58
Plasmatic selenium	Decreased	Unchanged	55
Potassium and Rubidium	Decreased	Unchanged	55
GSH-Peroxidase in plasma	Increased		43
Protein carbonyls	Increased	Increased	42, 43, 55
Blood GSH	Increased	Unchanged	12, 43
Blood SOD activity	Increased	Unchanged	35, 55
Exhaled nitric oxide	Mild increase	Decrease	8, 52, 56
RBC shape and physiology	Modified	Unchanged	15, 29, 32, 54, 59
RBC thiols	Decreased	Decreased	22, 32
RBC Glycophorin A, Band 3	Decreased expression		32, 59
RBC Membrane microviscosity	Increased	Unchanged	29, 54
RBC Glutathione peroxidase	Decreased	Decreased	22, 26, 55
RBC SOD activity	Increased/unchanged	Unchanged	26, 35, 43
RBC lipid profile changes, protein carbonyls	Increased	Unknown	13

dox-based signaling involved in cellular proliferation, senescence, or oxygen sensing.

Several nonphagocytic oxidases were proposed to function as host defense or proinflammatory enzymes based on the following evidence reviewed recently (31): (a) several are induced by proinflammatory cytokines, (b) several are involved in innate immune responses to microbial recognition through Toll-like receptors (TLRs), (c) several are expressed at high levels in epithelial cells, and (d) some are thought to function in partnership with known antimicrobial peroxidases. Although no studies have examined their expression or function in the context of COPD, these features, together with their expression patterns, suggest that several of these novel oxidases are potential sources of excess ROS in this disease. Both Nox2 and Nox4 function in vascular endothelial cells, whereas Nox1, a multicomponent oxidase closest to the phagocytic oxidase, is also TNF-α inducible and functions in vascular smooth muscle cells (30). Duox1, Duox2, and Nox4 are particularly interesting, based on their expression in the lung and their inducibility by cytokines that have roles in lung inflammatory disease. The dual oxidases (Duox1 and Duox2) were first identified as hydrogen peroxide generators that support thyroperoxidase in biosynthesis of thyroid hormones. High Duox expression was later detected in exocrine (salivary) glands and along mucosal surfaces of airways and the gastrointestinal tract. The mucosal isozymes were proposed to support the activity of lactoperoxidase (19), a well-known antimicrobial enzyme found in various exocrine secretions (milk, saliva, tears). Later work showed that the airway Duox-lactoperoxidase system is effective in killing *Pseudomonas aeruginosa* and *Staphylococcus aureus*, pathogens encountered in the lungs of cystic fibrosis patients (41). Several groups proposed other distinct roles for airway Duox1, including acid release and mucus hypersecretion in connection with oxidation of TNF-α converting enzyme (reviewed in 31). Duox1 and Duox 2 are differentially upregulated in human bronchial epithelial cells by distinct Th1 and Th2 cytokines (21): Duox1 by IL-4 and IL-13, and Duox2 by gamma-interferon, by exposure to polyinosine:polycytidylic acid or infection with rhinovirus, suggesting that Duox2 serves in antiviral responses. Interestingly, mice overexpressing gamma-interferon develop emphysema (64), because several NADPH oxidases are gamma-interferon induced (Nox1, Nox2, and Duox2), although other features of COPD (airway remodeling) were not seen in these animals.

Excess IL-4 and IL-13 production is detected in bronchial lavage lymphocytes from COPD patients, and IL-13 is associated with mucus hypersecretion and profibrotic processes (6, 66). A role for IL-13 is recognized in chronic allergic asthma (65), which shares some feature in common with COPD. Furthermore, mouse models in which IL-13 is conditionally overexpressed in lung epithelium display many features characteristic of COPD (inflammation, goblet cell hypertrophy, airway subepithelial collagen deposition, and metalloproteinase-dependent lung destruction) (16). Future work should explore whether excess IL-13 acts through Duox1 to promote these processes in COPD or asthma.

Initial interests in Nox4 were focused on the kidney and vascular cells. Recently, roles for Nox4 and the ROS it generates in TGF-β–mediated proliferative or hypertrophic responses of pulmonary artery and airway smooth muscle cells were suggested (60). TGF-β1 limits inflammation, while promoting tissue repair or fibrosis after chronic injury in several tissues, including the lung (66); its role in the pathology of the lung was reviewed recently (9). Observations in transgenic models suggest that TGF-β1, acting downstream of IL-13, could have roles in chronic asthma and COPD (9, 16). Some studies detected elevated TGF-β1 in the plasma and monocytes of COPD patients. Future work should explore whether Nox4 is a crucial mediator of airway remodeling or pulmonary hypertension observed in COPD and whether TGF-β1 induction of Nox4 also enhances ROS generation in peripheral tissues.

Although oxidants are commonly considered culprits in mediating tissue damage during inflammation (*i.e.*, through oxidative inhibition of α1-antitrypsin), other evidence suggests that the ROS derived from the phagocytic oxidase (Nox2) actually serve in limiting the activity of matrix metalloproteinase, thereby preventing tissue damage. Surprisingly, Nox2-deficient mice spontaneously develop emphysema at an early age, perhaps consistent with the finding that Nox2-deficient chronic granulomatous disease patients have dysregulated proinflammatory responses and excess granuloma formation (27). In contrast, enhanced activity of another novel NADPH oxidase, Nox3, was proposed to contribute to development of emphysema in TLR4-deficient mice (69). The lungs of TLR4-deficient mice exhibited increased oxidant stress, cell death, and elastin degradation leading to emphysema at 2 months of age. In this model, classic emphysema-related inflammatory mediators, matrix metalloproteases, and their tissue inhibitors remained unchanged, and no apparent influx of inflammatory cells was found in the lungs of these mice. The enhanced oxidant stress in these mice was attributed to increased Nox3 expression in lung tissues and in endothelial cells.

Other Nox family oxidases (Nox1 and Nox4) have also been associated with TLRs. Although it is not known whether any Nox isozyme alterations occur in human emphysema, these studies reveal novel innate immune and redox homeostatic mechanisms in which nonphagocytic oxidases can contribute to pulmonary oxidative stress and pathology related to COPD.

A ROLE FOR NITRIC OXIDE SYNTHASE, NITRIC OXIDE AND ITS RELATED OXIDATION PRODUCTS

Nitric oxide ($^{\cdot}$NO), a simple free-radical gas, elicits a diverse range of physiologic and pathophysiologic effects and plays a peculiar role in pulmonary diseases. $^{\cdot}$NO is normally present in the human exhaled breath and is significantly increased in asthma patients (8). $^{\cdot}$NO is synthesized from molecular oxygen and L-arginine by a family of enzymes known as nitric oxide synthases (NOS1–3). Significantly, in human airways, the main enzyme responsible for generating $^{\cdot}$NO is NOS2 (20), the most active NOS isoform, which in several other tissues is expressed only after induction by cytokines. This implies that the airways, even under noninflammatory conditions, are exposed to a robust $^{\cdot}$NO burst aimed to induce smooth muscle relaxation. Under chronic inflammatory conditions, however, the simultane-

ous presence of $^{\cdot}NO$ and $O_2^{\cdot -}$, as well as other ROS, can generate increased vascular permeability, cytotoxicity, and inflammatory cell infiltration. The cytotoxic properties of $^{\cdot}NO$ have been attributed to the formation of peroxynitrite ($ONOO^-$), a strong oxidant generated by the fast radical–radical reaction between $^{\cdot}NO$ and $O_2^{\cdot -}$. Peroxynitrite is able to oxidize all the most important cellular targets, including proteins, lipids, and DNA. One of the characteristic oxidative modifications induced by peroxynitrite on proteins is the formation of 3-nitrotyrosine; remarkably, a significant increase of 3-nitrotyrosine immunostaining in bronchial tissue from COPD patients has been reported (52). Moreover, a substantial proportion of the $^{\cdot}NO$ oxidizes in the presence of molecular oxygen and generates nitrogen oxides, which react with thiols to form S-nitrosothiols, compounds with potent relaxant activity. However, excessive formation of S-nitrosothiols may lead to enzyme inactivation, inducing in the cells a stress situation defined as "nitrosative stress." Tissues exposed to chronic nitrosative stress experience cell apoptosis, which has been observed in the parenchyma and airways of COPD and believed to be at the basis of the emphysema (23). Exhaled $^{\cdot}NO$ has been reported to be increased in COPD patients (8), although this finding was controversial, and the degree of elevation was apparently modest (2). One possible reason for the controversial results and the low exhaled $^{\cdot}NO$ level in COPD may be linked to the downregulation of $^{\cdot}NO$ due to the increased superoxide production, which is a characteristic of leukocytes from both smokers and COPD patients (see Table 1), and the possible formation of peroxynitrite.

In summary, compelling evidence indicates that $^{\cdot}NO$ and $^{\cdot}NO$-related oxidants play a relevant role in COPD pathology, but what about their role as far as the systemic disease? No clear answer to this question is known because oxidative changes induced by ROS/RNS are largely overlapping. Interesting enough, the RBC is the major scavenger of ROS/RNS and the most important "sink" of peroxynitrite (see references reported in 36), and it is conceivable that blood may be the most suitable tissue to highlight a systemic oxidant/antioxidant imbalance. This issue is not irrelevant to future therapeutic strategies aimed at regulating $^{\cdot}NO$ and/or $O_2^{\cdot -}$ synthesis through the control of NOS or Nox enzymes. However, in a recent challenging work, a comparison was proposed between changes observed in RBCs of COPD patients and those obtained *in vitro* by treatment of control RBCs with authentic peroxynitrite (32). Some of the oxidative modifications observed were similar, leading to the hypothesis that the involvement of peroxynitrite cannot be ruled out.

In any case, to relieve the pathologic picture of COPD disease, interventions aimed at the restoration of $^{\cdot}NO$-dependent endothelial vasoconstrictor–dilator balance seem to be promising (51). In this context, intriguing insights also come from a preliminary study carried out with sildenafil, which has been reported to improve hemodynamic parameters in COPD (1). This drug selectively inhibits the enzyme phosphodiesterase 5, thus acting downstream of the $^{\cdot}NO$-dependent signaling and enhancing cGMP-mediated relaxation.

Although, as outlined earlier, cigarette smoking is clearly the major environmental risk factor for COPD, the fact that chronic airflow obstruction will develop in only 15–20% of smokers clearly implicates genetic factors. For example, in consideration of the role of $^{\cdot}NO$, it has been reported that some alleles of NOS3, a constitutive NOS isoform, were overrepresented in COPD patients and associated with elevated nitrite and malondialdehyde levels (2). The COPD phenotypes may be remarkably relevant also in relation to the appearance of various oxidative-stress biomarkers. Differences in the course and severity of the disease between racial and ethnic groups, together with familial clustering and gender differences, have been described (3, 18) and critically reviewed (67), but it is still unclear whether a linkage with the appearance of oxidative-stress biomarkers exists (11, 35, 63).

THE RBC AS A CELLULAR BIOMARKER

A number of studies on RBCs are supporting the new concept that redox properties of these cells are indicative of the oxidative status of blood in several pathologies (see references in 38). In short, the RBC seems to be one of the most important tools of antioxidant defenses in the vasculature and, probably, for the whole organism. However, if the oxidative insult of the microenvironment overcomes the RBC defenses, this cell undergoes oxidative alterations (*e.g.*, change of rheologic/functional properties; see Fig. 1) that can still be stably detected at the periphery. This hypothesis is reasonable because this cell crosses the lungs once a minute, and it is conceivable that in the presence of intense and chronic oxidative stress, it is not possible to "repair" fully the damage. In COPD patients, RBCs show not only alterations in the activity of antioxidant enzymes (34) but also morphologic and functional alterations. In the blood of these patients, the appearance of several acanthocytic forms has been described (32, 59). Their deformability is a prerequisite for their "journey" through the blood vessels and is essentially due to (a) a peculiar microviscosity, (b) an extremely "flexible" cytoskeleton, and (c) the ability to adhere to each other without undergoing stable cluster (rouleaux) formation. All in all, these features lead to a cell plasticity that allows RBC movement in the blood flow (*i.e.*, characterizes their unique rheologic properties). All these features are lost once the cell is exposed to an oxidative insult (36, 46). Some concern has been expressed about the specificity and reproducibility of oxidative changes occurring in biologic fluids of patients with oxidative stress–related diseases, including morphofunctional alterations of the RBC; however, the choice of RBC morphofunctional alterations offers the advantage of working with a biomarker of paramount importance for vascular functions (39, 53).

What happens when an RBC crosses an ROS/RNS-rich microenvironment (depicted schematically in Fig. 2), such as that occurring in an inflamed vessel? A pro-oxidant environment may function as a "booster" of RBC aging and death (36, 46), with serious consequences for its rheologic and functional properties and, more important, may promote an RBC pro-oxidant behavior, thus contributing to vascular damage (38, 39).

The idea that the oxidative alterations of RBCs described earlier could be identified in the blood of COPD patients is emerging from the literature on the basis of two main concerns. One is pathophysiologic: the fact that patients with COPD are "asthenic" patients in whom severe limb muscle weakness and respiratory

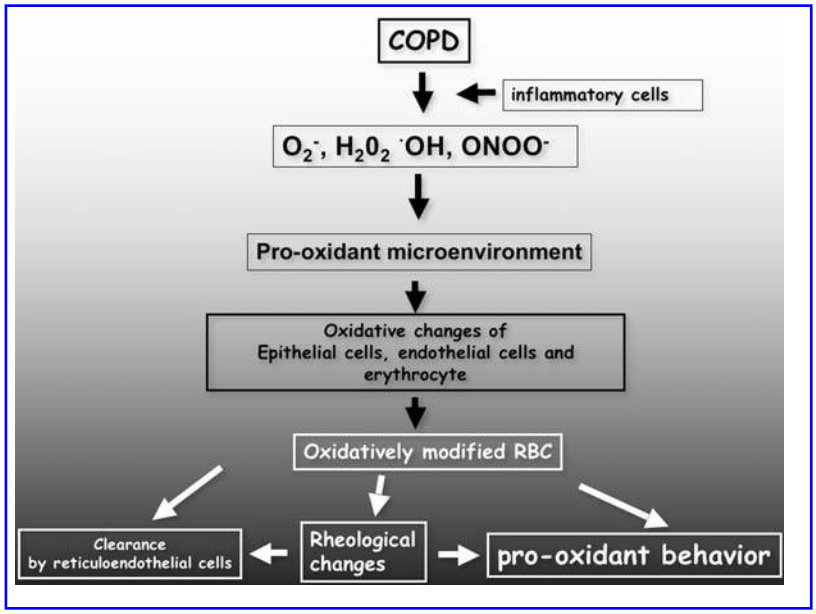

FIG. 1. Fate of RBCs exposed to oxidative insults overcoming RBC defense mechanisms.

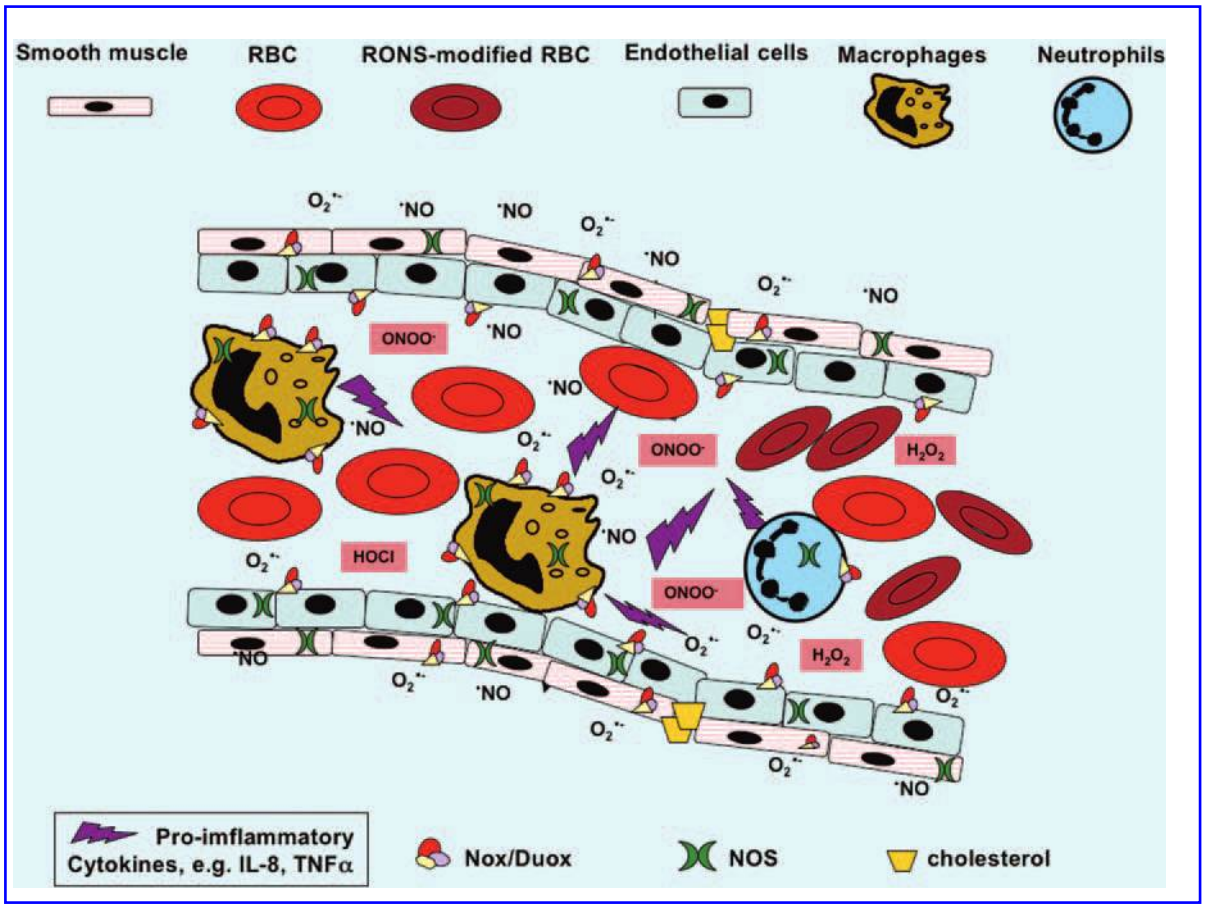

FIG. 2. Schematic representation of an inflamed vessel of the airway. NOS and Nox enzymes are the sources of $^{\cdot}NO$ and $O_2^{\cdot-}$ radicals. Deregulation/overproduction of these radicals generates reactive cytotoxic species such as peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and hydrogen peroxide (H_2O_2). Oxidatively modified RBCs are shown in brown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

muscle weakness due to chronic oxygen deficiency can accompany the course of respiratory disease, thus impairing their quality of life. The second derives from the finding that blood from patients with respiratory disease appears to be altered by overproduction of ROS/RNS, as suggested by some biomarkers (see Table 1), which may be proposed as real-time progression markers.

In conclusion, the assumption that COPD could be considered a redox-associated systemic disease represents an important challenge in the management of COPD. The search for progression markers is under way, with the aim to point out easy-to-do analytic cytology or biochemical tests that could help physicians in clinical practice, providing novel insights into the detection of biomarkers of diagnostic and prognostic value (61). Among these, in our opinion, could be considered Nox and NOS family enzymes or, intriguingly, the more abundant cell in the peripheral blood: the red blood cell. For instance, besides stable COPD, how COPD exacerbations could be defined and graded still remains unclear. Their mechanisms can include either bacteria or viral infections, but noninfective causes, such as air pollution and pulmonary embolus, are surely involved. Exacerbations are characterized, in the airways, by increased numbers of inflammatory cells (*e.g.*, neutrophils, cytokines, chemokines, and proteases) but, in the peripheral blood, increased concentrations of inflammatory cytokines and C-reactive protein have been detected (10). All these sources clearly indicate once more that oxidative imbalance can represent a hallmark of the disease that should be elucidated. Furthermore, it has been suggested that the systemic inflammation observed in COPD may play an important role in mediating the extrapulmonary complications of COPD, such as cardiovascular risk and lung cancer, the leading causes of morbidity and mortality for these patients (57). Hence, in this scenario, the development of valuable and innovative markers of lung damage, pulmonary or systemic inflammation, but also of the main hallmark of COPD (*i.e.*, oxidative imbalance bioindicators), seems to be mandatory for the refinement of treatments and for the evaluation of the progression and complications of the disease. In particular, in our opinion, the analysis of innovative peripheral blood biomarkers, including redox enzymes and cellular (*e.g.*, RBC) changes, could provide useful tools to discriminate among different clinical conditions in the long run.

ABBREVIATIONS

COPD, chronic obstructive pulmonary disease; Duox, dual oxidases; NOS, nitric oxide synthase; Nox, NADPH oxidase; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TLRs, Toll-like receptors.

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