Redox imbalance in the critically ill

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The majority of deaths amongst critically ill patients requiring intensive care are attributable to sepsis and its sequelae: septic shock, the systemic inflammatory response syndrome (SIRS) and the acute respiratory distress syndrome (ARDS). Clinically, sepsis/SIRS and ARDS are characterised by disordered vascular control, manifest as systemic hypotension and peripheral vasodilation refractory to intravascular volume resuscitation and vasopressor therapy; and pulmonary hypertension. Experimental and clinical evidence demonstrates that these patients suffer from severe oxidative stress. Thus, our own and other groups have shown that the vascular pathology of sepsis/SIRS and ARDS is initiated through the uncontrolled production of reactive oxygen (ROS) and reactive nitrogen species (RNS) which modulate inflammatory cell adhesion and cause direct injury to endothelium (Fig. 1).

Redox balance between anti-oxidants and pro-oxidants

In health, there is a balance between the formation of oxidising chemical species and their effective removal by protective anti-oxidants. Anti-oxidants are a diverse group of molecules with diverse functions. For example, they range from large highly specific proteinaceous molecules with catalytic properties to small lipid- and water-soluble molecules with non-specific scavenging or metal-chelating properties (reviewed by Gutteridge & Halliwell1). Anti-oxidants might, therefore, be defined as substances which, when present at low concentrations compared to those of the oxidisable substrate, significantly delay or inhibit oxidation of that substrate2. Anti-oxidants, therefore, control the prevailing relationship between reducing or oxidising (redox) conditions in biological systems. Such control offers two major advantages: (i) the ability to remove toxic levels of oxidants before they damage critical biological molecules; and (ii) the ability to manipulate changes, at the subtoxic level, of molecules that can function as signal, trigger or messenger carriers3,4.
Plasma and tissue anti-oxidants operate at primary, secondary and tertiary levels mainly as constitutive molecules. However, it is becoming increasingly clear that inducible anti-oxidants upregulated by oxidative stress can also play key roles in body protection.

The authors consider ‘primary’ defences to be those which prevent radical formation. The iron-binding properties of transferrin and lactoferrin fulfil such roles in extracellular fluids, since iron correctly attached to their high-affinity binding sites no longer catalyses radical formations. ‘Secondary’ defences are those which remove, or inactivate, formed reactive oxygen species (ROS). In some cases they may be enzymes such as the superoxide dismutases (SOD), catalase and glutathione peroxidase, or low molecular mass molecules such as vitamin E, ascorbate and gluta-
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thione (GSH). ‘Tertiary’ defences operate to remove and repair oxidatively damaged molecules and are particularly important for DNA integrity.

**Cellular anti-oxidant defences**

Oxygen metabolism occurs within cells, where anti-oxidants have evolved to deal speedily and specifically with reactive oxidants or reductants. As previously mentioned, these are likely to be proteinaceous catalysts, i.e. enzymes.

The superoxide dismutases (SODs) rapidly promote the dismutation of superoxide (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) and oxygen at a rate considerably faster than it occurs uncatalysed (Eq. 1)

\[
2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{Eq. 1}
\]

Three forms of SOD are present in the body; copper-zinc SOD (CuZn-SOD) in the cytoplasm, manganese SOD (Mn-SOD) in the mitochondria, and extracellular SOD (EC-SOD) the major form in the extracellular matrix.

Hydrogen peroxide, a product of the dismutation reaction, can be destroyed by two different enzymes, namely catalase (Eq. 2) and glutathione peroxidase (GSHPx), a selenium-containing enzyme requiring glutathione (GSH) (Eq. 3).

\[
2H_2O_2 \rightarrow O_2 + 2H_2O \quad \text{Eq. 2}
\]

\[
H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad \text{Eq. 3}
\]

During normal oxygen metabolism, enzymes such as these function as secondary anti-oxidants to eliminate toxic intermediates of oxygen inside the cell.

Minimizing intracellular radical reactions is an obvious primary anti-oxidant mechanism that must have evolved to restrict oxygen toxicity. A good example of this is cytochrome oxidase, the terminal oxidase of the mitochondrial electron transport chain which, while functioning catalytically, does not release reactive oxygen intermediates from its active centre.

The great advantage to the cell of having constitutive catalysts as anti-oxidants is that, like all catalysts, they are not consumed during their normal functioning.

**Membrane anti-oxidant defences**

Within the hydrophobic lipid interior of membranes, different types of lipophilic radicals are formed to those seen in the intracellular aqueous
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milieu. Lipophilic radicals require different types of anti-oxidants for their removal. Vitamin E (α-tocopherol), a fat-soluble vitamin, is a poor anti-oxidant outside a membrane bilayer but is extremely effective when structurally incorporated into it. Lipid soluble anti-oxidants are, therefore, extremely important in protecting membrane polyunsaturated fatty acids (PUFAs) from undergoing autocatalytic free radical chain reactions known as lipid peroxidation. The peroxidation of a PUFA leads to its destruction and the formation of a plethora of oxidation products such as lipid peroxides, carbonyls and carboxylic acids. Many of these are biologically reactive and may be used as signal molecules by the body.

The way in which a membrane is structured from its lipids appears to play an important role in decreasing its susceptibility to oxidative damage. Thus, structural integrity requires that the correct ratios of phospholipid and cholesterol are present as well as the correct phospholipids and their fatty acid side chains.

**Extracellular anti-oxidant defences**

Normally, enzymes such as the intracellular SODs, catalase and glutathione peroxidase are not present in extracellular fluids. Nevertheless, extracellular fluids are often subjected to fluxes of superoxide (O²⁻), nitric oxide (NO), hypochlorous acid (HOCl) and hydrogen peroxide (H₂O₂) by ‘activated’ phagocytic cells and some ROS can also arise by auto-oxidation reactions.

Blood is an effective anti-oxidant ‘buffering’ system with both plasma and red blood cells offering their own specialised protective systems. Red blood cells (RBCs) have an anion channel through which O²⁻ can enter the cell and be destroyed by CuZnSOD. Hydrogen peroxide is an uncharged molecule, which behaves much like water and can be destroyed by RBC catalase and glutathione peroxidase. Unlike plasma, RBCs contain high levels of GSH. Plasma contains both primary and secondary anti-oxidants and is usually a powerful inhibitor of free radical reactions. The question ‘what is the most important plasma antioxidant?’ is often asked. To this, there is no simple answer since the experimental findings change with the pro-oxidant used to bring about detectable oxidation. Thus, vitamin E is reported to be the most important, and one of the least important, plasma anti-oxidants, depending on the different reaction conditions used.

When plasma was first tested for its anti-oxidant activity towards a peroxidising brain homogenate, activity was found to be mainly proteinaceous. Detailed fractionation studies later revealed that almost all of this activity resided in two plasma proteins representing no more...
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than 4% of the total\textsuperscript{13}. In normal human plasma, these proteins were identified as transferrin and caeruloplasmin\textsuperscript{13,15}. Apotransferrin binds two moles of ferric ion per mole of protein with high affinity, producing a coloured complex absorbing at 460 nm. The iron transport protein transferrin is normally one-third loaded with iron and keeps the concentration of 'free' iron in plasma at effectively zero. Iron bound to transferrin will not participate in radical reactions, and the available iron-binding capacity gives it a powerful antioxidant property towards iron-stimulated radical reactions\textsuperscript{4}. Similar considerations apply to lactoferrin\textsuperscript{5} which, like transferrin, can bind two moles of iron per mole of protein, but hold onto its iron down to pH values as low as 4.0. The major copper-containing protein of human plasma is caeruloplasmin, unique for its intense azure blue colouration. Apart from its known response as an acute-phase reactant its biological functions remain an enigma. Suggestions that it plays a major role in iron metabolism as a ferroxidase enzyme (catalysing the oxidation of ferrous ions to the ferric state for binding to transferrin) have not been widely accepted (reviewed in Gutteridge & Stocks\textsuperscript{16}). However, the protein's ferroxidase activity makes a major contribution to the anti-oxidant activities of extracellular fluids\textsuperscript{113,16}. Haptoglobins are glycoproteins found in the α-1-globulin fraction of serum that respond as 'acute-phase' proteins. They bind haemoglobin (both oxy- and met-) in a 1:1 ratio to form a stable complex which has one of the strongest non-covalent protein bonds known. The normal level of circulating haptoglobin is sufficient to bind some 3 g of haemoglobin, making sure that no free haemoglobin is normally present in plasma. Free haemoglobin has the potential to stimulate lipid peroxidation\textsuperscript{17} as well as to undergo degradation with the release of reactive forms of iron\textsuperscript{18}. Binding to haptoglobin prevents, or decreases, both of these pro-oxidant properties of haemoglobin\textsuperscript{19}. Haemopexin is a plasma β-glycoprotein that binds haem tightly in a 1:1 ratio to form a pink-coloured complex. When delivering haem to cells, the haemopexin molecule is not degraded and returns to the circulation as an intact protein\textsuperscript{20} in much the same way as apotransferrin. Haem iron is a reactive form that promotes several radical reactions including lipid peroxidation, a process which is strongly inhibited by haemopexin\textsuperscript{21}.

In addition to these proteins, albumin should also be considered an important extracellular anti-oxidant. Albumin is a highly water-soluble protein with important binding, transporting, 'solubilizing', and osmotic properties. It has one high affinity copper-binding site but, like most other proteins, readily and non-specifically binds copper ions at many other sites. Albumin, therefore, effectively inhibits copper-stimulated radical damage in most systems. Albumin can also scavenge hypochlorous acid and peroxy radicals (RO\textsuperscript{•}\textsubscript{2}) and decrease damage done by iron salts (reviewed in Halliwell\textsuperscript{22}). The large amount of
albumin present in fluids, its oxidisable thiol group, its high turnover, and the resilience of the molecule to structural damage make it an important sacrificial, or secondary, anti-oxidant. When considering iron-binding proteins as primary anti-oxidants, it should be emphasised that they evolved for the conservation and transportation of iron in the body. An essential, but secondary, requirement is that, whilst doing so, they do not allow iron to express its powerful pro-oxidant properties.}

Plasma also contains a large number of low molecular mass molecules, many of which are redox active, that have been ascribed secondary anti-oxidant roles as non-specific scavengers of free radicals. These are consumed during their reactions with radicals examples being, uric acid, vitamin E, ascorbic acid and bilirubin (reviewed by Krinski). Several of these anti-oxidants are important vitamins which has, in the past, led to over-simplistic clinical trials being designed with the aim of investigating their ability to reverse life-threatening diseases.

Signalling by redox control

Redox reactive biological species

The oxidation-reduction (redox) potential of biological ions or molecules is a measure of their tendency to lose an electron (thereby being oxidised), and is expressed as $E_0$ in volts. The more strongly reducing an ion or molecule, the more negative is its $E_0$. It is becoming increasingly clear that control of redox balance by anti-oxidants plays an important role in cellular signalling.

Reactive oxygen species (ROS)

The oxygen molecule is used biologically to oxidise (burn) carbon- and hydrogen-rich molecules to obtain the chemical energy and heat essential for life. In the process of this, the oxygen molecule is reduced to water, by a stepwise addition of 4 electrons, giving rise to intermediate reactive oxygen species – some of which are free radicals. A free radical may be defined as any chemical species having one, or more, unpaired electron(s). The unpaired electron of a free radical is represented as a bold dot ('•'). The four-electron reduction of oxygen to water gives rise to the superoxide anion radical (O$_2^-$), hydrogen peroxide, and the hydroxyl radical ('•OH). The superoxide radical represented as O$_2^-$ is often shown as O$_2^-$ since it is less of a radical than molecular oxygen (having two unpaired electrons O$_2$**, which are not normally shown in this way). Superoxide is produced in numerous biological processes, particularly the electron transport chains of...
mitochondria and the endoplasmic reticulum (reviewed by Chance et al.\(^8\)). Production of superoxide by activated phagocytic cells is one of the most studied radical-producing systems\(^{24}\). When opsonized particles are contacted by neutrophils a ‘respiratory burst’ occurs, with oxygen uptake and the release of superoxide radicals into the phagocytic vacuole. Neutrophils in the presence of hydrogen peroxide, which is formed by the dismutation of superoxide, can also oxidise chloride ions (Cl\(^-\)) into the powerful oxidant hypochlorous acid (HClO) (Eq. 5).

\[
\begin{align*}
\text{H}^+ & \quad \text{H}_2\text{O}_2 + \text{Cl}^- \quad \rightarrow \quad \text{HClO} + \text{H}_2\text{O} \\
& \quad \text{Eq. 5}
\end{align*}
\]

Hypochlorous acid and hydrogen peroxide are reactive oxygen species (ROS), but they are not free radicals since they do not contain unpaired electrons. The enzymatic generation of reactive oxygen species by activated phagocytic cells has evolved as a purposeful contribution to host defence. The superoxide radical is not a particularly reactive species in aqueous solution and is not able to oxidatively damage most biological molecules. However, there are a few vulnerable sites within cells at which O\(^{2-}\) can do some direct damage (reviewed by Halliwell & Gutteridge\(^2\)). Any system producing superoxide would also be expected to produce hydrogen peroxide, by the dismutation reaction (Eq. 1).

\[
\begin{align*}
2\text{O}^{'-} & \quad 2\text{H}^+ \quad \rightarrow \quad 2\text{H}_2\text{O}_2 + \text{O}_2 \\
& \quad \text{Eq. 1}
\end{align*}
\]

This reaction (Eq. 1) is greatly accelerated when catalysed by the ubiquitous superoxide dismutase enzymes, which remove O\(^{2-}\) from solution at the expense of forming hydrogen peroxide. In the absence of transition metal ions, hydrogen peroxide is relatively stable and can diffuse across membranes in much the same way as water. Superoxide can react with hypochlorous acid to form hydroxyl radicals (‘OH)\(^{25}\), and both hydrogen peroxide and hypochlorous acid\(^{26}\) can react with reactive iron species to generate (‘OH) radicals. The reaction of iron salts with hydrogen peroxide and hypochlorite to generate a powerful oxidant was first described by Fenton in the 1890s\(^{27}\). We now know that the simple sequence represented in Equation 7, known as the ‘Fenton reaction’, involves higher oxidation states of iron and is considerably more complex than shown.

\[
\begin{align*}
\text{Fe}^{II} + \text{H}_2\text{O}_2 & \quad \rightarrow \quad \text{Fe}^{III} + \text{OH}^- + \text{‘OH} \\
& \quad \text{Eq. 6}
\end{align*}
\]

Hydroxyl radicals in free solution can attack most biological molecules at almost diffusion-controlled rates, but have little or no specificity in Fenton chemistry, they have considerable site-specificity because the
Fig. 2 The derivation of reactive oxygen species. NO\textsuperscript{+}, nitric oxide; Cu\textsuperscript{2+}, copper; Fe\textsuperscript{2+}, iron; Hb, haemoglobin; e\textsuperscript{-}, electron; and DNA, deoxyribo nucleic acid.

Molecular

oxygen

\[
\begin{align*}
\text{Molecular oxygen} & \quad \xrightarrow{e^-} \quad \text{Superoxide anion} & \quad \xrightarrow{e^-} \quad \text{Hydrogen peroxide} & \quad \xrightarrow{e^-} \quad \text{Hydroxyl radical} & \quad \xrightarrow{H^+} \quad \text{Water} \\
O_2 & \quad \text{NO}^+ & \quad H_2O_2 & \quad \text{OH}^- & \quad H_2O \\
\end{align*}
\]

\[
\begin{align*}
\text{peroxynitrite} & \quad \text{ONOO}^- \\
\text{H}^+ & \quad \text{HbFe} \\
\end{align*}
\]

Oxidation of proteins, lipids & DNA

Transition metals, (e.g. Cu\textsuperscript{2+}, Fe\textsuperscript{3+})

ferryl haem

Protein-, lipid- and DNA-based radicals, and oxidative changes to lipids, proteins & DNA

hydroxyl radical is formed close to where the iron is located (reviewed by Symons & Gutteridge\textsuperscript{[28]}).

Excessive biological production of superoxide can generate all the ingredients necessary for the formation of hydroxyl radicals. Superoxide can form hydrogen peroxide and release and reduce reactive forms of iron. This sequence is often described as the ‘superoxide-driven’ Fenton reaction (Fig. 2).

The reactive oxygens and free radicals so far discussed are low molecular mass inorganic molecules. However, organic oxygen radicals of biological polymers are equally important in biological systems with protein, carbohydrate, lipid and DNA free radicals constantly formed in the body. When the lipid is a PUFA, free radical attack results in a radical chain reaction known as lipid peroxidation.

Reactive nitrogen species (RNS)

Nitrogen gas accounts for 78% by volume of the earth’s atmosphere and because of its inert properties it is a major global anti-oxidant deterring combustion and other oxidative processes.
Three simple oxides of nitrogen are of current biomedical interest; nitrous oxide ($N_2O$), a colourless gas with anaesthetic properties (laughing gas), nitrogen dioxide ($NO_2$), a toxic brown coloured paramagnetic gas (free radical) which exists in equilibrium with its dimer dinitrogen tetroxide (peroxide) $N_2O_4$, and nitric oxide ($NO'$). Nitrogen dioxide is an environmental pollutant and may be produced in vivo from reactions of $NO'$. It is an initiator of lipid peroxidation. Nitric oxide is a colourless gas and a weak reducing agent, first recognised by Joseph Priestley in 1772. Biological interest in $NO'$ and other RNS has exploded since the recent observation that the vascular endothelium and other cells in the body produce small amounts of it from the amino acid L-arginine (reviewed by Moncada & Higgs). Nitric oxide is poorly reactive with most molecules in the human body (non-radicals), but as a free radical it can react extremely rapidly with others such as superoxide, amino acid radicals, and certain transition metal ions. The reaction between $NO'$ and superoxide ($K_2 6.7 \times 10^9 \text{M}^{-1}\text{s}^{-1}$) produces peroxynitrite ($ONOO'$), which is, itself, a powerful oxidant that can decompose to yield further oxidants with the chemical reactivities of $NO''$, 'OH and $NO_2^+$'. The exact chemistry of damage by ONOO$^-$ is a matter of considerable current debate. In addition to the putative ‘accidental’ generation of ROS/RNS in vivo, some are made deliberately. For example, $NO'$ has multiple physiological roles. Activated phagocytes generate $O^-'_2$, $H_2O_2$, $NO'$ and (in the case of neutrophils $HOCl$) as one of their many mechanisms for killing foreign organisms. $NO'$ and possibly its derivatives (e.g. $NO^+$) are widely used physiologically, whereas reactive RNS such as $NO_2^-$ and ONOO$^-$ may be too indiscriminately damaging. The interactions of RNS and ROS are also important. $H_2O_2$ activates NF-$\kappa B$ but $NO'$ inhibits activation. In vascular endothelium, $O^-'_2$ antagonises the action of $NO'$ and causes vasoconstriction which may represent a physiological mechanism for regulating vascular tone. Unfortunately, the rapid reaction of $NO'$ with $O^-'_2$ generates ONOO$^-$, a species possibly responsible for several of the cytotoxic effects attributed to excess $NO'$, such as destruction of Fe-S clusters in certain enzymes. To add to this complexity, $NO'$ modulates damage by ONOO$^-$. Peroxynitrite aggravates lipid peroxidation, but $NO'$ reacts fast with the peroxyl radicals (ROO$'$) that propagate this process (Eq. 7).

$$NO' + ROO' \rightarrow ROONO$$  \hspace{1cm} \text{Eq. 7}

If the resulting ROONO (organic peroxynitrite) species can be metabolised without the release of free radicals, then $NO'$ effectively inhibits lipid peroxidation. Clearly, the $NO'/ROS$ ratio is all important. Diminished availability of $NO'$ and increased ROS formation may be key events in the pathology of atherosclerosis.
Reactive iron species (RIS)

Within cells, there normally exists a pool of low molecular mass redox active iron which is essential for the synthesis of iron-requiring enzymes and proteins, and for the synthesis of DNA. This pool of iron is the target of iron chelators and is also a form of iron sensed by iron regulatory proteins. The amount, and nature of the ligands attached to this iron, remain unknown. However, a recently introduced fluorescence assay based on calcein may enhance our knowledge of intracellular iron pools. In contrast to the intracellular environment, extracellular compartments do not normally require low molecular mass iron. Iron-binding proteins such as transferrin and lactoferrin do not even remotely approach saturation in healthy subjects, retaining considerable iron-binding capacity which removes mononuclear forms of iron that enter cellular fluids. The differences between intracellular and extracellular compartments and their requirements for low molecular mass iron deserves special comment since it is iron in this form that is the most likely catalyst of biological free radical reactions. Inside the cell, low molecular mass iron need not pose a serious threat as a free radical catalyst due to the presence of specific defenses to safely and speedily remove all the \( \cdot O_2^\bullet\) and \( H_2O_2\) and organic peroxides (such as lipid peroxides) that could react with such iron. This is achieved by intracellular enzymes such as the superoxide dismutases, catalase and glutathione peroxidase and possibly also by thioredoxin-dependent \( H_2O_2\) removal systems. In the extracellular space, however, a different pattern of protection is apparent. Here, proteins bind, conserve, transport and recycle iron and, whilst doing so, keep it in non- or poorly-reactive forms that do not react with \( H_2O_2\) or organic peroxides. Proteins such as transferrin and lactoferrin bind mononuclear iron, whereas haptoglobins bind haemoglobin\(^{19}\) and haemopexin binds haem\(^{21}\). In addition, plasma contains a ferrous ion oxidising protein (ferrooxidase) called caeruloplasmin\(^{16}\). By keeping iron in a poorly reactive state, molecules such as \( O_2^\bullet\), \( H_2O_2\), \( NO^\bullet\), and lipid peroxides can survive long enough to perform important and useful functions as signal, trigger and intercellular messenger molecules\(^3\). During situations of iron-overload, plasma transferrin can become fully loaded with iron (100% iron saturation) and allow low molecular mass iron to accumulate in the plasma. Such iron, when present in micromolar concentrations, can bind to various added chelating agents such as EDTA, desferrioxamine and bleomycin that cannot abstract iron from transferrin. This non-transferrin bound iron can be associated with several ligands including citrate\(^{42}\) and other organic acids, and possibly albumin. Low molecular mass ligands for iron inside the cell are also a subject of considerable debate. ATP, ADP, GTP, pyrophosphates, inositol phosphates, amino acids and polypeptides have all been proposed.
In 1981, one of the authors introduced the 'bleomycin assay' as a first attempt to detect and measure chelatable redox active iron that could participate in free radical reactions. The assay procedure is based on the ability of the metal-ion binding glycopeptide antitumour antibiotic bleomycin to degrade DNA in the presence of an iron salt, oxygen and a suitable iron reducing agent. Data obtained using the bleomycin assay in extracellular fluids for levels of RIS have recently been confirmed by quantitating such iron by its ability to activate the enzyme aconitase.

**Signalling through anti-oxidant control of redox balance**

It has been suggested that certain ROS (and perhaps, by extension RIS and RNS), might be used as signal, messenger and trigger molecules. Increasing evidence of redox regulation of gene expression is ongoing; not only of oxyR (a bacterial reactive oxygen responsive transcription factor) and nuclear factor κB (NFκB) but also the role of thioredoxin and of active protein 1 (AP-1). If redox balance is considered to play a pivotal role in signalling cell functions, it becomes highly unlikely that short-term changes in anti-oxidants could influence this balance, perhaps explaining the poor record of anti-oxidant therapies to date.

**Intracellular iron signalling**

Cells normally accumulate iron via the binding of transferrin to high affinity surface receptors (TfR) followed by endocytosis. There is also a transferrin-independent pathway of cellular iron uptake that is said to involve a ferri-reductase and an Fe²⁺ transmembrane transport system (reviewed by De Silva et al.). The reductase is proposed to provide iron in soluble form to the membrane transporter. When non-transferrin bound iron appears in plasma, due to iron-overload or lack of transferrin (apotransferrinaemia), it is rapidly cleared by the membrane-bound transport system constitutively present on parenchymal cells; particularly those of liver, heart, pancreas and the adrenals. This system does not require endocytosis of a protein for iron delivery. The rate of synthesis of TfR and ferritin is regulated at the post-transcriptional level by cellular iron and co-ordinated by the iron-dependent binding of cytosolic proteins called 'the iron responsive element binding proteins' (IRE-BP; now known as iron regulatory proteins, IRP) which bind to specific sequences on their mRNA. It appears that low molecular mass iron is capable of acting as a signal to regulate ferritin and TfR synthesis in this way. In the absence of iron, IRP-I binds to the iron-responsive element (IRE) (the 3'-untranslated region of the TfR message contains a set of stem-loop structures termed IRE) stabilizing the transcript. When iron is present, the protein dissociates from the IRE and degradation of
the mRNA occurs. Recent work has shown that IRP-I is identical to the cytosolic enzyme aconitase. The protein functions as an active aconitase when it has an Fe–S cluster present or as an RNA-binding protein when iron is absent. Switching between these two forms depends on cellular iron-status.

Membrane signalling

Within the hydrophobic interior of membranes, lipophilic radicals are formed which are usually different from those seen in the intracellular aqueous space. Lipophilic radicals require hydrophobic anti-oxidants for their removal. α-Tocopherol, a fat-soluble vitamin, is a poor anti-oxidant outside a membrane but is extremely effective when incorporated into the membrane bilayer. Membrane stability and protection against oxidative insult depends very much on the way in which the membrane is assembled from its lipid components. Structural organisation requires that the ‘correct’ ratios of phospholipids and their fatty acids are attached (reviewed by Gutteridge & Halliwell and Halliwell & Gutteridge).

When a cell is damaged, or dies, it is highly likely that its membrane lipids undergo peroxidation. Tissue damage releases RIS and activates enzymes which catalyse peroxidation of polyunsaturated fatty acids, particularly linoleic acid, leading to a build-up of lipid peroxides. Peroxidation of membrane PUFAs produces a plethora of reactive primary peroxides and secondary carbonyls and it was suggested many years ago by one of the authors that lipid oxidation products such as these resulting from cell death could act as triggers for new cell growth. Through the detailed work of Esterbauer and colleagues, clearer insights into the biological reactivity of lipid oxidation products have emerged. 4-Hydroxy-2-nonenal (HNE), a peroxidation product of (n-6) fatty acids (when RIS are present), is a potent trigger for chemotaxis, can inactive thiol-containing molecules, and activate certain enzymes (reviewed in Esterbauer et al.).

As a general rule, low levels of ROS, and possibly reactive carbonyls, activate cellular processes whilst higher levels turn them off. The resting cell normally has a redox potential with a reduced state, and is progressively activated as oxidation increases. Too much oxidation deposes all function until eventually apoptosis or necrosis is triggered.

Extracellular signalling

Human body extracellular fluids contain little, or no, catalase activity and extremely low levels of superoxide dismutase. Glutathione peroxidases in both selenium-containing and non-selenium-containing forms are present in plasma but there is little glutathione substrate (1–2 μM). ‘Extracellular’ superoxide dismutases (EC-SOD) have been
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identified and shown to contain copper, zinc and attached carbohydrate groups. By allowing the limited survival of $O_2^-$, $H_2O_2$, lipid peroxides (LOOH) and possibly other ROS/RNS in extracellular fluids the body can utilise these molecules, and others such as NO$, as useful messenger, signal or trigger molecules. A key feature of such a proposal is that $O_2^-$, $H_2O_2$, LOOH, NO$^-$ and HOCl do not meet with reactive iron or copper and that extracellular anti-oxidant protection has evolved to keep iron and copper in poorly or non-reactive forms.

The major copper-containing protein of human plasma is caeruloplasmin, unique for its intense blue colouration. The protein’s ferroxidase activity makes a major contribution to extracellular anti-oxidant protection by decreasing ferrous salt-driven lipid peroxidation and Fenton chemistry.

Physiological functions of reactive oxygens and nitrogen

Although the excessive generation of ROS at the site of inflammation may contribute to cell injury, ROS formed in appropriate concentrations have important signalling roles in the control of a range of physiological processes. There is increasing evidence that $O_2^-$, $H_2O_2$ and ONOO$^-$ act as cell signalling molecules in the induction of a number of genes. Moreover, $H_2O_2$ is an important co-factor for a number of enzymes including guanylyl cyclase and cyclo-oxygenase. Current interest, however, is strongly directed towards the biological functions of the RNS NO$, now recognised as being a ubiquitous smooth muscle cell relaxant, with particular importance in the cardiovascular system. Moreover, NO$^-$ has potent anti-aggregatory and anti-adhesion effects on platelets and other blood borne elements. In most cells, NO$^-$ mediates its regulatory effects by binding to the haem of soluble guanylyl cyclase, which activates the enzyme to cause cGMP production. In healthy blood vessels, NO$^-$ is predominantly formed by a constitutive enzyme in endothelial cells (eNOS) which is activated by elevations in intracellular calcium. In addition, some blood vessels are innervated by nitrergic nerves which also contain a constitutive form of NOS (nNOS). In general, the release of NO$^-$ by eNOS and nNOS actively maintains blood flow without compromising cellular function.

In addition to its role in modulating vascular homeostasis, NO$^-$ is an important endogenous bronchodilator and is implicated in gastric emptying and peristalsis. Nerves in the central nervous system express high levels of nNOS, which has been implicated in a number of functions, including memory.

In addition to the constitutive forms of nNOS and eNOS, an inducible form exists (iNOS) which is expressed in white blood cells in response...
to pathogens, endotoxin or some cytokines. NO* formation by cells expressing iNOS tends to be relatively high and continuous. NO* released by immune cells, either directly or via combination with O²⁻ to form ONOO⁻, is an important anti-microbial agent. However, the inappropriate expression of iNOS systemically or at the site of inflammation can lead to over-production of NO*. Under similar conditions, there may well be an excess of O²⁻, leading to the formation of damaging ROS as discussed previously. Since excessive ROS production is thought to account for much of the tissue damage seen in diseases of the critically ill, therapeutic interventions designed to interfere with oxidative balance may prove useful in some patients.

Redox imbalance in the critically ill

In this section, we examine some changes that can occur in the redox balance of patients under the care of a critical care unit (ICU) during their illness.

Acute respiratory distress syndrome (ARDS)

The syndrome of acute respiratory distress in adults (ARDS) is characterised by refractory hypoxaemia secondary to non-hydrostatic pulmonary oedema and is associated with a wide variety of precipitating factors, often not directly involving the lung (reviewed by Macnaughton & Evans). Thus, ARDS can result from such diverse clinical conditions as sepsis, gastric aspiration, polytrauma, pancreatitis, haemorrhagic shock, severe burns, oxygen toxicity, and cardiopulmonary bypass. In spite of the increasing complexity and scientific basis of medical support, ARDS still carries a mortality rate of around 50%, little has changed from when it was first described. The precise mechanisms that lead to acute lung injury are at present unknown but recent evidence suggests that patients with ARDS are exposed to a severe oxidative burden from a variety of sources.

Antioxidant changes in ARDS

Primary antioxidants. In the preceding discussions, we defined and classified plasma antioxidants by their biological sites of action and their specialised functions. Here we discuss some of the antioxidant changes that have been reported to occur in the plasma, and bronchoalveolar lavage fluids, of patients with ARDS. Compared with
normal healthy control subjects, patients with ARDS (without multi-organ failure) show decreased levels of plasma iron-binding anti-oxidant activity\textsuperscript{62}. The decreased ability of plasma to protect against iron stimulated oxygen radical formation correlates with the percentage iron saturation of the transferrin. A small group of patients with ARDS, showing impaired liver function as part of their wider multi-organ failure, have low molecular mass iron present in their plasma\textsuperscript{63}, and a high iron saturation of their transferrin. In such cases, anti-oxidant protection is greatly decreased, or even lost\textsuperscript{63}. The primary plasma anti-oxidant, caeruloplasmin, is an acute phase protein, and, as such, concentrations increase in the plasma after tissue trauma. In animal experiments, plasma caeruloplasmin increases after expose to hyperoxia by a mechanism independent of the acute phase response\textsuperscript{64}. In two recent studies of patients with ARDS, plasma caeruloplasmin levels were reported as raised\textsuperscript{62}, and as normal\textsuperscript{65} compared to healthy controls. The latter finding is surprising in view of the known response of caeruloplasmin to trauma and hyperoxia. The iron oxidising anti-oxidant activity of ARDS plasma in the first study was, however, similar to that of control plasma, in spite of the measured higher caeruloplasmin protein levels present. The reason for this remains unclear, but may suggest that some caeruloplasmin is not fully functional as a ferroxidase\textsuperscript{62}. Caeruloplasmin is particularly susceptible to proteolytic damage and increased proteolytic activity in plasma or lung tissue\textsuperscript{66}, (where it is also synthesised), from patients with ARDS, may contribute to the apparent loss of activity.

\textit{Secondary anti-oxidants.} Numerous non-specific low molecular mass molecules present in plasma have been ascribed biological anti-oxidant properties. These include glucose, uric acid, bilirubin, ascorbic acid, vitamin E and thiols (reviewed by Krinsky\textsuperscript{23}). In addition, plasma also contains low concentrations of specific high molecular mass scavengers, such as EC-SOD, EC-glutathione peroxidase and catalase. Plasma from healthy individuals contains around 500 µmol/l of thiols mainly associated with the sulphydryl groups of plasma proteins. Plasma thiol values are, however, lower in patients with ARDS (around 300 µmol/l)\textsuperscript{67} and even lower than this when calculated for non-surviving patients\textsuperscript{67}. Interestingly, non-survivors have higher levels of plasma proteins compared with the survivors. The lung is a primary target for oxidant injury and damage to lung tissue leads to a loss of sulphydryl groups of both protein and non-protein molecules (reviewed by Paterson & Rhoades\textsuperscript{68}). Thiol groups are particularly susceptible to oxidation and can be destroyed by biological oxidants such as hydrogen peroxide, peroxynitrite, iron salts, peroxyl and hydroxyl radicals. Patients with ARDS have depleted levels of plasma, and red blood cell GSH (non-protein thiol)\textsuperscript{69}. The anti-oxidant properties of the drug N-acetylcysteine,
together with the observed depletion of thiols in patients with ARDS, have led to clinical trials designed to replace or protect vital thiol groups using this drug. However, to date, these have not demonstrated that thiol supplementation is of great benefit to patients with ARDS. Lung epithelial lining fluid contains high concentrations of GSH compared with plasma from the same individual. Most of the GSH (96%) is in the reduced form, and at levels some 140-fold higher than those of plasma. Normal human plasma GSH values reported in the literature are variable, probably due to the labile nature of the molecule and the non-specific methods used to measure it. However, it is likely that normal human plasma contains around 1–2 μmol/l. Using fresh single plasma samples randomly selected from 8 patients with ARDS, extremely low levels of ascorbate were found, ranging from < 1 to 5 μmol/l, compared with 49 ± 1 μmol/l in normal healthy controls. The findings were interpreted as evidence of increased oxidative stress in vivo and suggested that the low plasma ascorbate levels reflect increased neutrophil activity. α-Tocopherol is carried in plasma by lipoproteins, for example, in LDL there are around six molecules of α-tocopherol for each LDL particle. α-Tocopherol is a fat soluble anti-oxidant that offers little protection to the surrounding aqueous milieu. In three separate studies, plasma α-tocopherol levels have been reported to be low in patients with ARDS with values of 7.73 ± 0.54 mg/l (n = 12), 10.4 ± 3.5 mg/l (n = 25) and 4.0 mg/l (n = 6), compared with normal healthy control values of 11.46 ± 0.55 mg/l (n = 7), 14.9 ± 2.5 mg/l (n = 16) and 11.2 mg/l (n = 7), respectively. In a follow-up study, the same group concluded that α-tocopherol was not significantly lower in patients with ARDS when standardised to the total plasma cholesterol present. Hydrogen peroxide levels are increased in breath condensates of patients with ARDS undergoing mechanical ventilation, supporting the proposal that neutrophils, by producing ROS, play a major role in the oxidant stress that characterises the syndrome. However, only low or undetectable amounts of H₂O₂ are found in plasma extracts from patients with ARDS. When H₂O₂ destroying enzymes, such as catalase and glutathione peroxidases, were measured in the serum of patients with sepsis complicated by ARDS and appropriate controls, the former group had higher catalase activity which may explain the undetectable levels of H₂O₂ in plasma. Interestingly, there was no evidence to support red blood cell haemolysis as the origin of the increased serum catalase. Extending these studies to include serum manganese-containing superoxide dismutase (Mn-SOD), these researchers assessed enzyme levels and other protein makers and found that of 26 patients with sepsis, six who developed ARDS had higher serum levels of MnSOD and catalase activity.
Pro-oxidant changes

Patients with ARDS and multi-organ failure can saturate plasma transferrin, revealing RIS in their plasma. Unlike plasma from normal healthy controls, however, BAL fluids from the same controls contain RIS. This may in part explain why the lungs are so sensitive to oxidative damage from hypoxia, hyperoxia, inhaled oxidants and ‘activated’ phagocytic cells.

Patients who survive ARDS have more chelatable RIS in their lavage than controls, but patients who do not have none. This latter finding is compatible with increased alveolar capillary permeability leading to the transfer of plasma proteins to the lung, one of which (transferrin) binds the chelatable iron. Suggestions that the leak of plasma antioxidant proteins into the lung is beneficial in ARDS, may not be true since survival appears to depend on the severity of lung leak. Polyunsaturated fatty acids are highly susceptible to free radical mediated oxidation, and many of the peroxidic and aldehydic products formed have profound pharmacological effects on cells (reviewed by Esterbauer et al.). In an intensive care stay, patients with ARDS decrease their plasma concentration (%) of total linoleic acid, but increase their concentrations of oleic and palmitoleic acids. Such changes are highly characteristic of essential fatty acid deficiency disease and may be common to many conditions characterised by oxidative stress whereby ROS act as signal molecules to alter desaturase enzymes. If such changes are an adaptive response, it is difficult to understand why levels of oleic acid are so increased in ARDS when this mono-unsaturated fatty acid is used pharmacologically in animal models to induce ARDS.

Xanthine dehydrogenase (XDH) is a widely distributed enzyme which, upon oxidation of its thiol groups or following limited proteolysis, is converted to the oxidase (XOD) form. The oxidase enzyme utilises O₂ as a co-factor in the oxidation of hypoxanthine and xanthine forming the products, O₂⁻, H₂O₂ and uric acid. Increased levels of XOD or its substrates, therefore, increase the formation of ROS. Increased levels of XOD have been reported in the plasma of patients with ARDS, and more recently increased levels of plasma xanthine and hypoxanthine. Plasma hypoxanthine levels in non-surviving patients (37.48 ± 3.1 μM) were far greater than those in survivors (15.24 ± 2.09 μM), although both were markedly increased compared to normal healthy controls (1.43 ± 0.38 μM).

Redox imbalance in patients with ARDS has two major implications. Firstly, production of toxic levels of ROS, RIS and RNS leads to molecular damage of key molecules in cells. Secondly, sub-toxic increases in ROS, RIS and RNS can signal changes in cellular responses such as proliferation, apoptosis, and necrosis (Fig. 1).
Septic shock

Sepsis is a major cause of mortality in intensive care units. The release of endotoxin from the cell wall of Gram-negative bacteria is thought to initiate the major symptoms of septic shock, although Gram-positive bacteria (which do not produce endotoxin) are equally implicated in the attributable mortality. Sepsis is, of course, a major complication of, or may lead to, ARDS, and much of the literature deals with both conditions. In the following paragraphs we consider sepsis syndrome uncomplicated lung injury.

Anti-oxidant changes

Studies detailing changes in tissue or plasma anti-oxidants in patients with sepsis without lung injury are few. Most indicate that anti-oxidant imbalance is present in septic shock\textsuperscript{84,85}. Attempts to correct this by the administration of anti-oxidants has resulted in reported improvements in measured haemodynamic variables\textsuperscript{86}, although there is no evidence that survival rates are favourably influenced by such regimens.

Pro-oxidant changes

Recent reports that plasma RIS are elevated in sepsis\textsuperscript{86–88} were subsequently refuted\textsuperscript{89}; the later investigation finding no RIS and even decreased levels of total non-haem iron, consistent with the body’s known iron-withholding defence to microbial invasion\textsuperscript{90}. Plasma levels of thiols are lower\textsuperscript{89}, whereas hypoxanthine levels are normal (11.12 ± 0.69 μM) in patients with sepsis but no lung injury\textsuperscript{83}. Interestingly, levels of peroxides in the plasma appear to be some 3 times greater than normal\textsuperscript{91}.

Pharmacological implications and interventions

Albumin is often administered to patients with sepsis as fluid resuscitation. Recently, we observed that infused albumin significantly influenced the body’s thiol pool, sustaining an increase in plasma thiol levels that may represent a useful repletion of an important anti-oxidant\textsuperscript{92}. A recent discussion of the use of albumin administration in critically ill patients, however, concluded that it was associated with increased mortality\textsuperscript{114,115}. These data suggest that its continued use in certain critically ill patients requires urgent review.

Cardiopulmonary bypass (CPB)

Patients undergoing cardiopulmonary bypass for a variety of clinical reasons are routinely transferred at the end of surgery to the critical care
Redox imbalance in the critically ill

unit for periods of up to 48 h. Most bypass patients show signs of a systemic inflammatory response syndrome and have evidence of mild lung injury. However, 1–2% of patients develop ARDS.

**Antioxidant changes in CPB**

Epidemiological studies suggest that both diet and lifestyle contribute significantly to premature deaths from heart disease (reviewed by Gutteridge93). In particular, micronutrients in fruits and vegetables, several of which are anti-oxidant vitamins, appear to be important long-term protectors against the premature development of atherosclerosis. It is likely, therefore, that many of the patients receiving CPB surgery enter hospital with disturbed anti-oxidant/pro-oxidant profiles. Here, however, only those changes in primary anti-oxidants that occur during the course of surgery are considered.

Haemodilution, inherent in CPB, lowers the levels of all proteinaceous anti-oxidants in plasma. For example, iron-binding anti-oxidant protection (dependent upon transferrin concentration and its percentage saturation with iron) has been known to fall from 82% protection before surgery to 53% postoperatively. Similarly, iron-oxidising anti-oxidant protection, dependent upon caeruloplasmin, fell from 77% to 65% peri-operatively94. Furthermore, at a time when the concentration of plasma transferrin was decreasing, its saturation with iron increased from 40% to 57%95. In a follow-up study, transferrin saturation increased from 27% to 62% peri-operatively96. In 90% of these patients, there was a significant correlation between the percent increase in iron saturation of transferrin and alveolar capillary permeability observed using a radio isotopic technique96.

**Pro-oxidant changes during CPB**

When blood is circulated extracorporeally through plastic tubing and pumps, it causes severe shear stresses to blood cells and activates several enzyme cascades. As a result, red cells are lysed and release their contents and neutrophils are ‘activated’ to produce $O_2^-$ and $H_2O_2$. These ROS can react with free haemoglobin to form ferryl haemoglobin species and eventually disintegrate to release chelatable reactive iron species (RIS)18,95. In a recent study assessing the risk of iron release during CPB, one of the authors95 showed that all patients increased the percentage iron saturation of their transferrin, although, some (around 13%) went into a plasma iron-overload state95. Extending this study to assess the effect of blood cardioplegia regimens on this observation, the incidence of plasma iron-overload increased to 18% for those receiving cold blood cardioplegia and to 27% for those receiving warm blood cardioplegia98. The implication that the RIS detected in the plasma of
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Redox imbalance in the critically ill

iron-overloaded patients is deleterious comes from the finding that levels of 4-hydroxy-2-nonenal (a lipid peroxidation product of n-6 PUFAs) greatly increased when iron-overload occurred. Plasma total thiol levels are lower in CPB patients before surgery but rise significantly in those receiving blood cardioplegia. A likely source of these thiols is red blood cell lysis; a process which also produces RIS simultaneously. Additional oxidative stress is incurred when the aortic cross clamp is removed and reoxygenation of tissue follows the ischaemic period. Aberrant ATP catabolism, characteristic of ischaemia/reperfusion injury, follows causing a rise in xanthine and hypoxanthine levels. Hypoxanthine levels were found to be significantly higher in patients receiving warm blood cardioplegia and represents another potential source of ROS, since it is a substrate for the ROS-producing enzyme xanthine oxidase.

**Therapeutic implications**

**Acute respiratory distress syndrome**

There are two main aspects of ROS and RNS biology that have implications for the introduction of treatments for ARDS. Firstly, decreasing the damage incurred by ROS and secondly, introducing NO in inhaled form to redistribute pulmonary blood flow and improve oxygenation. Although, few clinical data exist in man, numerous reports have shown that ROS scavengers such as N-acetyl-cysteine or dimethylthiourea can reduce cell damage in animal models of ARDS. A second strategy examined in animal models has been the administration of various anti-oxidant enzymes, particularly SOD or catalase. A novel SOD-mimetic that also has catalase activity (EUK-8) has recently been shown to attenuate LPS-induced ARDS in pigs. Whether such an approach would be beneficial in man remains to be determined, but there is clearly potential for treatment with some form of anti-oxidant therapy.

Recently, patients with ARDS complicated by pulmonary hypertension have been treated with inhaled NO, which improves oxygenation by selectively increasing blood flow to well ventilated areas of lung. Significant benefits in oxygenation index have been shown in patients with ARDS treated with inhaled NO compared to placebo. However, since NO can contribute to oxidant stress (i.e. via ONOO-) there is the possibility that when it is inhaled by patients already exposed to high levels of oxidants, via endogenous pathways, the therapeutic benefits of improved blood flow are outweighed by increased cell damage. Indeed, evidence suggests that only around 50% of patients respond positively.
to inhaled NO\textsuperscript{103}. Whether non-responders represent a patient group where NO contributes significantly to oxidant damage remains to be established.

**Septic shock**

Treatments for septic shock are mainly supportive, typically centring around reversing the fall in systemic vascular resistance associated with the condition. After the demonstration that iNOS is expressed in blood vessels from experimental animal models of sepsis, attention was focused on NOS inhibitors which has been shown to lessen or completely reverse the associated fall in blood pressure\textsuperscript{104}. However, some reports indicated that NOS inhibition increased mortality in animal models of sepsis\textsuperscript{104}, possibly due to removal of the NO formed by eNOS and nNOS, which is considered ‘protective’, along with that produced by iNOS. This potential problem would be obviated by the use of specific iNOS inhibitors, which are currently under development. Alternatively, NO formed by iNOS in sepsis may be protective in constricted vascular beds where eNOS has been lost through endothelial injury (i.e. the pulmonary circulation). In order to address this possibility it would be necessary to provide a combined therapeutic approach, providing a NOS inhibitor together with a carefully titrated dose of a vasodilator (e.g. nitrovasodilator or prostacyclin). Indeed, using animal models of sepsis, the combined administration of a NOS inhibitor together with inhaled NO improves mortality compared to either treatment alone\textsuperscript{104}.

Other potential therapeutic approaches, related to ROS/RNS imbalance have concentrated on decreasing oxidative damage. There is now considerable evidence implicating ROS/RNS induced damage in the progression of organ dysfunction in sepsis. However, in similar fashion to ARDS, few data exist to demonstrate the effects of anti-oxidants in human sepsis. Nevertheless, studies using rodent hepatic cells in vitro have demonstrated the potential benefit of SOD-related drugs in sepsis\textsuperscript{105}, although, these benefits are not necessary applicable to the in vivo situation\textsuperscript{106}.

It seems, therefore, that a considerable amount of basic scientific investigation is required before a full understanding of the intricate consequences of ROS/RNS imbalance in the critically ill can be achieved. Specifically, three main advances are required: first, to be able to fully characterise the ROS/RNS status of individual patients within a disease cohort; second, to develop new anti-oxidants; and finally, to be able to manipulate constitutive and inducible anti-oxidant production before the onset of, or during the convolution disease states that afflict the critically ill.
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