Redox regulation of neutrophil apoptosis and the systemic inflammatory response syndrome

Daniel D. MELLEY, Timothy W. EVANS and Gregory J. QUINLAN
Department of Critical Care Medicine, Imperial College, Royal Brompton Hospital, Sydney Street, London SW3 6NP, U.K.

ABSTRACT
SIRS (systemic inflammatory response syndrome) may result from a wide variety of non-infective insults. Surgery is a recognized cause of SIRS, the onset of which can have adverse prognostic significance. Neutrophil activation is a key histopathological feature of SIRS, and neutrophil clearance through programmed cell death or apoptosis is an essential step in its resolution. Increasingly, it is recognized that ROS (reactive oxygen species), such as those generated by activated neutrophils during cardiac surgery, may have a regulatory role, influencing neutrophil lifespan and thus inflammation. In this review, we discuss the continuing importance of SIRS as a herald of inflammation and the role of neutrophil longevity in the resolution of inflammation, and we consider recent evidence for the regulation of neutrophil apoptosis by ROS.

SIRS (SYSTEMIC INFLAMMATORY RESPONSE SYNDROME)

Definitions
The host response to infection and other forms of tissue injury has been termed SIRS. Although controversy exists concerning the optimal defining criteria for SIRS, traditionally these have reflected changes in thermoregulation, cardiovascular and respiratory stability, and alterations in white blood cell count [1]. When SIRS is attributable to an identifiable infective process, it is termed sepsis. Sepsis complicated by pre-defined organ system dysfunction, through tissue or systemic hypotension, is regarded as severe [2]. Together, SIRS, sepsis and septic shock have been termed the ‘septic syndromes’.

The concept of SIRS was first explored in 1991 by a consensus conference convened by the American College of Chest Physicians and Society of Critical Care Medicine [2], bodies which were concerned that trials of novel immunomodulatory therapies were failing to demonstrate a mortality benefit. The convention recognized that acute severe illness of non-infectious aetiology shared clinical and pathophysiological features with sepsis caused by infectious organisms, and that the degree of immune dysregulation, rather than the specific trigger, was almost certainly more important in determining patient survival. The precise definitions of the sepsis syndromes [3,4] (Table 1) were intended to allow standardization of enrolment criteria for clinical trials, leading thereby to more consistent and comparable patient populations. It was also hoped that the identification of SIRS as a herald of immune dysregulation would favour prompt therapeutic intervention and thus improve survival.

Incidence of SIRS
SIRS is seen in association with a wide variety of non-infective insults and may afflict up to 33% of all patients requiring hospital admission [4]. SIRS is insult dependent, and is particularly common following surgery [5]. There is a progression between the different stages of the septic syndromes. Thus the prevalence of infection and bacteraemia increase with the number of SIRS criteria.

Key words: apoptosis, inflammation, neutrophil, systemic inflammatory response syndrome, surgery.
Abbreviations: ALI, acute lung injury; APAF-1, apoptosis protease-activating factor-1; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; CGD, chronic granulomatous disease; CPB, cardiopulmonary bypass; HIF, hypoxia inducible factor; IL, interleukin; •OH, hydroxyl radical; ROS, reactive oxygen species; SIRS, systemic inflammatory response syndrome; TNF, tumour necrosis factor.
Correspondence: Dr Gregory J. Quinlan (email g.quinlan@imperial.ac.uk).
fulfilled, and some 30% and 25% of cases respectively, evolve eventually to meet the defining criteria for sepsis and severe sepsis. Moreover, an increasing prevalence of eventual organ dysfunction (e.g. respiratory and renal failure, and disseminated intravascular coagulation) is observed with fulfilment of increasing numbers of SIRS criteria [5]. Finally, in a study examining specifically the incidence of SIRS in critical surgical illness, its presence was positively associated with mortality, organ failure and prognostic severity of illness scores (APACHE III) [6].

**Controversy and the SIRS concept**

Despite the widespread adoption of the concept of the sepsis syndromes since 1992, the majority of large-scale placebo-controlled trials of immunomodulatory therapy have failed to demonstrate a mortality benefit [7]. The concept of SIRS has been criticized as contributing, in part, to this lack of success [8]. Specifically, objections have cited the oversensitivity of the definition, leading to a high degree of heterogeneity among study populations and a lack of clinical relevance in units where the incidence of SIRS is apparently very high. Secondly, it has been suggested that identifying the specific underlying pathophysiological process is of greater importance when deciding upon inclusion criteria for clinical trials [9,10]. By contrast, others have suggested that sensitivity is an essential prerequisite for the function of SIRS as a herald of inflammation, whether it derives from infectious or non-infectious insults [11].

**SURGERY NECESSITATING CPB (CARDIOPULMONARY BYPASS) AND THE INCIDENCE OF SIRS**

SIRS following cardiac surgery, in the absence of infection, is a well-recognized entity. Although its incidence has varied from 10–55% in various clinical trials measuring inflammatory mediators [12–14], there have been no formal epidemiological studies. However, in light of the severity of the insult, SIRS following cardiac surgery might be expected to be an inevitable consequence [15]. Our own data reinforce that view, in that some 90% (of over 1200 patients) admitted sequentially to our Intensive Care Unit following surgery necessitating CPB fulfilled at least transiently the defining criteria for SIRS (S. Finney, D. D. Melley and T. W. Evans, unpublished work). Inflammatory activation following CPB is thought to arise via several mechanisms [1] (Figure 1). Firstly, ischaemia/reperfusion injury leads to the release of ROS (reactive oxygen species) and pro-inflammatory molecules (e.g. cytokines) into the circulation [16,17]. Secondly, activation of leucocytes [18], platelets [19], complement [20,21], clotting cascades [22] and other inflammatory mediators [23–26] may occur following the direct contact of blood with the extracorporeal bypass circuit. Finally, relative hypoperfusion of the splanchnic bed, both intra- and post-operatively, may lead to gut wall ischaemia, an increase in villous capillary permeability and translocation of enteral flora into the systemic circulation [27].

**ROS AND SIRS**

**ROS: definition and biological significance**

An oxygen free radical is a chemical species which contains one or more unpaired electrons [28]. Such species may be inorganic or organic in origin and are often, although not always, reactive in nature. Indeed, it is a common misconception that all oxygen free radicals are inherently unstable, especially given that ground state molecular oxygen is, by definition, a free radical. Most species, however, exhibit varying degrees of reactivity and
donate or receive electrons in energetically favourable reactions. Examples of reactive inorganic oxygen free radicals include O$_2^-$ (superoxide radical), which is only moderately reactive and is in general a better reductant than oxidant, and *OH (hydroxyl radical) which is an extremely reactive oxidant, being only limited in its reactivity with other molecules by an ability to diffuse from its site of production and react with them. There are also a range of oxygen-containing species which, although not free radicals, are important for oxygen-mediated electron transfer reactions. H$_2$O$_2$ is a notable example. In order to overcome the confusion regarding differing definitions of these related species, it is now more common to utilize the all-encompassing term of ROS when referring to them. The analogous term of RNS (reactive nitrogen species) is applied to nitrogen-centred species such as NO (nitric oxide). Some electron transfer reactions involving ROS require the presence of a catalyst, usually a variable valence transition metal, such as iron or copper. Indeed, iron (haem or non-haem) is found in association with the active site of most, if not all, proteins involved in oxygen transport, storage and metabolism. Aerobic metabolism results in the production of ROS as intermediates in the four-electron reduction of oxygen to water, which occurs in the mitochondria during ATP formation. ROS are also formed by a variety of other mechanisms, including the respiratory burst of inflammatory cells [29] and via the actions of cytosolic NADPH oxidases in other cell types [30]. Xanthine oxidase and aldehyde oxidase enzymes also produce ROS as by-products of catabolic processes. Hyperoxia and the effects of ischaemia/reperfusion, more correctly termed in this context reoxygenation, also increase whole-body ROS production. Biological processes involving electron transfer reactions, such as prostaglandin synthesis, can also lead to the formation of ROS by-products. Oxidative stress resulting from ROS production is a consequence of the mammalian aerobic existence but, under normal circumstances, is adequately controlled for by a range of defences, including compartmentalization, removal or repair of oxidatively modified biological molecules and via the actions of dietary, constitutive and inducible antioxidants. However, during certain disease processes and specific surgical procedures, such as surgery necessitating CPB, excessive ROS production can occur, thereby overwhelming these protective strategies and resulting in pronounced oxidative damage to biomolecules, the accumulation of toxic end-products and generalized cell/organ dysfunction. The formation of chemoattractant pro-inflammatory lipid peroxides within membranes is of particular importance in this regard [31,32]. Production of ROS by neutrophils in patients with the sepsis syndromes fulfils an antimicrobial function, but clearly has extracellular oxidative pro-inflammatory consequences.

More recently, the concept of ROS acting as key regulators and effectors of myriad cellular and tissue functions has emerged. Thus subtoxic levels of ROS can influence intracellular processes regulated by reductive and oxidative forces, thereby regulating numerous pro-inflammatory responses [33–36]. ROS production therefore not only has implications related to microbial cell killing and collateral oxidative damage, but also has potential autocrine and paracrine functions in the

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modulation of the inflammatory response, including neutrophil apoptosis, a process termed redox signalling.

**Oxidative stress and critical illness**

Oxidative stress seems to play a key role in the pathogenesis of the sepsis syndromes and their sequelae, including ALI (acute lung injury) and its extreme manifestation, ARDS (acute respiratory distress syndrome). Thus oxidative damage to plasma proteins and lipids is a feature of, and mortality predictor in, patients with established ARDS seen in association with a variety of conditions, including surgery necessitating CPB [31,37–39]. Secondly, deficiencies in antioxidant protection against the pro-oxidant catalytic effects of iron are detectable in these patients [40,41]. Thirdly, plasma iron mobilization (indicated by increased iron-loading of transferrin) is associated significantly with the development of ALI in a population of patients with SIRS following CPB [42]. Such patients had free or loosely bound iron, which is redox active and potentially pro-oxidant, detectable in plasma as a result of the surgical procedure [43]. Significant associations between iron levels and the specific marker of lipid oxidation, 4-hydroxy-2-nonenal [44], have been demonstrated in these patients, a result strongly suggestive of pronounced iron-catalysed oxidative stress. Indeed, free or loosely bound iron is measurable in plasma from patients with sepsis, ARDS and multi-organ failure [45]. Fourthly, markers of oxidative damage indicative of the presence of the •OH, peroxynitrite and hypochlorous acid are detectable in BAL (bronchoalveolar lavage) from patients with ALI [46,47], and deficient iron-binding antioxidant capacity and significantly increased levels of non-haem iron are found in BAL from those who fail to survive [48]. Such results obtained from observational studies in patients at risk of, and with established, ALI/ARDS indicate that the formation of ROS occurs in these populations at levels that overwhelm endogenous antioxidant defences.

**THE NEUTROPHIL AND SIRS**

The role of the neutrophil as a key cellular modulator of inflammation in the sepsis syndromes, and in the organ dysfunction (most commonly ALI) that may ensue, has been the subject of considerable research in recent years [49]. Neutrophils are among the first cells to be recruited to the site of any inflammatory insult. Activated neutrophils adhere to the vascular endothelium and transmigrate to the extravascular space along concentration gradients of chemokines. Locally mediated activation initiates the neutrophil oxidative burst, producing ROS. Protease enzymes and other pro-inflammatory mediators are also released by degranulation and have bactericidal and fungicidal properties which are relevant to the inflammatory reaction that characterizes the sepsis syndromes. ROS and proteases damage cells, extracellular matrix proteins and other macromolecules, whereas cytokine release maintains the influx of inflammatory cells, thereby perpetuating the response. Endothelial dysfunction arising in part through interaction with activated neutrophils is also thought to be critical to the development of the sepsis syndromes [50].

Increased neutrophil production and release from bone marrow is largely responsible for the neutrophilia seen in sepsis syndromes. However, prolongation of neutrophil lifespan and thus the length of time for which it remains a pro-inflammatory influence also occurs. By contrast, the removal of neutrophils from inflamed tissue is now recognized as a cardinal step in the resolution of inflammation [51,52]. Physiological cell death can be achieved by two mechanisms. Necrosis occurs as a response to extreme cellular stress, such as hypoxia, hyperthermia, viral invasion or exogenous toxins, and is characterized by mitochondrial swelling and failure, maintenance of chromatin, loss of membrane integrity with cellular swelling and eventual rupture. This leads to the release of cellular contents, including proteases and lysosomes, into the extracellular space causing further activation of inflammatory cells and perpetuation of inflammation. By contrast, apoptosis is an active process which permits the removal of cells without the promotion of inflammation [52] (Figure 2). It is a phenomenon seen in all tissues; for example, during cellular atrophy following withdrawal of hormones, growth factors or cytokines, and in tissue remodelling and repair. Apoptosis is characterized by cell shrinkage and disruption of the cytoskeletal architecture with ruffling and blebbing of the plasma membrane. Initially, mitochondrial and ribosomal function is maintained whilst the nucleus condenses. Ultimately, the cell fragments into a cluster of membrane-bound ‘apoptotic’ bodies which are cleared by neighbouring phagocytic cells, principally macrophages [53]. Importantly, macrophages which are induced to release pro-inflammatory mediators upon the ingestion of necrotic cellular material actually suppress inflammation through the release of the anti-inflammatory cytokine IL (interleukin)-10 following phagocytosis of apoptotic bodies [54,55]. Secondly, if apoptotic bodies are not cleared promptly, they lose membrane integrity and release their cellular contents in a process of ‘secondary necrosis’. Apoptosis is an inevitable and early event in unstimulated neutrophils, which normally remain in the bone marrow for 2 days following a 2-week period of maturation. Approx. 10¹⁰ neutrophils are released into the circulation daily, where they spend a further 6–10 h before undergoing diapedesis and transmigration into tissues. In the absence of any inflammatory stimulus, they remain for a further 2–6 days before undergoing spontaneous apoptosis. During infection, large increases in the numbers of effective cells are achieved rapidly
by the delay of tissue apoptosis and from the increased release of mature neutrophils from the marrow.

Hence delayed neutrophil apoptosis is pro-inflammatory and has been demonstrated to be part of the systemic inflammatory response [56] and also occurs following CPB [57]. Conversely, augmentation of neutrophil apoptosis is necessary for the resolution of inflammation. It is for these reasons that regulation of neutrophil apoptosis is seen increasingly as a potential target for immunomodulatory therapy. The molecular mechanisms regulating neutrophil apoptosis are complex and have been reviewed elsewhere [52,58].

**NEUTROPHIL APOPTOSIS**

**Initiation of neutrophil apoptosis**

Apoptosis can be triggered either by ligation of cell-surface receptors (the extrinsic pathway) or through the release of cytochrome c from the mitochondria (the intrinsic pathway), leading in turn to activation of caspases. The extrinsic pathway provides a mechanism for external pro-apoptotic anti-inflammatory signals to trigger cell death in target cells. The most widely studied signals are Fas ligand [59,60] and the pro-inflammatory cytokine TNF (tumour necrosis factor)-α [61,62], both of which interact with specific transmembrane receptors. Receptor-ligand binding leads to the conglomeration of intracellular death domains, which activate the apical caspase-8.

Caspases are a group of protease enzymes with a conserved cysteine at their active site. They cleave target proteins at aspartate residues (cysteine aspartate proteases). Caspases exist within the cytoplasm as zymogens (inactive procaspases). ‘Initiator’ caspases triggered by extrinsic or intrinsic pathways cleave further ‘executor’ caspases, which effect the morphological changes seen during apoptosis. Caspase targets include proteins involved in the regulation of apoptosis and apoptosis signal transduction, structural proteins and those essential for cell function, and those required for cellular repair and for regulating the cell cycle [63].

Neutrophils are programmed to undergo apoptosis spontaneously following extravasation into the tissues. However, rates of apoptosis may be increased, as in other cells, by ROS [64]. The mechanisms of this induction remain uncertain. High levels of pro-oxidant stress may cause DNA alteration and trigger p53, which classically induces apoptosis following genotoxic injury [65,66]. In several cell lines, up-regulation of Fas occurs following alterations of redox balance [67–69]. In neutrophils, recent studies suggest that death receptor clustering and the subsequent activation of caspase 8 are ROS dependent and may occur independently of Fas ligation in spontaneous apoptosis [70,71]. Alternatively, many cell signalling pathways are influenced by the redox environment, and signal transducers such as MAPK (mitogen-activated protein kinase) [72,73] and NFκB (nuclear factor-κB) [74] known to influence apoptosis may be important. Several groups have cited early mitochondrial dysregulation [75–77] as a key step in the induction of apoptosis by oxidant stress. In reality, the separation of apoptosis into intrinsic and extrinsic pathways is probably irrelevant biologically, as there is considerable crosstalk between the two. Thus death receptor activation is thought to promote the intrinsic pathway and mitochondrial signals may augment death domain signal transduction [78,79].

The activated neutrophil produces high volumes of microbicidal and pathogenic ROS. The induction of apoptosis by ROS may therefore be of fundamental importance to neutrophil removal from a site of inflammation, representing a potential mechanism of negative feedback in the inflammatory response. Following an encounter with, and subsequent phagocytosis of, a bacterial pathogen, the neutrophil oxidative burst leads to apoptosis not
only of the engulfing cell, but also of those in close approximation. The pro-apoptotic action of ROS in neutrophils following ingestion of Escherichia coli [80], Mycobacterium tuberculosis [81] and influenza viruses [82] supports this contention.

In vitro studies provide further evidence in support of this hypothesis. Thus exogenous H$_2$O$_2$ produced by xanthine oxidase- and glucose oxidase-generating systems induces apoptosis in neutrophils [83]. Secondly, endogenously produced ROS have been shown to be important in the activation of apoptosis by both Fas [60,71] and TNF-α [84]. Thirdly, neutrophils from patients with CGD (chronic granulomatous disease) lack the p component of NADPH oxidase and are unable to produce a membrane oxidative burst. These cells have lower intracellular H$_2$O$_2$ levels and decreased rates of spontaneous, Fas- and TNF-induced apoptosis when compared with neutrophils taken from normal individuals. Rates of spontaneous and Fas-induced apoptosis in neutrophils from patients with CGD can be restored to normal by the addition of H$_2$O$_2$. Fourthly, the addition of catalase, which reduces H$_2$O$_2$ to water, inhibits apoptosis in normal individuals [60]. Fifthly, pharmacological inhibition of intracellular NADPH oxidase has also been shown to decrease neutrophil apoptosis [81,85]. Finally, inhibition of intracellular NADPH oxidase has also been shown to decrease neutrophil apoptosis when compared with neutrophils taken from normal individuals.

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The role of mitochondria in neutrophil apoptosis

In eukaryotic cells, mitochondria compartmentalize ROS generated during the formation of ATP by oxidative phosphorylation, thus preventing deleterious oxidant reactions with other cellular substrates. However, mitochondria are now recognized to be of fundamental importance in triggering neutrophil apoptosis in response to oxidant stress [76]. Mitochondria were considered to be almost vestigial within neutrophils [87,88]. Their paucity within the cell following visualization by electron microscopy, the tendency of neutrophils to generate energy through glycolysis (beneficial in the low-oxygen environments in which they are often most active) and the apparent lack of effect of the mitochondrial poison cyanide supported this conclusion. It was believed that such short-lived cells, once mature and if unstimulated, had a limited requirement for continuous ATP formation. Free generation of ROS was thought to be confined largely to NADPH oxidase. Recent studies suggest that neutrophils possess a complex mitochondrial network almost exclusively dedicated to apoptosis. Mitochondrial membrane depolarization has been demonstrated to be an early event in neutrophil apoptosis, which may precede the activation of caspases and other molecular changes such as loss of plasma membrane symmetry and exposure of phosphatidylserine [87].

Spontaneous neutrophil apoptosis may rely upon the balance of pro- and anti-apoptotic members of the Bcl-2 family [58]. Relatively short-lived anti-apoptotic A-1, Mcl-1 and Bcl-Xi counteract the pro-apoptotic action of other members of the family. In the absence of survival signals and de novo synthesis, levels of Mcl-1 [89–91] and A-1 [92] fall relative to the more persistent pro-apoptotic factors, such that neutrophil apoptosis tends to commence spontaneously. Pro-apoptotic Bcl-2 homologues Bid, Bax, Bad and Bak are able to localize to the outer mitochondrial membrane where they alter its permeability. Classically, cytochrome c is released from the inter membrane space into the cytoplasm where it forms a complex with APAF-1 (apoptosis protease-activating factor-1) and caspase 9, together termed the apoptosome. In this intrinsic pathway of caspase activation, caspase 9 cleaves downstream caspases and initiates apoptosis. Neutrophils, which as discussed, generate only small amounts of mitochondrial ATP, contain very low levels of cytochrome c [93]. It is possible that the high levels of APAF-1 found in neutrophils sensitize the cell to cytochrome c release. ROS generation by the mitochondrial respiratory chain proximal to cytochrome c has been implicated in the maintenance of non-apoptotic neutrophil mitochondrial membrane function. Permeability changes induced by Bcl-2 family members may permit the release of these mitochondrial ROS into the cytoplasm [76] where they are able to promote alternative cell death pathways [94].

Exogenous ROS acting upon the mitochondrial membrane causing depolarization may themselves be the initial stimulus in the activation of the intrinsic pathway. There is evidence that A-1 [92] and, in other cell lines, Bcl-2 [95] have antioxidant functions, opposing the initiation of apoptosis. Mcl-1, which is the most closely studied family member in neutrophils, has been shown to be raised and correlates with lower apoptosis in patients with sepsis [96].

**REDOX MODULATION OF APOPTOTIC PATHWAYS**

**Redox balance, caspases and apoptosis**

At sites of inflammation, neutrophils encounter an environment in which the usual balance between pro-oxidant and antioxidant molecules may be upset. High levels of pro-oxidant species are generated by the local tissues and by neutrophils themselves (levels of H$_2$O$_2$ up to 100 µmol/l have been measured in abscess fluid) [97]. Most cells deploy a range of mechanisms for the maintenance of redox balance in the face of excessive pro-oxidant stress; many of these exist within neutrophils...
Systemic inflammatory response syndrome and neutrophil apoptosis

Figure 3  ROS may augment or inhibit both intrinsic and extrinsic apoptotic pathways

ROS released from mitochondria and following death receptor-mediated activation of NADPH oxidase promote caspase activation, whereas caspases themselves may be deactivated by redox modification of cysteine residues at their active sites.

[98]. More subtle modulation of redox balance may contribute to the physiological regulation of intracellular signalling, including several of the distal pathways involved in the execution of apoptosis [99] (Figure 3). The electron-accepting cysteine residue at the active site of both initiator and executor caspases is a potential target for redox modification. It has been suggested that both excessively oxidizing and reducing conditions may lead to caspase dysfunction [64,100]. Indeed, inactivation of caspases in neutrophils has been demonstrated following NADPH oxidase activation by PMA, suggesting that an alternate caspase-independent cell death pathway may follow the oxidative burst [101]. This would help to explain atypical morphological features seen in neutrophils, part way between apoptosis and necrosis, noted by some investigators. More recently, similar morphological changes have been demonstrated after pharmacological caspase inhibition following TNF-α/ROS stimulation of neutrophil apoptosis [94,102]. The clinical and pathological significance of these findings remains uncertain; indeed, other groups have reported morphologically normal apoptosis following PMA stimulation [103].

NO and apoptosis

Investigation of the role of NO in apoptosis has lent further support to the concept of redox regulation of caspase function. NO derivatives have been shown to trigger caspase-dependent apoptosis in several cell lines [104,105], as well as in myeloid cells [106–108]. In contrasting studies, the active site cysteine of caspases has been shown to be susceptible to reversible thiol S-nitrosylation following NO exposure [109–111]. This leads to caspase inactivation and delayed apoptosis [112], although it may predispose to necrosis. This S-nitrosylation may occur spontaneously in some cells as a further barrier to spontaneous apoptosis in that Fas receptor activation promotes cysteine de-nitrosylation and thus caspase activation [113]. In other cell lines, NO delays caspase activation by inhibition of apotosome formation subsequent to activation of the intrinsic pathway [114]. NO may also promote necrosis through disruption of mitochondrial electron transport [115]. NO concentration, free iron status and overall cellular redox balance have been shown to influence whether pro- or anti-apoptotic pathways are triggered following exposure to NO [116].

Neutrophil regulation of redox balance

The neutrophil maintains a number of systems for the regulation of redox balance, including catalase, superoxide dismutase and GSH [98,117]. In neutrophils cultured for more than 24 h these antioxidant substances become depleted, thus promoting a pro-oxidant and therefore pro-apoptotic state [118]. Separate investigation
with GSH-depleting agents has confirmed that altering the redox environment predisposes to apoptosis [119]. GSH depletion during activation of the respiratory burst has also been demonstrated and would facilitate apoptosis seen following phagocytosis [120]. Fas receptor activation leads to increased GSH efflux and apoptosis in non-neutrophil cell lines [121], whereas augmentation of intracellular GSH in neutrophils inhibits apoptosis [86]. The relative inability of mature neutrophils to promote de novo synthesis of protein may contribute to the rapid decrease of GSH seen with both physiological and pathological levels of oxidative stress. This fall in GSH and subsequent increase in ROS may contribute to the initiation of spontaneous apoptosis [71].

Transition metals and redox balance
Variable valency transition metals promote the formation of more highly reactive species, such as •OH from H₂O₂ [28]. H₂O₂ is freely permeable across membranes [122]. Therefore cellular compartmentalization of iron, the most abundant intracellular transition metal, plays a fundamental role in protection against oxidant damage. Neutrophil dysfunction, but not apoptosis, has been associated with iron overload and iron therapy [123–125], whereas chelators have been shown to inhibit neutrophil apoptosis following exposure to H₂O₂ [83]. In non-neutrophil cell lines there is abundant evidence to suggest that increased intracellular free iron is pro-apoptotic [126], exerting its effects through such diverse mechanisms as lysosomal [127] and mitochondrial dysfunction [128] and alteration of caspase activity [129].

In light of the freely diffusible nature of H₂O₂ and some other ROS, incorporation of iron into haem facilitates localization and specification of iron–oxygen interaction. Haem proteins, such as NADPH oxidase, NOS and the mitochondrial cytochromes, play a fundamental role in the physiological generation of ROS and have been implicated in the redox regulation of apoptosis. Free haem is also capable of inducing ROS and its metabolism by haem oxygenase may contribute to the anti-apoptotic action of this enzyme [130,131].

It has recently been shown that non-haem iron-containing metalloproteins, acting as oxygen sensors, are also central to the regulation of apoptosis. In many cell lines, hypoxia promotes apoptosis. By contrast, neutrophils, which often prosecute their antimicrobial role in inflamed tissues at low-oxygen tension, exhibit delayed apoptosis through activation of HIF (hypoxia inducible factor)-1α [132–134]. HIF-1α is inactivated by a series of iron-containing prolyl hydroxylases, the function of which is absolutely dependent on oxygen and iron. The absence of either species prevents HIF ubiquitination and removal and thus nuclear translocation ensues. Although HIF is known to regulate nuclear DNA transcription [135], the distal mechanisms of this hypoxic inhibition of apoptosis in neutrophils remain to be elucidated.

Resolution of apoptosis
Without phagocytosis by tissue macrophages, apoptotic bodies eventually undergo secondary necrosis. Apoptotic neutrophils have surface markers which allow recognition by macrophages and suppress pro-inflammatory signal production by such macrophages. It is becoming clear that oxidant species have a regulatory role even in this final clearance mechanism. Not only are ROS macrophage attractant and activators, but also the oxidation of neutrophil surface phospholipids improves recognition, uptake and immune suppression of macrophages [136,137].

SUMMARY AND CONCLUSION
Systemic inflammation in the absence of infection continues to contribute significantly to mortality and morbidity in patients following surgery. The clearance of neutrophils by apoptosis provides one mechanism through which the inflammatory response can be down-regulated. Redox active species, which are generated during sepsis syndromes, have been implicated in the regulation of several steps of the apoptotic mechanism. Thus initiation of both intrinsic and extrinsic pathways has been associated with increased levels of ROS, whereas antioxidants have a protective role. The executioners of programmed cell death, the caspases, have been shown to be prone to oxidative modification and inhibition, whereas modulation of cellular redox balance, for instance by GSH and iron, may augment or delay cell death. Finally, modification of external cell membranes by oxidation targets apoptotic neutrophils for removal by macrophages and thus the clearance necessary for inflammatory resolution.

Compartmentalization of ROS within cells and the contrast between levels generated under physiological and pathological conditions may help to explain their seemingly contradictory effects on initiation and inhibition of apoptosis. The complexity of pro- and anti-inflammatory signalling pathways involving ROS and the lack of more specific tools for their investigation and modulation in patients with sepsis, in part, explains the lack of success thus far in clinical trials of antioxidants. However, redox signalling remains fundamentally important in the physiological regulation of cellular metabolism and the dysregulation of such systems seen in systemic inflammation should remain a target for investigation and future therapy.

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