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REVIEW

Reactive oxygen species in acute lung injury

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Reactive oxygen species in acute lung injury. F. Chabot, J.A. Mitchell, J.M.C. Gutteridge, T.W. Evans. ©ERS Journals Ltd 1998.

ABSTRACT: The acute respiratory distress syndrome (ARDS) in adults is associated with a wide variety of precipitating factors, often not directly involving the lung, and has an associated mortality of 50–80%. ARDS is almost invariably associated with sepsis, either as an initiating factor or as a secondary complication, which increases the expression of a number of cytokines impacting upon several cellular systems. Specifically, activation of neutrophils sequestered in the pulmonary circulation by this process, causes the release of free radicals and reactive oxygen species (ROS), increasingly regarded as key substances modulating the endothelial dysfunction and disruption responsible for the principal clinical manifestations of the syndrome. Here we discuss briefly the pathophysiology of ARDS and its impact upon pulmonary vascular control; the biological origins of free radicals and other ROS involved, the mechanisms of their damaging effects, their contribution to the modification of pulmonary vascular control mechanisms in lung injury and possible therapeutic perspectives.
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The acute respiratory distress syndrome (ARDS) in adults is characterized by refractory hypoxaemia in the presence of bilateral pulmonary infiltrates on chest radiography [1]. ARDS is associated with a wide variety of precipitating factors, often not directly involving the lung, and has an associated mortality of 50–80%. ARDS can result from clinical conditions as diverse as gastric aspiration, polytrauma, pancreatitis, haemorrhagic shock, severe burns, oxygen toxicity and surgery involving cardiopulmonary bypass. ARDS is almost invariably associated with sepsis, either as an initiating factor or as a secondary complication, which *via* the action of bacterial lipopolysaccharides, increases the expression of a number of cytokines activating the complement and coagulation cascades. These changes impact upon several cellular systems, including circulating and resident pulmonary phagocytic cells. Activation of neutrophils sequestered in the pulmonary circulation by this process, causes the release of free radicals and reactive oxygen species (ROS), which are increasingly regarded as key substances modulating the pulmonary vascular endothelial damage that characterizes ARDS (fig. 1). The resulting endothelial dysfunction and disruption is responsible for the principal clinical manifestations of the syndrome. Here we discuss briefly the pathophysiology of ARDS and its impact upon pulmonary vascular control, the biological origins of free radicals and other ROS involved, the mechanisms of their damaging effects, their contribution to the modification of pulmonary vascular control mechanisms in lung injury, and possible therapeutic perspectives.

Pathophysiology of ARDS

Early studies examining the characteristics of pulmonary oedema fluid, and more recent isotopic studies have suggested that increased alveolar capillary permeability is ubiquitous in ARDS and lesser degrees of acute lung injury (ALI). Although the severity of hypoxaemia has some influence on outcome, it is not in itself an adverse prognostic sign and the majority of patients with ARDS die a nonrespiratory death, principally from multiple organ failure (MOF). It therefore seems likely that ARDS represents only the pulmonary manifestation of a panendothelial insult, which may result in interstitial oedema formation in most organ systems, leading to impaired tissue oxygenation, partly through a direct effect on diffusion, but also through adverse effects on microvascular control within the regional microcirculation.

Pulmonary circulation in ARDS

Increased pulmonary microvascular permeability is a reflection of damage to the pulmonary endothelium. As in the systemic circulation, endothelial cells influence pulmonary vascular tone, releasing several vasoconstrictor and vasodilator substances, as well as agents that affect the growth and the differentiation of cells in the vessel wall [2]. Many vasodilator agents exert their actions *via* endothelium-dependent mechanisms. Thus, when discussing the actions of drugs on pulmonary vessels, it is important to consider their effects on both endothelial and pulmonary vascular smooth muscle cells. In ALI/ARDS,

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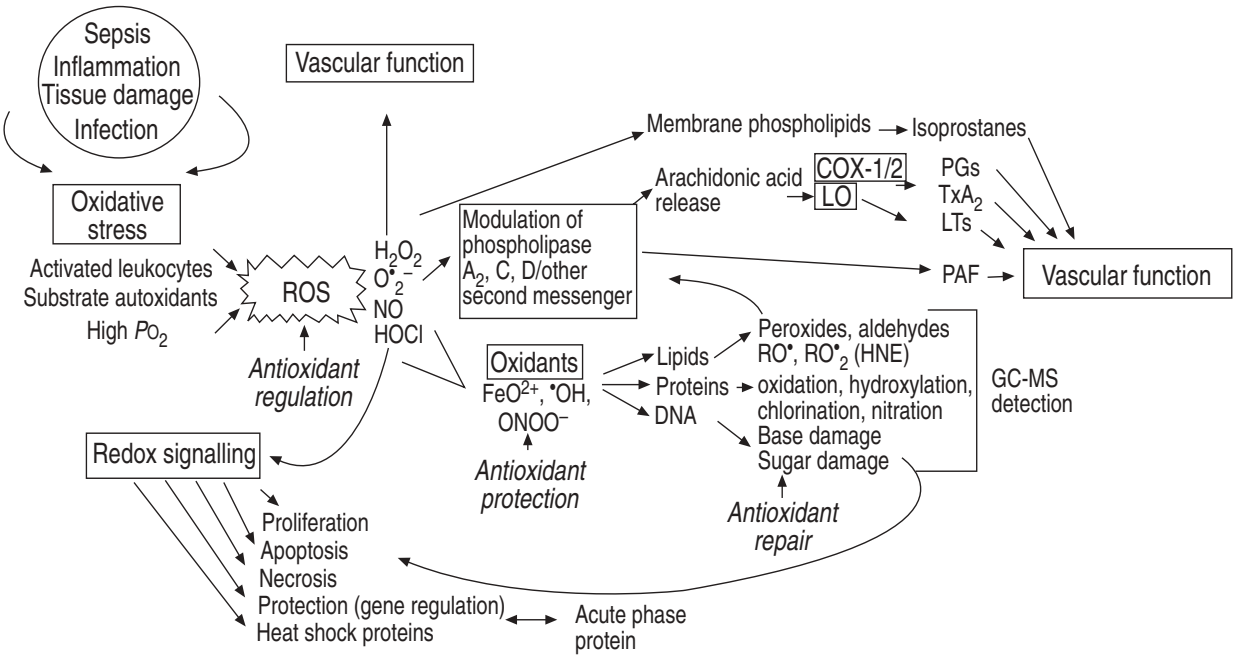

Fig. 1. – The possible role of reactive oxygen species (ROS) in modulating the effects of inflammation on vascular structure and function. P_{O_2}: oxygen tension; COX: cyclo-oxygenase products; LO: lipid oxidation; PGs: prostaglandins; TxA_2: thromboxane A_2; LTs: leukotrienes; PAF: platelet activating factor; H_2O_2: hydrogen peroxide; $O^{\bullet}_2{}^-$: superoxide anion; NO: nitric oxide; HOCl: hypochlorous acid; FeO^{2+}: iron oxide; $^{\bullet}OH$: hydroxyl radical; $ONOO^-$: peroxynitrite; $RO^{\bullet}$: alkoxyl; $RO^{\bullet}_2$ (HNE): peroxyl(4-hydroxy-2-nonenol); DNA: deoxyribonucleic acid; GC-MS: gas chromatography-mass spectrometry.

alveolar oedema formation may directly impair oxygenation, but it appears that ventilation (V')/perfusion (Q') mismatch may account for the majority if not all of the characterisitc refractory hypoxaemia. This in turn is almost certainly attributable to a loss of hypoxic pulmonary vasoconstriction (HPV), the physiological reflex that ensures V'/Q' matching occurs even in the presence of localized pulmonary damage. Moreover, there is evidence in both animal models and clinical investigations that patients with ALI/ARDS develop increased pulmonary vascular resistance (PVR), the extent of which may have prognostic significance. The ideal therapeutic intervention in ALI/ARDS would therefore diminish PVR, improve V'/Q' matching by reducing shunt and lead to a diminution in alveolar-capillary membrane permeability. Many attempts to pharmacologically manipulate the pulmonary circulation in patients with ARDS have therefore been made [3], but none has yet been shown to exert a favourable influence on mortality. Moreover, intravenously administered vasodilators usually cause gas exchange to deteriorate [4].

Free radicals in ALI/ARDS

Free radicals are usually reactive species, characterized by the possession of one or more unpaired electrons (e^-) (fig. 2). Their biological importance in modulating various forms of tissue injury, as well as in molecular signalling, is becoming increasingly clear. Thus, a number of reports
suggest that increased production of free radicals combined with decreased antioxidant capacity of pulmonary vascular tissue may contribute to the prognosis of patients with ARDS. They almost certainly contribute to the vascular damage seen in sepsis, or after the ischaemia/reperfusion inherent in the processes underlying ARDS, surgery necessitating cardiopulmonary bypass and lung transplantation procedures [for review see 5]. Free radicals are also likely to be involved in the regulation of vasomotor tone, by acting on endothelial and vascular smooth muscular cells. Whether they participate in the regulation of arterial tone under normal conditions is of current interest to us, but is, as yet, unknown.

Free radicals, ROS, and reactive nitrogen species (RNS)

Free radicals such as the radical superoxide ($O^{\bullet}_2{}^-$) and the hydroxyl radical ($^{\bullet}OH$) contain e^- whereas ROS such as hydrogen peroxide (H_2O_2) and organic hydroperoxides (ROOH) do not (fig. 2). Radicals can react with other

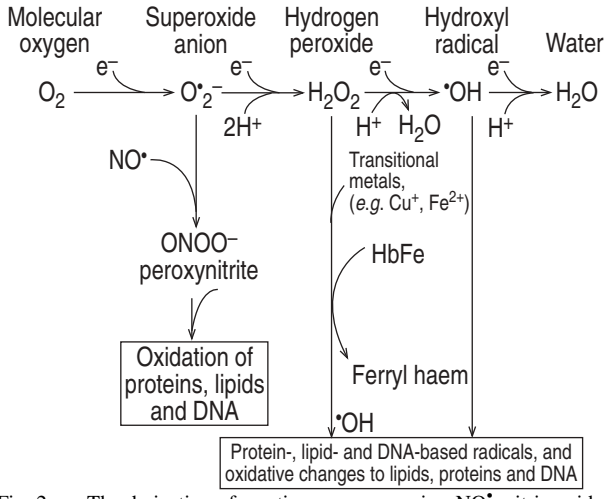

Fig. 2. – The derivation of reactive oxygen species. $NO^{\bullet}$: nitric oxide; Cu^+: copper; Fe^{2+}: iron; Hb: haemoblobin; e^-: unpaired electron; DNA: deoxyribonucleic acid.

radicals combining their e^- or, if sufficiently reactive, with nonradical molecules. When the latter is a polyunsaturated fatty acid, radical attack can lead to the initiation of a free radical chain reaction [for review see 6]. Since most free radicals in biological systems react rapidly with abundant molecular oxygen (O_2), a plethora of ROS have been identified (table 1). Univalent reduction of O_2, *i.e.* the addition of one electron to O_2, produces $O^{\bullet}_2{}^-$, a by-product of normal aerobic metabolism. $O^{\bullet}_2{}^-$ is a good reducing agent but a poor oxidising molecule whose fate, and reactivity depends greatly on environmental conditions within which it is formed. $O^{\bullet}_2{}^-$ is rapidly inactivated by the superoxide dismutases (SODs) [7], or by reacting with the free radical and endogenous vasodilator nitric oxide ($NO^{\bullet}$) (fig. 2). $O^{\bullet}_2{}^-/NO^{\bullet}$ interaction is even more rapid than that with SODs, and thereby limits any vasodilating effects mediated by $NO^{\bullet}$. Such reactions produce the powerful oxidant, peroxynitrite ($ONOO^-$) [8] referred to as a RNS (table 1). $ONOO^-$ may be intrinsically toxic, or may decompose to form other powerful oxidants, with reactivities similar to $^{\bullet}OH$ (fig. 2). However, the interaction between $O^{\bullet}_2{}^-$ and $NO^{\bullet}$ may not always be biologically deleterious. Several experiments have shown that $NO^{\bullet}$ may protect against oxidative damage when $O^{\bullet}_2{}^-$ is being generated [for review see 9].

Two molecules of $O^{\bullet}_2{}^-$ are reduced, either spontaneously, or by SOD-catalysed dismutation to form O_2 and H_2O_2, which is also widely formed by the direct two-electron reduction of O_2 during many enzymic reactions. H_2O_2 is an uncharged molecule that, unlike $O^{\bullet}_2{}^-$ can enter cells in much the same fashion as water (H_2O). H_2O_2 is a relatively stable molecule, unless transition metal ions (such as low molecular mass iron or copper) are present. Thus, in the presence of an iron salt, H_2O_2 is decomposed to yield the highly reactive $^{\bullet}OH$ by the Fenton reaction [10]. The importance of $O^{\bullet}_2{}^-$ in this reaction is to provide H_2O_2 by the dismutation reaction, and to act as a reductant of iron by converting ferric ions to the ferrous state (often referred to as $O^{\bullet}_2{}^-$-driven Fenton chemistry). $^{\bullet}OH$ are a major product of the radiolysis (homolysis) of H_2O by ionizing radiation [for review see 6], and are indiscriminate in their biological reactivity, attacking any molecule present at their site of formation. Hypochlorous acid (HOCl), a potent oxidant formed from H_2O_2 and chloride ions by myeloperoxidase released from neutrophil granules is increasingly recognized as a major damaging species in biological systems [11].

Table 1. – Reactive oxygen and nitrogen species

	Free radicals		Nonradicals	
Reactive oxygen species	$O^{\bullet}_2{}^-$	Superoxide	H_2O_2	Hydrogen peroxide
	$^{\bullet}OH$	Hydroxyl	HOCl	Hypochlorous acid
	$RO^{\bullet}_2$	Peroxyl	O_3	Ozone
	$RO^{\bullet}$	Alkoxyl	$^1O_2$	Singlet oxygen
	$HO^{\bullet}_2$	Hydroperoxyl	ROOH	Hydroperoxide
Reactive nitrogen species	$NO^{\bullet}$	Nitric oxide	$ONOO^-$	Peroxynitrite
	$NO^{\bullet}_2$	Nitrogen dioxide	NO_2^+	Nitronium cation
			NO^+	Nitrosyl cation
			ROONO	Alkyl peroxynitrite

Production and regulation of free radicals in cells

Free radicals are biologically produced in cells by electron transfer reactions, and serve as signalling and messenger molecules [6, 12]. Several enzymes even utilize free radical intermediates at their active sites during catalysis. Thus, $O^{\bullet}_2{}^-$ is produced by activated phagocytes as part of the microbial defence system [13]. During normal metabolism, $O^{\bullet}_2{}^-$ are constantly formed during the oxygenation of haemoglobin, and through leakage from electron transport chains. The accidental production of free radicals is kept to a minimum by efficient electron transfer, enzymic removal, scavenging and by keeping transition metal ions tightly sequestered. Such systems are co-ordinated to provide important biological antioxidant defence mechanisms. Protection can never be completely efficient and oxidative damage at a low steady state can always be detected. Free radical production in cells can be greatly increased by increasing O_2 concentrations in the microenvironment, and by administering "redox-cycling" drugs or toxins. Cellular sources of ROS are clearly many and include: the auto-oxidation and redox cycling of small molecules; generation by enzymes; leakage from electron transport chains; hypo- and hyperoxic states; and reoxygenation syndromes [6, 14].

Oxidant-antioxidant balance

In normal health, the formation of oxidants is balanced by their efficient removal by antioxidants [for review see 15], substances which when present at low concentrations compared with those of the oxidizable substrate, significantly delay, or inhibit oxidation of that substrate [6]. Plasma and tissue antioxidants can be classified into primary, secondary, and tertiary antioxidant defences [16]. Primary defences are best defined as those which prevent radical formation. The iron-binding properties of transferrin and lactoferrin fulfill such a role in extracellular fluids, since iron correctly attached to the high affinity binding sites of these proteins no longer catalyses radical formation. Secondary defences remove, or inactivate, formed ROS. In some cases these may be enzyme systems such as SOD, catalase and glutathione (GSH) peroxidase, or low molecular mass molecules such as vitamin E, ascorbate and GSH. Tertiary defences operate to remove and repair oxidatively damaged molecules, and are particularly important for deoxyribonucleic acid (DNA).

Three forms of SOD are physiologically present: copper-zinc (CuZn)-SOD in the cytoplasm; manganese (MT)-SOD in the mitochondria; and extracellular (EC)-SOD, the major form in the extracellular matrix. Depending on the conditions and concentrations, SOD can protect $NO^{\bullet}$ from inactivation by $O^{\bullet}_2{}^-$ [17], and is necessary for the release of biologically active $NO^{\bullet}$ [18]. The presence of SOD along the diffusion path of $NO^{\bullet}$ to vascular smooth muscle cells should also increase its biologically active half-life, and supports a function for EC-SOD as a mediator of $NO^{\bullet}$ activity in the maintenance of low pulmonary vascular tone [19]. The distribution of EC-SOD suggest that its specific roles may include the protection of the endothelial cell surface from oxidative stress. Cells have redox systems that maintain high concentrations of reduced GSH, which can be used to detoxify H_2O_2 and lipid hydroper-

oxides. GSH synthetase, peroxidase and reductase enzymes normally keep GSH levels high and oxidized GSH (GSSG) levels low. During periods of oxidative stress, GSH is converted to GSSG, which can be converted back to GSH by GSH reductase. Detoxification of H_2O_2 in this way leads to an accumulation of GSSH. Since high concentrations of GSSG can be cytotoxic, it is rapidly removed from the cell. Catalase is also an important enzyme that reduces H_2O_2 to H_2O, removing a key intermediate in the formation of $^{\bullet}OH$ and HOCl. The catalase content of the lung, however, is low [6, 20, 21], and other protective defences operate.

As previously mentioned, secondary defences include enzymatic and nonenzymatic molecules. The efficiency of nonenzymatic molecules may be limited, because they are often consumed during their scavenging roles. In cell membranes, α-tocopherol (vitamin E) plays an important scavenging role in protecting polyunsaturated fatty acids (PUFAs), as well as contributing to the structure of the membrane [22]. Many other molecules can intercept or "scavenge" free radicals, and these include: ascorbic acid; GSH; vitamin A; albumin; glucose; bilirubin; and uric acid, although their exact biological roles, as antioxidants, remain a topic of discussion.

Control of cellular antioxidant capacity

Oxidative damage results from oxidative stress when an imbalance in oxidant-antioxidant equilibrium develops [23], *via* an impairment in the regeneration of antioxidant capacity or in association with increased production of ROS. As proteins, antioxidant enzyme levels are clearly under genetic control, but in eukaryotic cells their expression is not well characterized. Superimposed upon this system is an inducible protective system involving oxidative stress, or heat shock proteins. It is likely that some of these antioxidant protection systems are regulated by cytokines. Thus, interleukin (IL)-1 and tumour necrosis factor (TNF)-α selectively induce Mn-SOD activity without affecting the expression of other antioxidant enzymes [24]. By contrast, in rats exposed to hyperoxia, the activities of various enzymes including Mn-SOD, CuZn-SOD, catalase, and GSH peroxidase are increased by IL-1 and TNF-α [25].

Involvement of ROS in acute lung injury

The purposeful contribution of free radicals to the normal physiological regulation of pulmonary vascular tone remains a topic of considerable research interest. However, there is considerable evidence to support a deleterious role for ROS in the pulmonary vascular abnormalities that characterize ARDS [for review see 16]. In animal models, oedema formation and vasoconstriction are observed when an oxidant generating system, such as xanthine oxidase is infused in rabbit lungs [26]. H_2O_2 appears most damaging to endothelial cells, and subsequently generates the $^{\bullet}OH$ [27–29]. In addition, $O^{\bullet}_2{}^{-}$ can, under appropriate conditions, react with $NO^{\bullet}$ to produce the powerful oxidant, $ONOO^{-}$ [8], which can nitrate proteins, DNA and lipids. In human ALI/ARDS, immunohistochemical staining of lung reveals nitrotyrosine residues, the magnitude of which correlates with the severity of lung injury. This suggests that $ONOO^{-}$ is an important oxidant in inflammatory lung disease [30]. Moreover, ROS may also indirectly contribute to the generation of ALI/ARDS by inactivating antiproteases and depleting antioxidants. In an isolated perfused animal lung model, both H_2O_2 and derived oxidants decrease lung anti-elastolytic activities and as a result, increased neutrophil elastase-mediated lung injury [31].

Damaging reactions of free radicals

Almost all biological molecules are attacked by free radicals possessing the reactivity of $^{\bullet}OH$. Cell membranes are rich in PUFAs, which are particularly susceptible to oxidative damage, resulting in a radical chain reaction known as lipid peroxidation. The oxidative destruction of membrane PUFAs is particularly damaging: firstly, it destroys membrane integrity; and secondly, it produces a plethora of peroxidic and aldehydic products which are highly cytotoxic [for review see 32]. Oxidative modification of proteins can lead to functional impairment or mark them for rapid destruction. DNA is a highly sensitive target for oxidative damage [for review see 33], since modification can lead to the introduction of mutagenic lesions. Pulmonary vascular and aortic endothelial cells exposed to 50 μM H_2O_2 display evidence of DNA damage [34]. This finding is consistent with the observation that cells in culture have redox active ferrous and copper ions associated with their DNA [35]. Nucleic acids are attacked at either the sugar or the base, giving rise to a large number of products, and strand breakage. In most cells, DNA damage causes the transient inhibition of protein synthesis and the arrest of cell growth in association with induction of stress genes and/or genes encoding antioxidant enzymes and proteins involved in the repair process [for review see 36]. The precise mechanism of alterations in gene expression after oxidative stress are not well defined. Transcriptional activation of several genes in response to oxidative stress may involve cytokines such as IL-1 and TNF-α [37], which are induced in various cell types exposed to ROS, and to upregulate expression of antioxidant genes [24, 25]. Cytokines play a complex role in free radical-induced tissue injury, since they can disturb the oxidant/antioxidant balance, by activating inflammatory cells, and by inducing antioxidants [25].

Effects of ROS on pulmonary vascular endothelial cells

It is increasingly apparent that the pulmonary circulation is not merely a passive conduit involved in gas exchange, but is a complex system composed of highly differentiated cells with specialized functions that play key roles in health and disease. Endothelial cells play a key role in regulating vascular tone [2, 38] and pulmonary endothelial dysfunction is a hallmark of ARDS [39]. Considerable evidence indicates that ROS may mediate many forms of endothelial injury [40]. In several whole animal and isolated organ models, endothelial injury appears to be neutrophil-dependent [41, 42]. Activated neutrophils can affect endothelial cell morphology and metabolism, and when "primed" by endotoxin, can initiate

endothelial cell membrane injury. ROS appear to mediate neutrophil-dependent endothelial injury in both *in vivo* and *in vitro* models. Arguments for the role of neutrophil-derived ROS include: the necessity of physical contact between neutrophils and endothelium; the inability of neutrophils from patients with chronic granulomatous disease to cause the endothelial response; the demonstration of lipid peroxidation products coincident with the injury; and inhibition of the neutrophil effect by antioxidants [39]. ROS originating within endothelial cells may also mediate endothelial injury in the absence of neutrophils, specifically in hyperoxic and ischaemia-reperfusion injuries, and indirect injury to the endothelial cells by endotoxin [39]. In alloxan-induced lung injury, endothelial swelling, vesiculation, and vacuolization are induced by H_2O_2 and the subsequent generation of $^{\bullet}OH$ [43]. Electrolysis has been used to study the effects of free radical injury on the intact pulmonary circulation [44], by allowing the delivery of several highly potent oxidizing species directly to the endothelial luminal membrane. Such an approach avoids the necessity for prior ischaemia or neutrophil activation in order to generate radicals. Electrolysis induces selective pulmonary endothelial injury with impaired acetylcholine (endothelial)-dependent vasodilatation, which can be prevented by certain free radical scavengers [44].

The precise biochemical mechanisms involved in endothelial dysfunction remain unknown, but are associated with impaired biosynthesis and release of $NO^{\bullet}$. When ROS alter the endothelial release of $NO^{\bullet}$, pathophysiological changes characteristic of ARDS are seen [45]. The generation of H_2O_2 and other ROS can activate phospholipase (PL)A_2; the initiating step in the production of arachidonic acid metabolites [26, 46]. Oxidative stress is also associated with elevation of intracellular calcium ion [47]. $O^{\bullet}_2{}^{-}$ inhibits synthesis of prostacyclin, but not thromboxane (Tx)A_2, and may be an endothelium-derived contracting factor [48]. The interaction of $O^{\bullet}_2{}^{-}$ and $NO^{\bullet}$ limits vasodilation, but in the process generates $ONOO^{-}$. Thus, oxidant stress appears to influence vascular reactivity by altering the production, release or effect of endothelially-derived paracrine factors through changes in calcium signalling within endothelial cells [40].

Effects of ROS on pulmonary vascular smooth muscle

The direct effect of ROS on pulmonary vascular smooth muscle is less clear. $O^{\bullet}_2{}^{-}$ has a weak contractile effect [48, 49], probably due to inactivation of $NO^{\bullet}$ [50]. Moreover, chemical interaction between $NO^{\bullet}$ and $O^{\bullet}_2{}^{-}$ leads to the formation of $ONOO^{-}$ [8], which has complex biological reactivity. Thus, it relaxes bovine pulmonary arteries, in part by nitrosylating tissue GSH which subsequently releases $NO^{\bullet}$ over prolonged time periods [51], but persistent production of $ONOO^{-}$ can cause depletion of thiols, and hence antioxidant protection, eventually leading to oxidative injury and impairment of physiological function. In isolated perfused rat hearts, $ONOO^{-}$ causes coronary vasodilatation. Following repeated exposure to high concentrations, vascular dysfunction and inhibition of relaxation to other vasodilator compounds develops [52]. By contrast, $ONOO^{-}$ is an effective dilator of pulmonary arteries *via* polyadenosine 5'-diphosphoribose synthase (PARS) activation [53]. By contrast, H_2O_2 causes relaxation of precontracted isolated bovine intrapulmonary arterial rings, independent of endothelially-derived mediators [54]. Conflicting reports exist regarding the action of ROS on pulmonary arterial smooth muscle. High dose H_2O_2 causes reversible endothelially-independent contraction of rat pulmonary arterial smooth muscle, accompanied by some damage to the vascular smooth muscle [55]. $O^{\bullet}_2{}^{-}$, H_2O_2 and activated neutrophils induce contractile responses in pulmonary arteries. Neutrophil-induced contraction is mediated through the release of cyclo-oxygenase products, whereas that caused by ROS, is additionally modulated through protein kinase C activation [56, 57].

Biological origins of ROS involved in tissue damage

Production of ROS by inflammatory cells and cytokines

A large number of ROS are produced by inflammatory cells located in the airways and circulation. Activation of neutrophils with the release of ROS in endothelial cell monolayers [58], isolated perfused lung [59] and in whole animals [59, 60], causes increased pulmonary vascular permeability. When phagocytes are exposed to appropriate stimuli, they form large quantities of $O^{\bullet}_2{}^{-}$, an important precursor of other more reactive species. Stimuli involved in the activation of the phagocytes include bacteria, cytokines such as TNF-α, platelet activating factor (PAF) and endotoxin [21]. Neutrophil responses, including ROS production, secretion of granule contents, and adherence to the endothelium, can be enhanced by prior exposure to endotoxin [61] and cytokines [62, 63]. Recently, evidence suggests H_2O_2 is produced by adherent granulocytes when intact rat lung is treated with endotoxin [64].

Production of ROS stimulated by endotoxin

The importance of neutrophil priming by endotoxin has been observed in several experimental models [65]. In rabbits, administration of low dose endotoxin does not cause lung injury, but neutrophils pretreated with endotoxin and infused in animals, are more frequently retained within the lungs than untreated cells [65]. Endotoxin may enhance production of oxidants by neutrophils *via* stimulation of monocytes, activation of complement, and following endothelial cell injury. Moreover, endotoxin primes monocytes for oxidant [66] and cytokine (TNF and IL-1) release, the latter priming neutrophils for enhanced oxidant production *in vitro* [67]. Activation of complement by endotoxin can initiate and propagate lung injury. Complement fragments can promote the release of $O^{\bullet}_2{}^{-}$ from primed neutrophils, stimulate monocytes and macrophages and increase the conversion of xanthine dehydrogenase to xanthine oxidase [68]. The latter may be an important source of oxidant in lung injury, since it is concentrated within endothelial cells, is released by direct stimulation following cell injury [39] and has been implicated in lung injury resulting from numerous insults including: ischaemia-reperfusion, hypovolaemia, hyperoxia; and hypoxia [69, 70]. Following the exposure of bovine pulmonary artery endothelial cells to endotoxin *in*

vitro [71, 72], early evidence of intracellular free-radical generation has been observed. Direct exposure of endothelial monolayers to endotoxin causes enzyme release and morphological changes, which can be inhibited by certain antioxidants [39]. Endotoxin-induced lung injury in sheep is, in part, neutrophil-dependent [71], and infusion of N-acetylcysteine [73] or catalase [74] attenuates the pathological changes, providing further evidence that ROS, released from activated neutrophils contribute to lung damage. Moreover, infusion of catalase has been reported to decrease lung lipid peroxidation after endotoxin administration [74]. Importantly, the contribution of oxidants to endotoxin-induced lung injury are, in part, species-dependent. Moreover, paradoxically endotoxin can initiate responses induced by pulmonary O_2 toxicity that render tissues resistant to oxidant stress. Thus, rats treated with small amounts of endotoxin achieve a survival advantage following exposure to hyperoxia compared to controls. Tolerance to hyperoxia was dependent upon SOD activity [75], which is known to be increased by endotoxin in cultured bovine and porcine pulmonary endothelial cells [76, 77]. Furthermore, pretreatment of animals or cells with TNF and IL-1 prior to endotoxin exposure decreases pulmonary levels of oxidized GSH and lung injury [78] and increases the level of antioxidant enzyme which may contribute to the increased survival of cytokine-pretreated rats [25]. Additional studies in porcine pulmonary arterial endothelial cells have demonstrated induction of $O^{\bullet}{}_2^-$ dismutase messenger ribonucleic acid (mRNA) in response to TNF-α [77]. Tolerance to hyperoxia is associated with an increase in the lungs of antioxidant enzymes such as $O^{\bullet}{}_2^-$ dismutase, GSH peroxidase and reductase [79].

Production of ROS during ischaemia/reperfusion

Reperfusion of tissue following a period of ischaemia leads to reoxygenation injury and microvascular dysfunction. Pulmonary ischaemia-reperfusion causes increased microvascular permeability [80, 81], although the precise biochemical mechanisms involved are complex and remain incompletely defined. A role for ROS is likely, in that certain antioxidants attenuate injury in isolated lungs following ischaemia-reperfusion [80–82], although the results are sometimes conflicting in intact animal models [83]. Exposure of bovine aortic endothelial cell monolayers to hypoxia and reoxygenation causes increased permeability and release of $O^{\bullet}{}_2^-$ and $^{\bullet}OH$ [84, 85], which is prevented by pretreatment with SOD or catalase [84]. In isolated perfused rabbit lungs, $O^{\bullet}{}_2^-$ release increases after ischaemia-reperfusion [82]. The role of ROS in ischaemia-reperfusion has also been examined by detecting oxidation products of target molecules, and by determining the consumption of tissue antioxidants [for review see 5]. Thus, in an isolated, perfused lung model of ischaemia-reperfusion, certain markers of lipid peroxidation correlate with changes in lung permeability [86]. In rabbit lungs, exposure to hypoxia-reoxygenation decreases lung GSH content, but increases GSSG [87]. $NO^{\bullet}$-derived oxidants such as $ONOO^-$ are thought to be partly responsible for tissue damage in various models of oxidative injury [8], including pulmonary ischaemia-reperfusion [88]. However, exogenous $NO^{\bullet}$ may also prevent ischaemia reperfusion-induced microvascular injury [89]. Although activated neutrophils and other phagocytes sequestered in the pulmonary circulation, are potential sources of ROS during ischaemia-reperfusion injury, they may also be generated by endothelial cells themselves. For example, reoxygenated bovine aortic endothelial cells produce both $O^{\bullet}{}_2^-$ and $^{\bullet}OH$ [85]. Tissue ischaemia increases conversion of xanthine dehydrogenase to xanthine oxidase, which in turn produces ROS during oxidation of its substrates [90]. Allopurinol attenuates lung injury after exposure to ischaemia-reperfusion *via* xanthine oxidase inhibition [81]. The role of endothelium-derived ROS in generating tissue damage was confirmed in a model of heart-lung transplantation, in which endothelial cells appeared to produce 20–40% of the free radicals detected, and in which allopurinol decreased tissue damage [91]. Xanthine oxidase increases membrane permeability of bovine endothelial cells to albumin, and alters their structure [92] *via* the generation of ROS [85].

Activated neutrophils may amplify ischaemia-reperfusion damage in the lung, but their presence is not necessary to initiate the injury, or to allow its full expression, if the degree of ischaemia and resulting hypoxia is sufficiently severe [93]. In some models exposed to a mild ischaemia-reperfusion, neutrophils may be required to trigger the events that result in detectable cell injury [93]. Thus, stimulated neutrophils can convert xanthine dehydrogenase to xanthine oxidase in rat pulmonary artery endothelial cells [94], suggesting that neutrophils recruited to ischaemic microvascular beds can reinforce the injurous process [80]. However, other factors, such as calcium, energy substrates, complement, cytokines, arachidonate metabolites and cytochrome P-450 are also implicated in ROS generation in pulmonary ischaemia-reperfusion injury, although data are somewhat conflicting [93]. During ischaemia, depletion of adenine triphosphate (ATP), and interruption of the sodium-calcium exchanger, results in an influx of calcium that activates proteases capable of destroying the cytoskeleton and inducing the irreversible transformation of xanthine dehydrogenase to xanthine oxidase [93]. Calcium accumulation has been implicated in the inhibition of mitochondrial electron transport and in the increased formation of H_2O_2 [93]. The importance of calcium in modulating ischaemia-reperfusion injury has been demonstrated using verapamil, which improved oxygen transfer and pulmonary haemodynamics more than hydrallazine, suggesting that calcium-channel blockade rather than vasodilatation was responsible for the beneficial effects observed [95].

ROS derived from oxygen therapy and toxins

Free radicals modulating pulmonary vascular control in ALI/ARDS can arise from the therapeutic use of O_2 and chemicals such as paraquat and bleomycin [for review see 20]. When O_2 is supplied to the lungs at concentrations greater than those present in air at normal atmospheric pressure, tissue injury and pulmonary hypertension develop. *In vivo* exposure to hyperoxia for 7 days increases the sensitivity of isolated pulmonary arteries to the vasoconstrictor prostaglandin F2α, and diminishes the ability of acetylcholine to relax preconstricted pulmonary vessels [96]. O_2-induced toxicity is manifested in the pulmonary

arterial vascular bed and in the alveolar septa leading to modifications in cell barrier function, with interstitial and alveolar oedema [97]. An increased concentration of inspired O_2 leads to enhanced intracellular production of $O^{\bullet}_2{}^-$ and H_2O_2 in the lungs, suggesting that hyperoxic toxicity is due to excessive production of ROS, probably by lung mitochondria [98]. Recent studies confirm that pulmonary endothelial cells exposed to 30 min hyperoxia produce free radicals of mitochondrial origin [99]. Hyperoxia also appears to amplify the susceptibility of lung tissue to neutrophil-mediated oxidant damage [100]. The pathophysiological significance of ROS toxicity is supported by investigations demonstrating that antioxidant depletion increases mortality in animals exposed to hyperoxia [101], whereas endotoxin [75] and cytokines [24, 25, 78, 102] induce antioxidant levels and prolong survival. As discussed above, the regulation of pulmonary antioxidant enzymes following hyperoxia is modified by variable changes in gene expression, production of immunoreactive proteins, and enzyme activity [103]. In human umbilical vein endothelial cells, the regulation is also complex and appears to be exerted at different levels [104]. Significant resistance to hyperoxia-induced oxidative stress has been demonstrated in pulmonary endothelial cells overexpressing Mn-SOD [105].

Redox cycling xenobiotics are metabolically activated by intracellular reductases, before they can transfer electrons to O_2 to form $O^{\bullet}_2{}^-$. The popular herbicide paraquat, when taken accidentally or deliberately, is highly toxic to lung tissue and causes severe ARDS [106]. Paraquat has the ability to undergo a single electron reduction to form a stable radical in the absence of O_2 [107]. The paraquat radical reacts avidly with O_2, reconstituting the cation and in the process, generating $O^{\bullet}_2{}^-$ [108]. The radical cycle continues so long as O_2 and a source of electrons are available. The glycopeptide antitumour antibiotic bleomycin is particularly toxic to lung tissue, which has a poor ability to degrade the drug. When bleomycin is delivered by intratracheal instillation, into animal models it rapidly causes ARDS. Bleomycin has a high affinity for DNA, hence its use as an anticancer agent, and in the presence of iron forms a DNA-bleomycin-iron complex. Studies with cultured endothelial cells have demonstrated the requirements for O_2 and iron to allow completion of the bleomycin redox cycle, that causes cell injury [109]. Alloxan, a diabetogenic agent that damages pancreatic tissue, can also induce lung injury *via* ROS production. Certain antioxidants significantly prevented the development of endothelial cell damage [43].

Contribution of free radicals to clinical ARDS

The precise mechanisms that lead to lung damage in ARDS are unknown, but recent investigations suggest such patients are exposed to a severe oxidative burden from a variety of sources and that their antioxidant activities are not optimal to combat this challenge [for review see 110]. Table 2 reviews evidence suggesting that severe oxidative stress is experienced by patients with ARDS. Although many reports support the importance of ROS in modulating lung injury, direct evidence for their production in the pulmonary circulation is difficult to obtain. H_2O_2 produced by adherent granulocytes has been observed in the pulmonary vascular endothelium of rats challenged with endotoxin, but did not cause lung oedema [64]. Patients with ARDS receive aggressive therapeutic support, including mechanical ventilation, positive end-expiratory pressure and high O_2 concentrations. Such an aggressive approach has been thought necessary to maintain O_2 transport, but may be implicated in exacerbating the primary injury.

There are several clinical conditions in addition to ARDS in which the lung is subjected to temporary ischaemia-reperfusion, including lung transplantation and surgery involving cardiopulmonary bypass. The latter is associated with a small but significant incidence of severe alveolar-capillary injury presenting as ARDS [128]. Prospective ultrastructural and functional studies suggest, however, that minor degrees of cellular injury after cardiopulmonary bypass are invariable [129, 130]. Increased pulmonary transvascular protein flux after canine cardiopulmonary bypass is mediated in part by neutrophil-derived ROS [131], which have also been detected in patients undergoing bypass for myocardial revascularization [132, 133]. ROS generation during cardiopulmonary bypass can be due to contamination of the circulating blood by endotoxin which primes the neutrophils [62]. In piglets, cardiopulmonary bypass impairs pulmonary $NO^{\bullet}$ production, resulting in pulmonary vasoconstriction and right ventricular dysfunction; these changes can, apparently, be decreased by antioxidants [134]. Cardiopulmonary by-pass procedures introduce several distinct phases of oxidative stress, that lead to the damage of proteins and lipids in the circulation. For example, extracorporeal circulation of blood activates neutrophils and regulating cascades leading to $O^{\bullet}_2{}^-$ and H_2O_2 production, and to the lysis of red blood cells. H_2O_2 can react with released haemoglobin to free ROS [135]. Additional iron release occurs in patients receiving warm blood cardioplegia [136]. Reoxygenation injury is also a feature of surgery involving cardiopulmonary bypass (CPB) and occurs when the aortic cross-clamp is released. Recent work has shown that ischaemia-reperfusion during CPB leads to an increase in plasma hypoxanthine levels, particularly when warm blood cardioplegia is used [137], further increasing the potential for ROS generation.

Some degree of hypoxia in organs stored for transplantation is difficult to avoid. The use of antioxidants in lung preservation fluids has therefore been of interest. Many of the basic constituents of the perfusates used in flush perfusion already contain such agents [138]. In lung transplantation, a number of antioxidants have been added to preservation regimens, either in the perfusate, or as a pretreatment, or at the time of reperfusion. These approaches have shown reduced oedema formation and improved function [91, 138, 139]. The contribution of oxidants to lung rejection has been considered. During episodes of acute rejection, endothelium-dependent vasorelaxation to agonists, such as adenosine diphosphate (ADP), are decreased in pulmonary arteries of allotransplanted lung. Recent experiments designed to define responses of pulmonary arteries to rejection-activated leukocytes, and to identify possible mediators of those responses, have suggested that mononuclear cells cause vasoconstriction. In rings with intact endothelium, mononuclear cells activated by acute pulmonary rejection caused greater contraction, and this was decreased by the addition of SOD and catalase [140]. Thus, antioxidants may have a beneficial role

Table 2. – Some evidence that patients wtih acute respiratory distress syndrome (ARDS) are under severe oxidative stress

	Finding	Comments	Refs
Evid. of increased oxidants			
	Increased H_2O_2 in exhaled breath	Breath vapour condensate from normal subjects contains little H_2O_2	111 112
	Inactive α_1-antiproteinase in BAL	Probably damaged by ROS, RNS, or reactive chlorine species	113
	Decreased levels of GSH in lung lavage fluid, and RBC's	Increased levels of GSSG found	114 115
	Loss of plasma thiol groups	Nonsurvivors of ARDS often have lower thiol levels	116
	Increased plasma protein carbonyl groups	Highly suggestive of oxidative damage by RIS	116
	Presence of catalytic iron in the plasma	Present when ARDS patients are in multiorgan failure	117
	Presence of catalytic iron in BAL fluid	BAL fluid from normal volunteers contains RIS, as does BAL from ARDS survivors. Nonsurvivors, however, show no RIS in BAL, but high transferrin levels due to leak from the plasma	118
	Increased lipid peroxidation products in plasma	Increased TBA reactivity	119
		Increased 4-hydroxynonenal	121
		Decreased linoleic and arachidonic acids	122
	Increased plasma xanthine oxidase activity	Maybe released from injured tissues after oxygenation injury to the lung	123
	Increased plasma hypoxanthine	Indicative of hypoxia, and aberrant ATP catabolism during ischaemia-reperfusion	124
	Nitrotyrosine formation	Immunostaining of lung tissue from ARDS patients revealed nitrotyrosine suggestive of damage by $ONOO^-$ or other RNS	8
		HPLC and GC-MS techniques show increased plasma levels of nitrotyrosine in patients with ARDS compared to controls	124
	Orthotyrosine formation	HPLC and GC-MS techniques show increased levels of plasma protein orthotyrosine, suggestive of increased $^{\bullet}OH$ formation	125
	Chlorotyrosine formation	HPLC and GC-MS techniques show increased levels of plasma protein chlorotyrosine which correlates with myeloperoxidase activity in patients with ARDS	125
Evid. of decreased antioxidants			
	Low levels of plasma ascorbate	Possibly destroyed by oxidants	126
	Low levels of plasma α-tocopherol	Appear to be low when not standardized to lipid content of plasma	127 119 121
	Decreased plasma caeruloplasmin (Cp) "ferroxidase" activity	Plasma Cp protein levels often elevated but ferroxidase activity per unit of protein is decreased	120
	Low transferrin levels in plasma, with increased percentage saturation with iron	Iron-binding antioxidant activity of plasma is low due to loss of iron-binding capacity	120

Evid.: evidence; H_2O_2: hydrogen peroxide; BAL: bronchoalveolar lavage; ROS: reactive oxygen species; RNS: reactive nitrogen species; RIS: reactive iron species; GSH: glutathione; GSSG: oxidized GSH; RBCs: red blood cells; TBA: thiobarbitaric acid; ATP: adenine triphosphate; $ONOO^-$: peroxynitrite; GC-MS: gas chromatography-mass spectrometry; HPLC: high-performance liquid chromatography; $^{\bullet}OH$: hydroxyl radical.

not only in allowing organ preservation for longer periods, but also in diminishing graft rejection, as has been previously suggested for other organs.

Removal of a thrombotic obstruction in a major pulmonary vessel following thromboendarterectomy, or exceptionally after thrombolytic therapy for acute embolism [141], or in re-expanding lung after a large quantity of fluid or air is rapidly aspirated from pleural effusion or pneumothorax which has been present for several days can lead to ischaemia-reperfusion injury. Endothelial injury has been observed in ARDS after vascular thrombosis has been resolved, probably exacerbated by high inspired levels of O_2 [142].

Therapeutic perspectives

Over a number of years the pharmacological use of SOD and catalase as intervention antioxidants in human diseases have proved to be ineffective. However, in a number of animal models they are protective. For example, the infusion of SOD attenuates endotoxin-induced lung injury in guinea-pigs [143] and sheep [144]. Treatment with SOD significantly decreases endotoxin-induced rises in pulmonary artery pressure, pulmonary vascular resistance and microvascular permeability [144]. SOD also prevents the effects of TNF-α on the vasorelaxant response to acetylcholine and TNF-induced decreases in nitrovasodilator activity [145]. However, in TNF-induced lung injury [146], SOD attenuates the pulmonary hyper-

tension but fails to block the increase in pulmonary vascular permeability [144]. Infusion of catalase in sheep attenuates the pulmonary oedema generated by air emboli [147]. The use of SOD is limited by its short circulating half-life (5–6 min) and its limited capacity to penetrate cells [6, 143]. In order to improve the effectiveness of SOD it has been targeted to the cell surface and had its circulatory half-life increased. Heparin-binding derivatives [148] achieve the former and polyethylene glycol (PEG)-SOD the latter. PEG-SOD also has a greater uptake by endothelial cells, compared with native SOD, and can result in increased intracellular SOD activity. Liposome encapsulation has been employed to improve the intracellular utilization of both SOD and catalase. It increases intracellular SOD concentrations in cultured aortic endothelial cells, which then display augmented resistance to hyperoxia [149]. The use of enzyme supplementation introduced many problems, and excess SOD may even be toxic, perhaps through enhanced genearation of H_2O_2 [150]. Pharmacological approaches to inhibit free radical generating systems, such as iron chelation or xanthine oxidase inhibition are possible. Iron chelation with desferrioxamine offers protection in several models of ARDS [150]. Allopurinol has been used in ischaemia-reperfusion injury as an antioxidant to inhibit xanthine oxidase activity, and hence tissue damage [90]. Dietary supplementation of patients with ALI/ARDS with nonenzymatic antioxidants such as vitamin E, N-acetylcysteine [151] and dimethylthiourea (DMTU) has also been investigated. Cultured endothelial cells treated with vitamin E undergo less injury after exposure to stimulated neutrophils or H_2O_2 [152]. Vitamin E deficient rodents have increased susceptibility to oxidative stress after exposure to endotoxin [153], and resulting tissue injury can be decreased by vitamin E treatment [154]. However, in a prospective, double blind, placebo controlled trial in 66 patients with ARDS, N-acetylcysteine failed to improve survival or the indices of lung damage [155]. DMTU can react with $^{\bullet}OH$, H_2O_2 and HOCl *in vitro*, has a long biological half- life and distributes well across cell membranes. DMTU decreases oxidant-induced injury in several experimental designs, such as in cultured endothelial cells [156], and isolated perfused lungs [59]. Additionally, DMTU infusion into canulated pigs attenuates the pulmonary membrane permeability alterations resulting from endotoxaemia [157]. However, DMTU has limited capacity to scavenge ROS, and high concentrations of DMTU are cytotoxic. There is increasing evidence to suggest that $NO^{\bullet}$ may be an effective scavenger of ROS [158] and may inhibit iron-containing enzymes such as xanthine oxidase [159]. Recently, $NO^{\bullet}$, has been used as a vasodilator to treat patients with ARDS [160]. In nine patients, $NO^{\bullet}$ administered by inhalation for 40 min produced a significant decrease in the mean pulmonary artery pressure, and in the shunt fraction. Seven patients were subsequently treated with inhaled $NO^{\bullet}$ for 3–53 days, during which time the investigators were able to decrease the inspired O_2 concentration by 15% [161]. Genetic control and induction of antioxidants, and of oxidative stress proteins by pharmacologically altering their synthesis hold great promise for understanding and controlling oxidant-mediated processes. Increases in antioxidants that occur in rats exposed to hyperoxia [101], cytokines [25] or endotoxin [75] are most likely under genetic control. Cytokine or endotoxin administration to rats, increases lung Mn-SOD mRNA within 4 h. In parallel, lung Mn-SOD protein and activity increase after 24 h, and remain elevated for several days [102]. Moreover, bovine pulmonary endothelial cells cotransfected with the Mn-SOD expression vector, overexpress Mn-SOD and show a twofold increase in survival when subjected to oxidant stress, compared to control cells [105].

Conclusion

Reactive oxygen species contribute to diseases associated with pulmonary vascular lesions, such as lung injury in both human subjects and relevant animal models. In many cases their contribution to molecular damage is obvious by the footprints they leave behind. Their more subtle contribution to physiological processes by acting as signal, messenger and trigger molecules is however, less well understood. Antioxidant supplements have to date a poor record in treating life-threatening diseases, and future developments are more likely to come *via* genetic manipulations.

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