Iron signalling regulated directly and through oxygen: implications for sepsis and the acute respiratory distress syndrome

G. J. QUINLAN, Y. CHEN, T. W. EVANS and J. M. C. GUTTERIDGE
Unit of Critical Care, Royal Brompton Hospital and National Heart and Lung Institute, Imperial College School of Medicine, Sydney Street, London SW3 6NP, U.K.

ABSTRACT

Reactive oxygen species produced at toxic levels are damaging species. When produced at sub-toxic levels, however, they are involved as second messengers in numerous signal transduction pathways. In addition to these findings, we can add the concept that iron (often viewed as the ‘villain’ in free radical biology) can also be considered as a signalling species. Iron is intimately involved in the regulation of its own storage, compartmentalization and turnover. During adult respiratory distress syndrome (ARDS) and sepsis, such regulation may be aberrant or altered in some predisposed way. Such changes may have profound implications for tissue damage, and for the modulation of the inflammatory response in these patients. The search for a genetic predisposition in patients that leads to the development of ARDS associated with abnormalities in iron turnover and signalling would seem to be an important and logical progression for studies into the disease. These may lead eventually to the design of effective treatment regimens that involve the control of iron.

CLINICAL PERSPECTIVE

The majority of deaths among critically ill patients requiring intensive care are attributable to infection and its vascular sequelae: sepsis, septic shock, the systemic inflammatory response syndrome (SIRS) and (eventually) organ system failure. The commonest organ thus afflicted is the lung, and some 40% of patients with sepsis develop acute lung injury or its extreme manifestation, the acute respiratory distress syndrome (ARDS). ARDS is responsible for up to 50000 deaths per annum in the U.S.A., and acute lung injury results in mild respiratory impairment necessitating intensive care for thousands more. Sepsis/SIRS and acute lung injury/ARDS are therefore closely linked from the epidemiological and pathophysiological viewpoints: lung injury in all its manifestations represents the pulmonary component of a pan-endothelial insult that characterizes sepsis/SIRS. Together, these conditions cause an estimated 100000 deaths per annum in the U.S.A. alone and represent by far the commonest causes of prolonged admission to an intensive care unit (ICU).

INTRODUCTION

There is good evidence to suggest that levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are elevated in critically ill patients, and that such...
species cause molecular damage and contribute to the pathogenesis of both ARDS and sepsis [1]. Evidence to support a role for ROS and RNS in these conditions comes almost entirely from experimental animal models, and from observational studies in patients in whom characteristic markers of oxidative damage have been measured [2,3]. ROS- and RNS-mediated molecular damage represent extreme events in the disease process. Recent evidence, however, suggests that the production of these reactive species at sub-toxic levels can also have profound biological consequences through their functions as second messengers and redox signal molecules.

Transition-metal ions, particularly iron in the form of low-molecular-mass (LMM) biological complexes, are capable of catalysing the formation of a variety of inorganic ROS [4], such as the hydroxyl radical (•OH) from hydrogen peroxide (H$_2$O$_2$) (Fenton reaction) and from hypochlorous acid (HOC1).

The hydroxyl radical is an exceedingly aggressive species that reacts with most biological molecules at near diffusion-limited rates. During this process, it leaves behind patterns of oxidation characteristic of its presence [4]. Evidence suggests that the hydroxyl radical may act as a signalling species for the up-regulation of cellular processes, including transcription [5] and apoptosis [6]. The present reviewers, however, are more cautious in ascribing a direct purposeful biological role to such a reactive species. Iron can also react with organic ROS to form products that modulate cellular responses (reviewed in [7]).

A key feature of LMM Fe is that it regulates its own cellular uptake and turnover through the iron-regulatory proteins (IRPs), and, together with ROS and RNS, mediates signal transduction pathways (redox signalling). In this short review we discuss some of the main concepts of redox signalling involving iron, with special emphasis on their relevance to critical illness.

**REDOX SIGNALLING AND THE ACTIVATION OF NUCLEAR FACTOR-κB (NF-κB)**

The redox state of any biological molecule in defined in terms of $E_{\text{red}}$ in volts (V). The more strongly reducing an ion or molecule, the more negative is its $E_{\text{red}}$. The sum of these oxidative and reductive processes defines the redox balance within that environment. Under normal circumstances, most biological environments are in a reduced state, maintained in cells by thiols (R-SH) such as glutathione (GSH), and by NADH and NADPH. The same is also true of extracellular fluids such as plasma, where high-molecular-mass protein thiols provide reducing equivalents, in addition to numerous LMM compounds such as ascorbate, urate and glucose. Oxidative stress can alter the overall redox balance and lead to a more oxidizing, or a less reducing, environment.

Cells have evolved to deal with oxidative stress by using constitutive and inducible antioxidant systems, and by removing and repairing damaged molecules. In extreme circumstances, protective mechanisms such as these can become overwhelmed, which results ultimately in oxidative damage, involving profound biological changes. For example, cells exposed to oxidative stress invariably show damage to their DNA. The cell contains a nuclear, enzyme poly(ADP-ribose) polymerase, which is activated by DNA strand breaks and may participate in DNA repair or, more likely, in other cell signalling processes. If the oxidative insult is sustained, excessive activation of poly(ADP-ribose) polymerase leads to the depletion of tissue stores of NAD$^+$ and ATP, resulting in cell death by either apoptosis or necrosis.

Severe oxidative stress results in oxidative damage to most types of biological molecules, often leading to a loss of their biological function, as well as to the increased accumulation of toxic oxidation products [4]. Increased ROS production seems to be an inevitable consequence of most disease processes, since evidence of oxidatively damaged lipids, proteins, carbohydrates and DNA is seen in a variety of clinical conditions [8]. Oxidative damage represents an extreme event resulting from oxidative stress, whereas production of ROS and RNS at sub-toxic levels may have more subtle implications. The concept is emerging that sub-toxic levels of ROS and RNS play key roles in redox signalling. A more obvious example of redox signalling relates to the phenomenon of preconditioning. When an organism is exposed to sub-lethal stress due to a deleterious agent such as hyperoxia, followed by a recovery period, and then re-exposed to higher levels of hypoxic stress, the organism becomes better able to tolerate such stress. Several groups have shown that pre-exposure to sub-lethal hyperoxia leads to significant protection against re-exposure to lethal hyperoxic stress. The protective mechanisms involved implicate the up-regulation of antioxidant enzymes [9], demonstrating a sensing and signalling function for the ROS produced during hyperoxia. Observations suggest that numerous cellular functions are controlled by redox regulation at the transcriptional level. The most notable of these systems is activation of the nuclear transcription factor NF-κB, first identified in B-cells in 1986 [9a] but now recognized as ubiquitous. In the cytoplasm, NF-κB exists as an inactive form bound to its inhibitor IκB. Upon activation, the transcription factor migrates to the nucleus, where it binds to κB binding sites in the promoter sequences of a variety of genes. The formation of numerous gene products is associated with NF-κB activation, including cytokines, cell adhesion molecules, acute-response proteins and NF-κB itself (reviewed in [10]). Numerous chemical agents, such as inflammatory mediators and ROS, appear to activate NF-κB. The nature of the stimuli for and gene products produced by this transcription factor strongly suggest that it is
involved in modulating both immune and inflammatory responses.

Oxidants may, both directly and indirectly, be principal regulators of NF-κB activation, although this viewpoint is the subject of current debate. In support of a key role for oxidants as activators, cytokines such as tumour necrosis factor-α (TNF-α) have been shown to mediate NF-κB activation via a hydrogen peroxide second messenger system [11]. Organic hydroperoxides may also be involved in cytokine-mediated NF-κB activation, since it has been demonstrated, using interleukin-1 (IL-1), that the peroxide will not activate NF-κB in endothelial cells overexpressing phospholipid hydroperoxide glutathione peroxidase [12]. Further, in a rat model of IgG immune-complex-induced lung injury, damage is mediated by cytokines (TNF-α and IL-1β) via ROS-dependent mechanisms involving oxidants generated independently of neutrophil activation [13]. Iron has also been implicated in NF-κB activation using a rat model of liver injury [14]. Ligation of the common bile duct resulted in increased levels of markers of oxidative damage. Hepatic macrophages showed increased levels of TNF-α and IL-6 mRNAs, together with NF-κB activation. Iron chelation therapy effectively blocked these effects in the in vivo model, as well as in cultured macrophages pretreated with lipopolysaccharide. Recent studies have indicated that haem oxygenase-driven haem turnover is important for iron priming of hepatic macrophages for NF-κB activation and for the expression of pro-inflammatory genes in alcoholic liver injury [15]. Iron has also been implicated as an activator of NF-κB and AP-1 (activator protein-1, another transcription factor) in hydrogen peroxide-induced apoptotic cell death of oligodendrocytes [16]. This may represent an example of iron acting as an important signal molecule, a concept that is discussed in greater detail below. Challenging animals with endotoxin causes changes in cellular signal transduction and gene expression [17], some of which may be mediated through changes in redox balance. Endotoxin probably elicits changes in the redox balance by increasing cellular levels of superoxide and hydrogen peroxide [18], both of which can activate NF-κB as second messengers. In support of this concept, IL-8 gene expression, mediated by NF-κB activation in response to endotoxin treatment of human astrocytoma cells, has been found to be redox-regulated through a glutathione-dependent pathway [19].

Activation of NF-κB occurs by removal of the inhibitory IκB subunit. In this process, redox-sensitive kinases phosphorylate IκB, thereby labelling the inhibitory subunit for removal by a 26 S proteasome complex. Hydrogen peroxide appears to be the principal ROS responsible for the activation of NF-κB, but roles for superoxide (O$_2^-$) [20], the hydroxyl radical [15,16], organic peroxides [12] and RNS [21] are still being considered. Recent studies have also implicated thiols as redox sensors involved in NF-κB activation. For example, addition of the thiol-containing antioxidant S-allylcysteine prevented both hydrogen peroxide- and TNF-α-induced NF-κB activation in human T lymphocytes [22]. Additionally, thiol-modifying agents have been shown to inhibit NF-κB activation in response to a TNF-α challenge [23], and the antioxidant dimethylthiourea protected against sepsis in rats by inhibiting activation of NF-κB [24]. Furthermore, it has been demonstrated that free thiol groups are important for the DNA binding of active transcription factors, including NF-κB [25]. It is proposed that binding may be modulated by the ratio of oxidized to reduced glutathione within cells, and by a redox-sensitive thiol group on the p50 subunit of the transcription factor [26]. When this thiol group is nitrosylated by exposure to peroxynitrite, the DNA-binding ability of p50 is decreased 4-fold [27]. Indeed, nitric oxide may regulate expression of nitric oxide synthase (NOS) type II by preventing binding of NF-κB to DNA [28], as does the thiol-binding agent selenite. It appears that NF-κB can be redox-regulated at two levels, i.e. through activation and through DNA binding. Both of these processes appear to involve the participation of cellular redox-sensitive thiol groups. Clearly, NF-κB represents only one of numerous transcription factors that have been found to respond to redox regulation; others include AP-1, p53 and CREB (cAMP-response-element-binding protein) [25,29,30]. This type of signal transduction may represent a common mechanism for the transcription of a diverse array of gene products (for review, see [31]), co-functioning with protein tyrosine kinase, protein tyrosine phosphatase, lipid and Ca$^{2+}$ signalling pathways (reviewed by Kamata and Hirata [32]). The role of ROS and RNS in signalling and vascular control has been reviewed recently [33].

**Iron signalling in sepsis and acute respiratory distress syndrome**

**Iron Utilization and Control**

Iron is an element essential to most aerobic life forms, and is required for the synthesis of iron-containing proteins and DNA. Iron-containing proteins function as catalysts, such as the oxidases, oxygenases and certain antioxidants, and also as transporters of oxygen and electrons. Since iron is poorly absorbed and is excreted by the body, mechanisms have evolved to retain and recycle it, using iron transport and iron storage proteins.

**Iron Transport**

Extracellular iron transport is carried out by the mainly liver-synthesized protein transferrin. In normal human plasma, transferrin is only 25–30% loaded with iron, and the available iron-binding capacity represents a sub-
stational antioxidant (and antimicrobial) capacity [34], since iron correctly bound to transferrin is no longer redox active. Plasma also contains a copper-containing protein called caeruloplasmin which responds as an acute-phase reactant. Caeruloplasmin has several oxidase activities in vitro, but, with the exception of its ferroxidase activity [34] and glutathione peroxidase-like activity [35], these appear to be of no physiological importance. The ferroxidase activity of caeruloplasmin converts reactive ferrous ions (Fe\(^{2+}\)) into the less reactive ferric state (Fe\(^{3+}\)). This enzymic iron-oxidizing (ferroxidase) activity of caeruloplasmin has been considered to be protective [34], because Fe\(^{3+}\) can be loaded into transferrin at physiological pH. Lactoferrin is an iron-binding protein that is released by activated neutrophils into the extracellular space. Like transferrin, lactoferrin binds, with high affinity, 2 mol of Fe\(^{3+}\) per mol of protein. Unlike transferrin, however, lactoferrin can hold on to its iron at lower pH values. Extracellular fluids also contain proteins that specifically bind, transport and conserve haem-containing molecules. Thus haemopexin binds haem iron, and the haptoglobins bind haemoglobin. In the process of retaining and recycling iron, these proteins also remove pro-oxidant forms of iron, and so act as antioxidants [36,37]. When we consider iron-binding and iron-oxidizing molecules in extracellular fluids, we see that, while functioning as iron-recycling proteins, they also keep iron in non- or poorly reactive fluids. The latter have a role in the regulation of iron mobilization [38]. We now address this aspect of iron signalling.

**TRANSFERRIN RECEPTORS (TfRs) AND FERRITIN**

Most cells acquire the iron they need by using TfR to facilitate the uptake of iron-loaded transferrin. Once inside the cell, iron can be stored safely inside the protein ferritin. TfRs are expressed on the surface of iron-requiring cells, where they bind transferrin for receptor-mediated endocytosis. Iron is released from transferrin within the cell by an acidification process, which weakens the affinity of iron for transferrin. In human serum, a soluble TfR is found [39], which is generated by proteolytic cleavage of the extracellular domain of cellular TfR. In rat liver, the strong correlation between iron release and levels of soluble TfR suggests the latter have a role in the regulation of iron mobilization [40].

Ferritin is an intracellular iron-storage protein composed of 24 subunits. The resultant protein shell encloses a core of stored iron (up to 4500 molecules of iron can be stored per ferritin molecule). The proportions of these 24 subunits can vary widely between ferritins of different tissue origin and in various disease states (reviewed in [41]). In human ferritins the H (heavier) subunit is more acidic and predominates in heart tissue, whereas the L (lighter) subunits predominates in liver and spleen. The H-chain subunit has a ferroxidase centre that is necessary for iron uptake by the ferritin molecule. Deletion of the H-chain gene in mice leads to early embryonic lethality. Ferritin is found in human serum, although it has no H-chain isoforms [42]. Receptors for serum ferritin have been reported in different cell types, including liver cells, lymphocytes and lipocytes, suggesting that serum ferritin may have distinct roles in the transfer of iron between different cells, and in the regulation of cellular proliferation.

**IRPs**

The expression of ferritin and of TfRs is regulated post-transcriptionally by the IRPs. Two IRPs, IRP-1 and IRP-2, have been identified [43] as monomeric cytosolic proteins of molecular masses 90–95 kDa and 105 kDa respectively. When IRPs are activated they bind to iron-responsive elements (IREs) located in untranslated areas of TfR and ferritin mRNAs [44]. IREs are stem–loop structures located in the untranslated 5' region of ferritin mRNA and in the 3' region of untranslated TfR mRNA [45]. The binding of IRPs to IREs on ferritin mRNA is triggered by low cellular iron levels; this results in an inhibition of ferritin synthesis, because the mRNA is prevented from ribosomal association. Conversely, IRP binding to IREs on TfR mRNA prevents proteolytic degradation by targeted endonucleases, so stabilizing the mRNA. This results ultimately in increased synthesis of TfR. When cellular iron levels are high, the IRPs are inactivated, and the reverse of the above situation occurs (Figure 1). For a recent review of the IREs and the role of oxygen and iron in ‘on/off’ signalling, see [46]. Several studies have demonstrated links between iron uptake or release and ferritin synthesis. For example, it has been demonstrated that increased dietary iron intake results in depressed liver ferritin synthesis [47]. In addition, ischaemia/reperfusion injury leads initially to ferritin degradation and iron release, which then acts as a signal for renewed ferritin mRNA synthesis, in order to re-establish ferritin levels [48].

The mechanisms by which the IRPs are activated are diverse and still incompletely understood. Activation of IRP-1 involves an iron–sulphur cluster [((3Fe–4S)\(^+\) + Fe\(^{3+}\)(4Fe–4S)\(^2+\)]) which, depending on its iron status, acts either as an RNA-binding protein or as a...
cytoplasmic aconitase [49]. IRP-2, however, does not show aconitase activity, and nor does it possess an iron–sulphur cluster. Evidence suggests that IRP-2 activity is regulated by proteasome-mediated degradation, a process that involves an iron-dependent oxidation mechanism and requires a unique amino acid sequence containing three cysteine residues [50]. Haem also appears to be capable of mediating IRP-2 degradation [51]. The regulation of the IRPs by LMM iron represents the clearest known example of iron acting as a biological signal molecule. Many questions still remain to be answered, however, since molecules other than iron can activate IRPs. Thus some oxidants and inflammatory mediators elicit opposing responses of IRP-1 and IRP-2 [52], as does hypoxia [53]. There also appear to be tissue-specific variations in IRP-1 and IRP-2 levels [54], and in certain cell lines IRP-2 activity has been shown to be the sole mediator of iron turnover, demonstrating that both IRPs are not required in order to maintain iron homoeostasis [55]. Cell and animal experiments also suggest that activation of IRP-2 is more rapid than that of IRP-1 [56].

The importance of IRPs to advanced life forms is shown by their highly conserved sequence identity between species [44,54,57]. Further, in support of this, IRP-1 mutant cell lines that bind IREs regardless of cellular iron content (and are therefore unable to maintain cellular iron homoeostasis) exhibit diminished survival rates [58]. Presumably, uncontrolled iron accumulation, leading to oxidative stress, and serious molecular damage accounts for the increased mortality.

We have already mentioned that iron is not an exclusive signal for IRP activation, since several oxidants also activate these proteins. There is confusion, however, as to the precise role of these various factors in activation. For example, when cell lysates are exposed to ROS generated by xanthine oxidase, IRP binding activity is inhibited, but the situation is reversed by the addition of ROS scavengers (superoxide dismutase or catalase) [59]. The addition of ascorbate to cells in culture that have been challenged with iron results in an increase in ferritin synthesis, suggestive of a down-regulation of IRP activity [60]. Experiments with ascorbate often cause confusion, particularly when reactive iron species are present, since, depending on the concentration of ascorbate present, it can act as either an antioxidant or a pro-oxidant. When cells are treated with hydrogen peroxide, or starved of iron, they show greater IRP activation in response to \( \text{H}_2\text{O}_2 \) than to a lack of iron. Such a response is not seen, however, when cell extracts are used. Also, IRP induction by hydrogen peroxide can be blocked by protein phosphatases, suggestive of a stress-induced kinase activation pathway, which appears to be distinct from the iron induction pathway [61]. There are suggestions that IRP activation by extracellular ROS is mediated by an as yet unidentified insoluble membrane-associated component [62]. Indeed, it has been demonstrated that activation of IRP-1 differs in response to extracellular or intracellular oxidative stress, and that, in the case of intracellular ROS generation, it does not result in disruption of the iron–sulphur cluster [63]. Recently, murine fibroblasts...
subjected to quinone-mediated intracellular oxidative stress were found to lose both IRP binding ability and aconitase activity. This post-translational loss of both enzymic and genetic functions appeared to be unrelated to the Fe–S cluster switch [64]. The involvement of redox-sensitive thiols in IRP activation has been shown for IRP-1, since Cys-437 must be free and in a reduced form for activation and RNA binding to occur [65]. Similarly, IRP-2 activation has also been shown to be dependent on redox-active thiols, since RNA binding can be inhibited by thiol alkylating agents or by oxidation [66]. Iron does not appear to be involved directly in hydrogen peroxide-mediated IRP activation, at least through the Fenton reaction [67]. Hypoxic inactivation of IRP-1, however, can be abolished if the cells are pretreated with the iron chelator desferrioxamine, suggesting that iron is acting as a signal molecule under these conditions [68]. Opposing effects have been reported during hypoxia experiments using different cell lines [69]. In these studies hypoxia was found to strongly enhance IRE–IRP-1 binding, but to decrease cytosolic aconitase activity.

RNS also activate IRPs. For instance, synthesis of nitric oxide has been shown to lead to inhibition of mitochondrial and cytoplasmic aconitases, while increasing IRP binding to mRNA [70]. Furthermore, human cell lines in culture show diminished ferritin expression and increased Tfr synthesis when NOS is active, an effect reversed by the NOS inhibitor N\textsuperscript{G}-methyl-L-arginine [71]. Additionally, endothelial cells exposed to iron in the presence of NO donors exhibit a down-regulation of ferritin synthesis, accompanied by a longer-term increase in sensitivity to oxidants [72]. These and other studies suggest that actively functioning NOS can increase IRP binding activity [73–75]. Once again, however, different patterns of responses are seen to cytokine-dependent NO production for IRP-1 and IRP-2, whereby small increases in IRP-1 binding activity occur together with a pronounced decrease in IRP-2 binding [76].

Proteins other than Tfr and ferritin can be regulated by IRPs, if their mRNAs contain IREs. These proteins include mitochondrial aconitase, a key enzyme in the tricarboxylic acid cycle. Indeed, modulation of dietary iron intake in rats affects the levels of liver mitochondrial aconitase, which appears to involve iron signalling by IRPs and is suggestive of a novel role for this enzyme in cellular iron metabolism [47,77]. Similarly, \( \delta \)-aminolaevulinate synthetase, the rate-limiting enzyme in erythroid haem synthesis, also appears to be regulated by IRPs [78], as does synthesis of transferrin, since an IRE has been identified on the 5’-untranslated region of transferrin mRNA [79]. A recent report suggests that the haem precursor \( \delta \)-aminolaevulinic acid is capable of inducing the activation of IRP-1 via a ROS-mediated mechanism [80]. IRPs have now been implicated in functions including the intracellular and extracellular turnover and storage of iron (and its implications for antioxidant functions in the body), as well as metabolic processes mediated through the tricarboxylic acid cycle.

**HAEM OXYGENASE (HO)**

An additional source of intracellular LMM Fe, not directly acquired via Tfrs, arises from the action of constitutive (HO-2) and inducible (HO-1) HOs. These
enzymic isoforms catalyse the oxidative cleavage of discarded haem to release biliverdin, LMM Fe and carbon monoxide in equimolar amounts (Figure 2). HO-1 is also known as heat-shock protein 32 (HSP-32). Since induction of HO-1 occurs in response to a variety of stimuli that cause direct or indirect oxidative stress in the host, it is also regarded as a stress-response protein. HO-1 and HO-2 are located in the endoplasmic reticulum of numerous tissues and cells (for reviews, see [81,82]). Recently, a third HO isomer has been described in endoplasmic reticulum obtained from rat brains [83]. This HO-3 enzyme has a considerable amount of sequence identity with HO-2, but is not as efficient at catalyzing the breakdown of haem to biliverdin [83]. Its precise biological function(s) at present remains unknown [83].

Regulation of HO-1 expression occurs either by increased transcription or by increased mRNA stabilization. Several transcriptionally active regulatory sites, including domains for NF-κB, AP-1 and heat-shock factor (all oxidatively activated transcription factors), have been identified in the promoter region of the HO-1 gene (reviewed by Choi and Alam [82]). Indeed, AP-1 has been identified in animal models as the transcriptional regulator for both lipopolysaccharide- and pyrrolidine dithiocarbamate-mediated HO-1 expression [84,85]. Factors thought to be involved in the regulation of HO expression and/or activity are listed in Table 1.

It has been proposed that induction of HO-1 represents a protective stratagem to defend against oxidative stress, and there is experimental evidence to support this view [86–88]. The mechanisms by which HO-1 elicits these protective effects are at present unclear. Early work suggested that the removal of pro-oxidant haem and the formation of the antioxidant bilirubin was responsible [89] (Figure 2). More recently, direct protective effects have been suggested by experiments using an HO-1 gene deletion mouse model [90]. These authors suggested that HO-1 releases LMM Fe into the extracellular space, and that this decreases the toxic effects of intracellular LMM Fe accumulation.

ROS and RNS production is a feature of many inflammatory diseases, and such species can activate IRPs (both IRP-1 and IRP-2). When this occurs, ferritin synthesis is decreased, while the cellular uptake of LMM Fe from extracellular iron-containing transferrin is increased. As an iron storage protein, ferritin has an iron-detoxifying function (antioxidant); however, under certain conditions, superoxide [91] or stimulated polymorphonuclear leucocytes [92] may cause the release of some of the iron from ferritin, with pro-oxidant implications. The amount of iron released is likely to determine whether a signalling or a damaging response is elicited. For example, cells transfected with HO-1 showed a cytoprotective response for reactive iron at low levels of HO-1 expression, whereas at high levels of transfection no protection was seen [93].

The third product that is formed during HO-catalysed haem degradation is carbon monoxide (CO), which can be demonstrated in the lungs of critically ill patients as well as in normal healthy controls [94]. This LMM gas molecule has second messenger vasodilatory properties similar to those of NO (reviewed in [95]). Biological signalling by CO may represent an important function of HO-1 induction. Indeed, recent studies suggest that CO formation may be as beneficial to the patient as NO formation [96,96a]. Interactions between the production of CO and NO may indicate a regulatory network involving NOS and HO [97]. As discussed above, numerous reports suggest that induction of HO represents a protective stratagem. There are, however, some recent findings that do not fully support the hypothesis that HO-1 is solely antioxidant in character. For example, induction of ferritin protein after lipopolysaccharide challenge appears to be independent of synthesis of its RNA, and of HO activity [98]. Secondly, in an HO-2 gene deletion mouse model, induction of HO-1 leads to increased oxidative injury and mortality during hyperoxia, due to mechanisms that appear to involve the accumulation of redox-active iron [99]. In our laboratory we have demonstrated that the pro-oxidant activity of haem iron is greatly increased when HO (probably both HO-1 and HO-2) releases LMM Fe from it [100]. Bilirubin formed in equimolar amounts to LMM Fe was unable to prevent an overall pro-oxidant response, presumably because it was consumed stoichiometrically during the reaction, while LMM Fe remained catalytically
HO-1 induction is increased in the presence of haem, pulmonary endothelial cells are subjected to hyperoxia, the lungs of iron-overloaded rats [102]. When human pulmonal vascular endothelial cells are subjected to iron overload show a rapid increase in HO-1 activity [106]. A further adverse effect of HO-1 induction may be the production of peroxynitrite in response to CO challenge. Vascular endothelial cells release this oxidant upon exposure to CO. The mechanism for this release appears to involve an increase in the steady-state levels of NO due to competition for cellular binding sites [107]. CO can also inhibit surfactant production by human type II pneumocytes by inducing the formation NO and cytokines [108], further emphasizing how susceptible the lung is to a variety of oxidant challenges.

The role of HO-2 in mammalian systems is less controversial than that of HO-1, and it seems to be mainly protective [99], by recycling body iron through haem catabolism. HO-2 has two other haem-binding motifs in addition to its active site, which may also serve antioxidant function by removing pro-oxidant haem iron [109].

Iron salts can act as a signal for HO-1 induction [102,110], and appear to elicit a rapid response in the lungs of iron-overloaded rats [102]. When human pulmonary endothelial cells are subjected to hyperoxia, the induction of HO-1 is increased in the presence of haem, and even more so when LMM Fe is present [111]. Although many molecules can up-regulate HO-1, the above findings suggest that iron signalling is important for up-regulation of this stress protein. Based on these observations, it is reasonable to speculate that other stress inducers of HO-1 may effect up-regulation by mechanisms that ultimately involve iron. The participation of iron, both as an inducer and as an end-product of HO-1 activity, may also have implications for the actions of the IRPs, at least via iron-mediated pathways of regulation. This again may have wider implications in terms of other antioxidant functions controlled by the IRPs, such as iron binding (transferrin) and cellular iron storage (TfR and ferritin). These functions may represent additional antioxidant and protective functions that are attributable to HO-1 induction, similar to those already proposed for ferritin as an antioxidant [112].

### FURTHER EXAMPLES OF IRON SIGNALLING

Compared with the wealth of knowledge on calcium signalling, iron signalling is a novel concept, with few well defined examples. In the case of IRP-1, with its Fe–S cluster, regulatory mechanisms involve the direct participation of iron [113] or indirect participation through the formation of ROS. Such ROS may be either inorganic (i.e. $O_2^−$, $H_2O_2$, $OH^−$ and $HOCl$) or organic (such as lipid hydroperoxides and cyclic endoperoxides). In the presence of iron, lipid peroxidation can lead to the formation of numerous reactive aldehydes, such as 4-hydroxy-2-nonenal (reviewed in [4]). This $n=6$ fatty acid peroxidation product exhibits many profound biological effects (reviewed in [7]), and has recently been shown to inhibit NF-$κB$ activation by preventing IkB phosphorylation [114]. Indeed, the events described above have been shown to involve Jun N-terminal kinase, $c-jun$ and $c-fos$, with iron-dependent formation of hydroxyl radicals and lipid peroxidation products as up-regulators of this pathway. Such actions of 4-hydroxy-2-nonenal, and other iron-catalysed lipid peroxidation products, may therefore be indicative of an indirect iron signalling response.

Furthermore, iron signalling has recently been implicated as a key step in mediating the effects of UV B irradiation, which leads to increases in matrix-degrading metalloprotease and stromelysin-1 mRNA levels in human dermal fibroblasts [115]. Cellular iron levels also profoundly influence the levels of NOS mRNA formed in murine macrophages, suggesting that there is a role for iron signalling in NO production [116]. Iron overload in rats, caused by a high dietary intake of iron, leads to the accumulation of iron in the liver and increased activities of the antioxidant proteins Mn-dependent superoxide dismutase and $γ$-glutamyl transpeptidase (a key enzyme in the synthesis of glutathione) [117]. This appears to be
thought to be a key apoptotic cell signal [122], although as well. Cytochrome and clearly plays an important part in iron signalling. The finding that normal healthy control subjects have redox-active LMM Fe [105]. Even more surprising was the severe lung leak seen in non-survivors, which would allow plasma transferrin to enter the BAL fluid and bind all the LMM Fe [105]. Even more surprising was the finding that normal healthy control subjects have redox-active LMM Fe in their BAL fluids [105]. The physiological reason for this is not clear, but it would perhaps explain why the lungs are so susceptible to inhaled toxins and pollutants, which can cause damage through ROS formation.

Surgery necessitating cardiopulmonary bypass results in the development of ARDS in around 1.5% of cases [125], and leads to increased iron saturation of transferrin and also to the presence of redox-active iron in the plasma of a proportion of patients [126]. Furthermore, an association between the development of lung injury and the iron loading of transferrin has been established in these patients [126]. It seems likely that iron may be a feature of the ROS-mediated damage found in such patients, although other iron-independent mechanisms are clearly involved. Recently it has been demonstrated that the uptake of iron by A549 cells (alveolar type-1-like cells) is modulated by the inflammatory cytokines TNF-α and IL-1β [127]. Furthermore, the form in which the iron is presented to the cells determines the levels of uptake. Enhanced iron uptake occurs mainly in the presence of non-transferrin-bound iron and TNF-α, and is associated with increased cytotoxicity [127], a finding that may have implications for lung injury in ARDS and sepsis. Interestingly, anti-inflammatory cytokines such as IL-4 and IL-13 have also been shown to regulate iron metabolism in activated macrophages from mice [128].

Iron overload is a serious condition that occurs chronically in various clinical conditions, such as idiopathic haemochromatosis and thalassaemia, as well as physiologically in the neonate [38]. Work from our laboratory has identified a transient plasma iron overload that is characteristically seen during cardiopulmonary bypass surgery [129] and in patients with ARDS complicated by end-stage multi-organ failure [104]. Since most cardiopulmonary patients convalesce normally, the damaging effects of transient iron overload are ill-defined, but appear to include increased levels of oxidative stress [130]; in animal models the up-regulation of the stress protein HO-1 is also seen [102].

Although most critically ill individuals do not show plasma iron overload, they do exhibit abnormal extracellular iron chemistry. Levels of the iron-binding protein transferrin are decreased in patients with ARDS [103, 131], and the protein shows significantly increased levels of iron saturation compared with that in non-ARDS patients and normal healthy controls [103]. Levels of the iron-oxidizing protein caeruloplasmin were reported to be unaltered in the plasma of patients with ARDS [131], but other studies suggested that this acute-phase protein is elevated in the plasma such patients although its iron-oxidizing activity is compromised [103]. These plasma proteins were reported to be present in BAL fluid obtained from patients with ARDS, and were suggested to act as protective antioxidants [132]. This was not, however, borne out by findings described above.

Iron signalling in sepsis and acute respiratory distress syndrome

IRON AND CRITICALLY ILL PATIENTS: ARDS AND SEPSIS

ROS are produced continuously in the body as by-products of normal aerobic respiration and other purposeful biochemical processes. However, when an organ or tissue is placed under oxidative stress, ROS production can overwhelm the body’s endogenous antioxidant defence systems and lead to tissue damage. Such situations are commonly seen in critically ill patients, in whom ROS production is greatly increased by inflammatory cells and the endothelium, due to ischaemia/reperfusion injury, and as a result of ventilatory support with high inspired oxygen concentrations. The current practice of using inhaled NO therapy to support patients with ARDS could also lead to increased oxidative damage. Numerous studies have confirmed that patients with ARDS show clear evidence of increased oxidative damage to lipids, proteins and DNA [123, 124]. Our own studies have shown that there are profound abnormalities in the extracellular iron chemistry of these patients, and even full iron saturation of transferrin, leading to iron overload [103, 104]. When bronchoalveolar lavage (BAL) fluid from patients with ARDS was examined for iron content, surprising results were obtained. Patients who survived had redox-active LMM Fe in their BAL fluids, but those who did not survive had no such iron. They did, however, have high levels of transferrin, which was not fully iron-loaded [105]. The most likely explanation for this is the severe lung leak seen in non-survivors, which would allow plasma transferrin to enter the BAL fluid and bind all the LMM Fe [105]. Even more surprising was the finding that normal healthy control subjects have redox-
The role of macrophages in iron turnover in the lower respiratory tract has been reviewed recently [133]. Further evidence for the involvement of iron and iron regulation in patients with ARDS comes from findings which suggest that elevated plasma ferritin levels may be a useful predictor of the onset of lung injury [134]. Ferritin is normally regarded as an intracellular iron-storage protein. It can, however, be measured in plasma, and such measurements are used to assess labile iron stores [135]. The increased levels of plasma ferritin found in patients likely to develop ARDS [136] may in part represent cellular leakage from damaged cells. There may, however, also be implications associated with altered iron signalling via the IRPs. There are no data concerning lung TfR levels in patients with ARDS, although ongoing work in our laboratory (G. J. Quinlan, Y. Chen, T. W. Evans and J. M. C. Gutteridge, unpublished work) using a rat model of sepsis suggests that TfRs are under transcriptional control, and that lung responses differ from those in the heart (Figure 3). These findings may have implications for patients with ARDS, and in particular for the rapid lung damage seen early in the development of this syndrome.

Although iron regulation and turnover in the critically ill patient is now being investigated extensively using cell culture and animal models, human studies are considerably more difficult. The future challenge is to see whether the iron biochemistry seen in critically ill patients is a cause or consequence of altered iron signalling, and to what extent, if any, the ability to intervene and change these signalling processes might benefit patients with ARDS and sepsis.

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