Biochem. Soc. Symp. **66**, 149–166
(Printed in Great Britain)

14

Mitochondrial dysfunction in sepsis

Mervyn Singer[1] and David Brealey

Bloomsbury Institute of Intensive Care Medicine, University College London Medical School, Rayne Institute Building, University Street, London WC1E 6JJ, U.K.

Abstract

The current mainstream view of organ failure induced by sepsis revolves around inflammation and loss of vascular control. However, there has been a resurgence in interest in bioenergetic failure due to mitochondrial dysfunction. This concept is not new — studies date back 30 years; however, the data have been highly conflicting with findings of either decreased, increased or unchanged mitochondrial activity and/or nucleotide levels. These studies are virtually all based on non-human cells, isolated perfused organs or *in vivo* animal models that have received a variety of insults ranging from mild to severe, and monitored for different durations ranging from minutes to weeks. As a generalization, there does appear to be depression of mitochondrial function with longer-duration models of greater severity. This is confirmed by the scanty human data currently available. This chapter provides an overview, and attempts to relate the biochemical changes to the clinical condition. The potential roles of nitric oxide, intracellular calcium and reactive oxygen species are highlighted.

Introduction

Sepsis is the clinical scenario stemming from an exaggerated whole-body inflammatory response to infection. It is characterized by a high cardiac output, low blood pressure, temperature abnormalities, lactic acidosis and organ dysfunction [1]. There is a continuous spectrum of disease ranging from that requiring little medical intervention to full intensive-care support with mechanical ventilation, renal-replacement therapy, vasoactive drugs and blood-product replacement. Sepsis is the commonest insult that can trigger this response; a similar picture can occur with severe trauma, burns, pancreatitis and many other exogenous insults. A classification system [2] (Table 1) has

[1]To whom correspondence should be addressed.

Table 1 Clinical definitions of systemic inflammation and sepsis

After Bone et al. [2].

Condition	Description
Systemic inflammatory response syndrome (SIRS)	The systemic inflammatory response to a variety of severe clinical insults. Requires two or more of: • core temperature $>30°C$ or $<36°C$ • heart rate >90 beats/min • respiratory rate >20 breaths/min or $P_aCO_2 <4.3$ kPa • white blood cell count $>12000/mm^3$ or $<4000/mm^3$ or $>10\%$ immature neutrophils
Sepsis	The systemic response to infection. Requires SIRS criteria in the presence of proved or suspected infection
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion or hypotension
Septic shock	Sepsis associated with hypotension (despite adequate fluid resuscitation) and perfusion abnormalities

been devised to allow clinicians and researchers to distinguish between septic and non-septic aetiologies, the degree of severity, and to provide some consistency in audit and research studies. An Italian multicentre study [3] of 1101 patients in 99 intensive-care units reported mortality rates of 27, 36, 52 and 82% for patients with the systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock, respectively. A 1987 study suggested that sepsis affected 175.9 per 100000 population, and that it was the 13th biggest killer in the U.S.A. [4]. The incidence is rising as a result of more interventional medical care in other areas, and growing populations of the elderly and immunosuppressed.

Current therapy is, in general, supportive of the failing organ(s) until these organs (hopefully) recover adequate function [1]. In terms of specific treatment, while infection can usually be cured very rapidly (within a few days) by surgical drainage and/or antibiotics, no treatment has been conclusively demonstrated to control sequelae resulting from the inflammatory response [5]. This can lead to progressive deterioration in the clinical condition into a syndrome of multi-organ failure, the mechanisms for which are still ill understood. As more of the inflammatory pathways are unravelled, so the story becomes more complex.

The inflammatory response is (or is, at least, currently thought to be) mainly under macrophage control. The macrophage becomes activated by contact with micro-organisms, or their constituents, such as lipopolysaccharide (endotoxin) — part of the cell wall of Gram-negative bacteria. The macrophage then secretes large amounts of pro-inflammatory cytokines, such as tumour

necrosis factor (TNF) and interleukin (IL)-1. Their usual role is to induce a localized area of inflammation to contain and combat the infection. This is achieved by a chemotactic effect on other cells of the immune system, including neutrophils, monocytes and lymphocytes, drawing them into the localized region of infection. Specialized adhesion molecules are up-regulated on the endothelium in these areas of inflammation to allow attracted cells to marginate and cross the vessel wall. These cytokines augment neutrophil function by making them more efficient microbe killers and by increasing the rate of lymphocyte clonal expansion. There is increased capillary permeability allowing more fluid and high-molecular-mass proteins (e.g. antibodies, complement) to leave the circulation, resulting in the characteristic swelling (oedema) that surrounds areas of inflammation. Other body defence mechanisms are activated by these cytokines. The complement cascade enhances phagocytosis by neutrophils or monocytes and can also directly destroy some bacteria. The coagulation system is activated to form a pro-thrombotic state. This in turn leads to activation of fibrinolysis and the kinin system, which results in increased vascular permeability and vasodilatation.

Eicosanoids (prostaglandins, thromboxanes and leukotrienes), derived from membrane phospholipids via arachadonic acid, are formed by various cells (e.g. endothelial cells, macrophages, mast cells). Their actions are complex, with varying degrees of vasoconstriction and dilatation being induced in different areas of the microvasculature. They probably modulate other active compounds. Other inflammatory mediators released include histamine, 5-hydroxytryptamine and platelet-activating factor. In recent years, the role of nitric oxide (NO) in sepsis has become highlighted [6]. High levels of NO production, assumed but not conclusively proven to be due to generation of the inducible NO synthase enzyme, are found in sepsis. Its mitochondrial effects will be discussed later; however, it also has a potent vasodilating effect through increasing cyclic GMP levels. All the above systems interconnect to increase blood flow and capillary permeability, and to attract immune-effector cells to the area of inflammation.

This response is usually self-regulating; once the triggering infection has been cleared, the level of inflammation subsides. The macrophages deactivate, the inflammatory mediators are broken down, there is increased production of anti-inflammatory cytokines (e.g. IL-10 and transforming growth factor-β), and thus a resolution of inflammation. The precise switching mechanism is unknown.

If this process becomes more generalized, either as a result of a more aggressive infectious insult or a loss of control of the inflammatory response, the patient becomes systemically unwell — septic. There appears to be a genetic-susceptibility component, often with exaggerated up-regulation of pro-inflammatory cytokines such as TNF. This has been shown in meningococcal meningitis [7], severe malaria and leishmaniasis [8] and sepsis [9]. There is a variation within the TNF promoter region that increases the risk of contracting severe malaria seven-fold [8]. Stuber et al. demonstrated that certain allelic forms of TNF are associated with a poorer outcome in patients with

severe sepsis [9]. The response to infection also seems to depend upon the type of human leucocyte-associated antigen (HLA) expressed on the cell surface.

Massive uncontrolled release of pro-inflammatory mediators leads to generalized vasodilatation and increased capillary permeability, resulting in loss of fluid from the circulation. This often leads to hypotension, requiring large amounts of fluid and vasopressor agents to resuscitate the patient and restore vital organ perfusion. The capillary leak leads to a generalized interstitial oedema, which can further impair tissue oxygenation. Aggregated white cells and platelets occlude the microcirculation. Activated neutrophils also release lysosomal enzymes (e.g. catalase, elastase) and free oxygen radicals, resulting in local damage with endothelial disruption. The body attempts to heal itself, leading to a widespread but disorganized fibrotic reaction, further impairing tissue oxygenation and organ function.

The above processes can be identified histologically in many organs, e.g. lung, liver and gut. The pulmonary manifestation is labelled ARDS, the acute respiratory distress syndrome. This usually results in severely impaired gas exchange, necessitating mechanical ventilation. For reasons still unknown, different organs — or combinations thereof — are affected in different patients. Sometimes ARDS may predominate, whereas in others severe hypotension may be the major problem. Most septic patients develop single organ dysfunction, though a third will develop multiple organ dysfunction. Outcome depends upon the number and duration of organ failures; a 90% mortality is associated with three or more organ failures compared with a 33% mortality for single organ failure [10].

Over the course of the illness, which may require intensive care for weeks if not months, the patient becomes pyrexial and hypercatabolic with increased gluconeogenesis and protein catabolism leading to hyperglycaemia and generalized muscle wasting. After the period of exaggerated inflammation, the patient enters a period of relative immunosuppression, coined by Bone [11] as the compensated anti-inflammatory response syndrome (CARS). There are elevated plasma levels of anti-inflammatory mediators (e.g. IL-10) and reduced levels of HLA-DR expression on monocytes [12]. This makes the patient more prone to secondary infections, which can then trigger further inflammatory responses.

The development of CARS may be one of the reasons for the high-cost failure of numerous targeted anti-inflammatory therapies [5]. Appreciation of the role of endotoxin, cytokines and other mediators and effectors led to the development of specific inhibitors and modulators of these identified factors. Large multicentre trials on a variety of anti-endotoxin antibodies, anti-TNF antibodies, soluble TNF-receptor antibodies, IL-1-receptor antagonists etc., have failed to show any clinical benefit. Timing of administration is important; giving an anti-inflammatory agent during the immunosuppressed phase may be injurious, whereas attempting to block endotoxin after macrophage activation may also be fruitless. Other attempts at more generalized suppression of inflammation (e.g. steroids) [13], removal of mediators (e.g. high-volume haemofiltration) [14] or boosting defence mechanisms (e.g. antithrombin-III administration) [15] have also failed to show consistent benefit.

The organs sometimes fail despite seemingly adequate perfusion, suggesting direct cellular dysfunction rather than reduced delivery of substrate — including oxygen. There have been essentially two competing theories as to why organs fail in sepsis. One theory states that there are localized defects in organ blood flow as a result of microvascular shunting of blood, and that nutrient capillaries become obstructed by micro-thrombi and aggregated neutrophils [16]. The opposing view is one of direct cellular dysfunction [17]. It is likely that both contribute to the development of multiple organ dysfunction.

There is also considerable debate as to whether tissue hypoxia actually exists in sepsis. It is now recognized that lactic acidosis does not necessarily equate with anaerobic metabolism and that other mechanisms, including accelerated glycolysis, an increase in the deactivated form of pyruvate dehydrogenase and hydrolysis of ATP, may also generate hydrogen ions [18]. Hotchkiss and Karl developed a septic rat model [19]; despite adequate macrovascular oxygen delivery, they found no evidence of bioenergetic failure or tissue hypoxia in rat muscle, liver, heart and brain using *in vivo* ^{31}P nuclear magnetic resonance spectroscopy, [^{18}F]fluoromisonidazole and microfluorometric enzymic techniques. However, a contrary view is expressed by Fink in a well-referenced review [17].

Body oxygen consumption falls progressively with increasingly severe sepsis [20]. As over 90% of total oxygen consumed by the body is by the cytochrome oxidase (complex IV) enzyme of the mitochondrial respiratory chain, it is reasonable to infer that an increasing degree of mitochondrial dysfunction is present in such critically ill patients. Whereas cardiovascular insults, such as haemorrhage and heart failure, result in a decreased supply of substrate to meet the demands of cellular respiration, in sepsis the cardiac output is often elevated.

A further finding unique to the septic state is a rise in tissue oxygen tension (P_{O_2}). Whereas P_{O_2} usually falls in line with the severity of the haemorrhagic, cardiogenic or hypoxaemic insult [21,22], it is elevated in sepsis provided adequate resuscitation has been accomplished. This has been demonstrated in animals and organs as diverse as rat bladder [23,24], pig gut mucosa [25] and human skeletal muscle [26]. The latter patient study found decreased P_{O_2} with severe heart failure, normal P_{O_2} with localized infection and raised P_{O_2} in sepsis which fell towards normal as the patient improved [26].

The seemingly paradoxical rise in P_{O_2} could be explained by decreased utilization of oxygen by the respiratory chain. This provides additional indirect evidence for mitochondrial dysfunction; however, as will be described, the literature presents a rather confused picture, with mitochondrial function being variously described as depressed, normal, or even increased, in the septic state. In part, this discrepancy is methodological with a wide variety of animal models, isolated perfused organs, cell suspensions and isolated mitochondria being investigated with an impressive array of techniques, over variable durations, and at variable time periods following the septic insult.

Three issues arise: (i) does sepsis produce mitochondrial dysfunction?; (ii) are there decreased levels of ATP and/or reduced rates of ATP synthesis?; and

(iii) what are the proposed mechanisms of sepsis-induced damage to mitochondria?

Until recently, human data have been notably absent but some of our preliminary investigations will be described that add additional weight to the hypotheses that sepsis leads to mitochondrial dysfunction, that this is temporally related to multiple organ dysfunction or failure, and that NO and its congeners, notably peroxynitrite ($ONOO^-$), are strongly implicated in its causation.

Mitochondrial ultrastructural damage in sepsis

Ultrastructural mitochondrial and cellular damage is well recognized in sepsis [27–34]. After 24 h of Gram-negative sepsis, heart mitochondria consistently showed morphological changes with loss of structural integrity noted in both cell and mitochondrial membranes [33]. Endothelial-cell damage, interstitial oedema and swelling of mitochondria were observed in porcine skeletal muscle after 18 and 48 h of endotoxaemic shock [34]. After 48 h, the muscle fibre diameters had increased and swollen mitochondria and segmental necrosis of muscle fibres were frequently observed. In contrast to ischaemic injury, early phagocytosis was not seen. Regenerative changes were not detected either.

Welty et al. [35] took serial skeletal muscle biopsies in baboons before bacterial challenge with *Escherichia coli*, then at 12 and 24 h after injection, and at death. The organelles became enlarged with distorted cristae and electron-lucent areas within the matrix. With advanced injury the inner mitochondrial membrane became fragmented. Quantitative morphometric analysis showed significant increases in cristal-membrane surface density and intermembrane space with a decrease in matrix staining density. They felt this damage would be severe enough to compromise muscle oxidative metabolism.

Mitochondrial respiration in sepsis — decreased, no change or increased?

Numerous studies have examined this question with conflicting results. In general, animal or organ studies on mitochondrial function taken within a few hours of the insult usually show no change in activity, although some report an elevation, whereas others find a depression in activity. On the other hand, studies lasting more than 6–12 h more frequently show depressed activity. A few studies have assessed temporal changes and often report a progression from elevation or no change in activity to depressed activity.

Decreased activity

Over 30 years ago, several groups independently described the inhibitory effects of endotoxin upon mitochondrial respiration, energized processes and fatty acid oxidation [36–40]. These findings were confirmed by Kilpatrick et al. [30], who exposed cultured mouse neuroblastoma cells to endotoxin and

demonstrated rapid changes in the oxidation of intramitochondrial pyridine nucleotides, a decline in the cellular [ATP]/[ADP] + [P_i] ratio (where P_i is inorganic phosphate), alterations in mitochondrial morphology and increasing membrane permeability to both high-molecular-mass matrix enzymes and K^+ ions. In further studies [41,42] the same group sought possible causes for these changes. Endotoxin administration resulted in a 54% decrease in flux through pyruvate dehydrogenase (PDH) which was reversible by dichloroacetate, an inhibitor of PDH kinase. Only 44% of the total PDH complex in endotoxin-treated cells was in the active (non-phosphorylated) form as compared with 72% in control cells. All these effects were reversible if the endotoxin was washed off the cells within 10–15 min, but cellular damage — both morphological and functional — became irreversible if the endotoxin remained in contact with the cells for 30 min or more.

Mitochondria obtained from septic or endotoxic models demonstrated significant decreases in respiratory control index [43–45], state-3 respiration [43-46] and state-4 respiration [45].

Mela et al. performed a number of investigations on mitochondrial function and ultrastructural change in rat and guinea pig models [40,47–51]. Kidney and brain mitochondrial oxygen utilization, ATP synthesis and Ca^{2+} transport activity were significantly depressed 24 h after induction of sepsis [48]. High-dose steroids prevented this deterioration in mitochondrial function and completely abolished the 60% mortality seen in untreated animals. In a follow-up study [49], ATP synthase, adenine nucleotide translocase and carrier-mediated Ca^{2+}-transport enzymes were specifically and adversely affected. A further study [51] utilized a chronic rat model of sepsis created by the formation of a subcutaneous abscess with animals being killed after either 1, 2 or 3 weeks. In contrast with their earlier findings, no significant differences were found in state-3 respiratory activities of liver mitochondria isolated from septic or sham control rats. However, they did demonstrate sepsis-induced fuel shifts in muscle mitochondria, although mitochondrial respiration was not uncoupled at any time and muscle energy charge remained unaltered.

Close correlation was found between the severity of shock and hepatic energy state [52]. Rats with irreversible shock showed a marked deterioration in adenylate system parameters, whereas animals with reversible shock had an unchanged energy state. Schaefer et al. examined changes *in vivo* in cytochrome aa_3 redox state in endotoxaemic rats using near-infrared spectrophotometry [53–55] and concluded that endotoxin induced direct (or rapidly mediated indirect) impairment of oxidative phosphorylation. Impaired intestinal oxidative phosphorylation occurred as early as 5 min post-endotoxin with a significant shift towards reduced cytochrome $a + a_3$. Intestinal oxygen consumption, however, remained unchanged [53]. The fall in intestinal cytochrome $a + a_3$ redox state was endotoxin-dose dependent [54], and correlated with falls in blood pressure and flow, although tissue oxyhaemoglobin (HbO_2) levels and systemic oxygen consumption remained unchanged. Endotoxin also induced decreases in brain cytochrome aa_3 redox state [55]; however, in this organ, it was related to impaired blood flow and was proportional to the decrease in tissue HbO_2.

Similar findings were made in a primate model of hyperdynamic Gram-negative sepsis [56]. Although oxygen delivery and consumption hardly changed over 24 h, progressive changes in cytochrome $a + a_3$ redox state occurred within a few hours. This correlated with mitochondrial structural changes. An early defect in oxygen provision to mitochondria followed by a progressive loss of functional cytochrome $a + a_3$ was proposed. Kang et al. [57] found morphological damage to mitochondria with a significant increase in intracellular Ca^{2+} content and depletion of cytochrome *c* oxidase.

No change

Clemens et al. [58] found that both oxidative phosphorylation capability and oxygen consumption of mitochondria isolated from livers of septic rats were similar to controls. No significant alterations in state-3 and state-4 rates, or respiratory control index were found in muscle, kidney or liver mitochondria in short-term experiments (<6 h) [59–62].

Initial increase, later decrease

Hirai et al. studied 23 pigs with peritonitis [63]. Over 7 days, liver mitochondrial enhancement was observed, but the energy charge level was barely maintained. After 10–14 days, the energy charge level fell to 0.68, and there was a marked decrease in mitochondrial function. A high positive correlation was noted between cardiac index and liver mitochondrial phosphorylation activity ($r = 0.85$, $P<0.01$).

This initial increase in respiratory activity followed by a later decrease was also found by Ohtoshi et al. in septic rats [64]. Mitochondrial phosphorylation activity was enhanced at 6 h ($P<0.001$), but decreased at 18 h. Hepatic energy charge levels fell from 0.84 to 0.77 at 12 h ($P<0.01$) and to 0.60 at 18 h ($P<0.001$). The mitochondrial redox state increased significantly at 12 and 18 h after induction of sepsis. The ketogenic capacity of the liver was inhibited, although the liver could still maintain gluconeogenesis at relatively normal levels until a more advanced stage of sepsis.

Mizobata et al., using magnetic resonance spectroscopy *in vivo*, also found that the duration of infection modified mitochondrial oxidative capacity in rat skeletal muscle with increased phosphocreatine (PCr) breakdown, decreased PCr/ATP ratios and an incrementally decreased PCr/P_i ratio (7% at 24 h post-induction of sepsis, 23% at 48 h) [65]. Of note, the maximal oxidative ATP synthesis rate at 24 h was accelerated in septic animals.

Increased activity

Dawson et al. [66] found enhanced hepatic, cardiac and skeletal muscle mitochondrial function in rats injected with a lethal dose of endotoxin. However, this was an unusual study as mitochondrial function (respiratory control index, ADP/O_2 ratio and protein levels) was serially determined during a 4 h post-mortem period. Whereas hepatic mitochondria ceased to function within 30 min of the time of death, cardiac and skeletal muscle mitochondria functioned normally for up to 4 h in both septic and control animals. Mitochondria from the septic animals had a significantly higher respiratory

control ratio (RCR). Poderoso et al. also found that liver slices from septic animals had a 60% greater oxygen uptake than controls, although this was markedly dependent on the oxygen supply. Mitochondrial oxygen uptake was nearly 30% more with malate plus glutamate as substrate, but not with succinate [67].

Mixed findings

Membrane permeability of liver mitochondria from septic rats increased 10–100-fold over that of controls [68]. However, mitochondrial oxidative phosphorylation activity (using glutamate as substrate) was enhanced throughout all periods until death and was associated with concomitant increases in RCRs. The authors suggested that both hyperfunctioning and deteriorated mitochondria co-exist.

Piantadosi's group recently reported measurements in both isolated hepatocytes and liver mitochondria taken 16 h after the onset of sepsis in rats [69]. Endogenous respiration by isolated cells was decreased, though cyanide-resistant (non-mitochondrial) respiration was unaffected. Maximal oxygen consumption in ADP-supplemented hepatocytes was decreased with succinate as the substrate, but not with malate plus glutamate. However, maximum state-3 oxygen consumption by isolated liver mitochondria increased up to 35% during sepsis using either succinate or malate plus glutamate as substrate. Differences were identified in morphology with mitochondrial swelling seen in most of the septic livers, as well as increased oxidative stress. They too proposed that hepatocytes contain a mixed population of injured and hyperfunctional mitochondria during sepsis.

In summary, there are highly conflicting results but models of sufficent severity and duration of insult do, in general, demonstrate inhibition of respiratory enzyme activity. In whole-animal models, or in mitochondria or cells isolated from septic or endotoxaemic animals, a duration of at least 6 h is often necessary to detect impaired activity.

Is the rate of ATP synthesis/ATP concentration affected during sepsis?

These questions yield the most equivocal answers. In part this is related to the difficulties in measuring ATP and the relatively sparse data available. However, variations in model severity and in organs studied would also appear to influence the findings.

Kilpatrick et al. exposed cultured mouse neuroblastoma cells to endotoxin and found a decline in the cellular [ATP]/[ADP] + [P_i] ratio [30]. The rate of ATP synthesis was initially maintained, but declined after longer exposure to endotoxin. Mela and Miller [48] found a significant fall in ATP synthesis in mitochondria isolated from kidney and brain taken 24 h post-injection of endotoxin into rats and guinea pigs. A follow-up study suggested ATP synthase activity was diminished [50]; however, they subsequently found no uncoupling of mitochondrial respiration in muscle and liver samples taken from a chronically septic rat model [51]. Asher et al. made similar observations

[61] in liver and kidney mitochondria isolated from endotoxic rats 5 h after injection, while Kopprasch et al. [52] found the rate of ATP synthesis was unaffected irrespective of whether their endotoxic rats were in reversible or irreversible shock. A contrary result was obtained by Mizobata et al. [65], who found that the maximal rate of ATP synthesis was accelerated in septic animals. However, Shiomi et al.'s study of endotoxic rats [70] demonstrated reduced hepatic ATP contents and oxygen consumption with an associated decrease in mitochondrial membrane potential.

The cellular ATP level is an important determinant for apoptotic cell death [71]. The mitochondrial membrane potential, the driving force for ATP synthesis, falls with apoptosis whereas its maintenance prevents cell death. Oxidative stress, NO and peroxynitrite, Ca^{2+}, proteases and nucleases mediate apoptosis and are all increased in sepsis. One can speculate that as maintenance of ATP levels is necessary for survival, the cell, on exposure to exogeneous insults, may initially attempt to protect itself by reducing metabolic activity and thus ATP requirements. This 'hibernation' may then terminate either when the milieu normalizes, following release of a new mediator, or following production of new enzyme(s). A major insult may overwhelm any protective mechanisms, initiating permeability transition pore opening, mitochondrial swelling with membrane disruption, further ATP depletion and cell death.

Potential mechanisms of sepsis-induced damage

Recent studies place considerable importance on the intramitochondrial production of free radicals and NO, though cytokine-mediated effects, tissue hypoxia *per se*, depletion of endogenous anti-oxidants, alterations in intracellular Ca^{2+} and depression of other metabolic pathways should also be considered.

Reactive oxygen species production

Numerous studies have suggested increased production of reactive oxygen species (ROS) in sepsis — both in mitochondrial, cell and animal models, and in patients [72]. Apart from any direct intracellular effects of sepsis, the combined consequence of vasodilatation, microvascular flow obstruction and redistribution, endothelial leak, hypovolaemia and interstitial oedema may all contribute to tissue hypoxia and ROS generation. Upon resuscitation and reperfusion, ROS production may be enhanced further.

ROS are also formed in mitochondria [73]. These include superoxide, hydrogen peroxide, hydroxyl radicals and NO. Apart from dose-dependent injurious effects, leading to oxidation of nucleic acids, proteins and lipids, they also appear to act as physiological modulators of some mitochondrial functions. For example, H_2O_2, which originates in mitochondria, predominantly from superoxide dismutase, will stimulate Ca^{2+} release from intact mitochondria. Stimulation of mitochondrial ROS production followed by an increase in Ca^{2+} release and re-uptake (Ca^{2+} cycling) will result in apoptosis and necrosis, and may be a significant contributor to ischaemia/reperfusion injury.

Llesuy et al. [46] produced sepsis in rats by caecal ligation and puncture (CLP) and measured spontaneous chemiluminescence of adductor muscle and liver at 6, 12, 24 and 30 h after the insult. Muscle chemiluminescence showed a maximal increase of about two-fold after 6–12 h, while liver chemiluminescence increased by about 80% after 24 h. In muscle the activities of the anti-oxidant enzymes superoxide dismutase, catalase and glutathione peroxidase were maximally diminished (by 46–83%) after 12 h. In liver, only catalase activity showed a 52% decrease after 24 h of sepsis. The rate of hydrogen peroxide production of muscle mitochondria was increased 2.5-fold after 12 h of sepsis. These changes occurred in parallel with inhibition of active mitochondrial respiration. Sakaguchi et al. [44] gave endotoxin to mice and also found concurrent depression of mitochondrial respiration with greatly increased generation of superoxide anion.

ROS generation was detected in liver mitochondria taken from CLP-septic rats [62]. Whereas inhibition of electron transport at complex I with rotenone had no effect on hydroxyl radical production, the combination of rotenone plus cyanide was effective. Referenced to the mitochondrial respiration rate, both hydroxyl radical ($OH^{\cdot}$) and H_2O_2 production were greater in septic liver mitochondria. However, state-3 and state-4 respiration rates were no different from controls. The authors postulated that enhanced mitochondrial oxidative stress in sepsis, by liver mitochondria utilizing FAD-linked but not NAD-linked substrates, was related to alterations in the activity of complex II of the electron-transport chain.

Cytokine-induced mitochondrial damage

Cytokine release is well documented in sepsis. In particular, TNF and IL-1 release have been intensively studied; therapeutic trials based on blockade of these cytokines or their receptors by specific antibodies have been attempted, albeit without success. Although cytokine release is known to intiate numerous infllammatory system cascades, its effects on mitochondria have only recently been investigated.

After administering TNFα and IL-1β to rats (12 and 24 h) there was a hypermetabolic hepatic mitochondrial state with increases in respiratory control index and oxidative phosphorylation rate, albeit with a significant decrease in hepatic energy charge [74]. Kurose et al. [75] demonstrated that Kupffer cell-derived mediators altered the mitochondrial oxidative phosphorylation of hepatocytes taken from endotoxaemic Wistar rats. Oxidative phosphorylation was assessed by rhodamine-1,2,3 fluorescence, a dye used to indicate mitochondrial membrane potential. After 2 h, a marked decrease in fluorescence was observed. After 4 h, there was a further fall in fluorescence, an increase in NO production and mitochondrial membrane barrier dysfunction. In cultured rat aortic smooth muscle cells interferon-γ and TNFα synergistically inhibited complex I and II activities of the mitochondrial respiratory chain [76].

Calcium

High levels of intracellular Ca^{2+} activate many potentially destructive enzymic pathways (e.g. proteases, phospholipases, endonucleases) that affect

cell function and may result in cell death. Intracellular Ca^{2+} accumulation may thus play an important role in the development of multiple organ failure [77]. Ca^{2+} may participate in free radical formation in the liver during endotoxaemia [44].

Sepsis-induced elevations in cytosolic Ca^{2+} have been well described [57,64,78,79], although Ohtoshi et al. found markedly higher Ca^{2+} levels in septic liver mitochondrial fractions [64]. Deaciuc and Spitzer found a slight, but significant, increase in total hepatic Ca^{2+} content with sepsis [80]; although a decrease was noted in Ca^{2+} content in the microsomal fraction, there was an increase in mitochondrial content. They concluded that sepsis induced discrete alterations of Ca^{2+} fluxes and compartmentalization within the cell. Kasden and Kirkpatrick found no significant difference in either RCR or mitochondrial Ca^{2+} content between mitochondria from septic and control rats incubated in a Ca^{2+}-free medium [81]. Liver RCR, however, increased significantly with Ca^{2+} or endotoxin pretreatment, whereas muscle mitochondrial RCR decreased significantly with Ca^{2+}.

A fall then a recovery in mitochondrial membrane potential was accompanied by a rise and fall of cytosolic Ca^{2+} levels [82]. Killing of the cells was reduced when the cytosolic Ca^{2+} was chelated, or when the cyclic uptake and release of Ca^{2+} (Ca^{2+} cycling) by the mitochondria was prevented. The authors proposed that NO killed cells by de-energizing mitochondria, thereby flooding the cytosol with Ca^{2+}. In a later study [83] they found that peroxynitrite stimulates a specific Ca^{2+} release pathway, which operates when oxidized mitochondrial pyridine nucleotides are hydrolysed in a Ca^{2+}-dependent manner to ADP-ribose and nicotinamide. Subsequently, they showed [84] that mitochondrial NO synthase activity was (i) associated with the inner mitochondrial membrane but not with the matrix fraction, (ii) constitutively active, exerting substantial control over mitochondrial respiration and membrane potential and (iii) further stimulated when Ca^{2+} was taken up by mitochondria.

NO

NO production is probably higher in sepsis than in any other clinical condition. Inhibition of its synthesizing enzymes (e.g. by N^G-monomethyl-L-arginine [L-NMMA]) or its effector pathway (e.g. by Methylene Blue) has been shown to produce rises in blood pressure and reduction in vasopressor requirements. It has various actions, including vasodilatation, prevention of platelet aggregability, cytotoxic effects (including damage to DNA) and reversible mitochondrial inhibition. Its congener, peroxynitrite, formed by reaction of superoxide with NO, is a more potent oxidant with irreversible inhibitory effects.

NO has been shown to compete with oxygen at cytochrome oxidase and to reversibly inhibit this enzyme activity, even at nanomolar concentrations [85]. This effect of NO is more potent at lower oxygen tensions and weaker at high oxygen tensions; ATP synthesis is thus inhibited in an oxygen-concentration-dependent manner [86–88]. The mitochondria may well operate as an oxygen sensor with NO being an important regulator of mitochondrial energy metabolism, decreasing ATP synthesis in low-oxygen-supply situations.

Table 2 Inhibition of mitochondrial enzymes by NO and peroxynitrite

Complex activity inhibited	Reference
NO inhibition	
Complex I, aconitase	[90]
Complexes I and II, aconitase	[91]
Complexes I and II	[76]
Complex IV	[92]
Complex IV	[93]
Complexes II–III and IV	[94]
Complex IV	[95]
Complex IV	[88]
Complex IV	[96]
Complexes II and IV	[97]
Complexes III and IV	[89]
Peroxynitrite inhibition	
Mitochondrial aconitase	[98]
Complexes I and II, mitochondrial ATPase	[99]
Complexes II+III, IV	[100]
Complexes I–III	[96]
Complexes I and II, not complex IV	[97]

Brown suggested that the apparent K_m for oxygen of mitochondrial respiration is thus elevated and that this could potentially make the respiration rate sensitive to the oxygen level found in physiological and pathophysiological condition [85]. The supranormal tissue oxygen tensions achieved in septic patients or animals [23–26] may thus decrease the inhibitory effect of NO on mitochondrial respiration but would have no effect on the irreversible inhibition by peroxynitrite. NO and peroxynitrite have also been shown to have variable effects upon other respiratory chain enzymes (Table 2).

Poderoso et al. suggested that NO inhibits mitochondrial electron transfer at cytochrome oxidase but also at the ubiquinone–cytochrome *b* region of the respiratory chain, and that this led to increased oxygen production, reacting with NO to form peroxynitrite [89]. This removes NO and thus reactivates the previously inhibited cytochrome oxidase. At physiological concentrations of NO, a combination of inhibition of electron transfer, NO-induced oxygen production and peroxynitrite formation may all participate in the regulatory control of mitochondrial oxygen uptake.

Piantadosi's group recently reported [69] that inducible NO synthase induction in hepatocytes after 16 h of sepsis was variable, and that minimal oxidation products of NO were detected. A contrary finding was made by Shiomi et al. in endotoxic rats [70]; inhibition of the inducible but not the constitutive form of the enzyme resulted in recovery of oxygen consumption and ATP levels, and reversed the decrease in mitochondrial membrane potential.

There has been recent clinical interest in blocking either the synthesis of NO or its actions (e.g. with Methylene Blue) in hypotensive septic patients

requiring large doses of vasopressors. A large multicentre study of L-NMMA has recently been prematurely terminated with an increased number of deaths, especially in those patients with low cardiac outputs. Kaneda et al.'s study of another non-specific inhibitor, N^G-nitro-L-arginine methyl ester, in endotoxaemic rabbits also found deleterious effects upon cardiac output and pulmonary circulation with further falls noted in hepatic ATP content [101]. This was contrary to their earlier findings with a NO scavenger, carboxy-PTIO, where hepatic ATP concentrations improved to control values [102].

The reduced form of glutathione (GSH) is the most abundant cytosolic thiol that will easily react with NO [103,104]. The inhibitory effect of NO on respiration was suppressed by GSH, L-cysteine and *N*-acetylcysteine, but not by oxidized glutathione. Cellular GSH might play an important role in the regulation of energy metabolism and in protecting against respiratory chain damage.

Other findings

An impaired capacity for gluconeogenesis and ketogenesis has been found in the livers of septic rats [105,106], although an increased hepatic mitochondrial fatty acid oxidative capacity was found in early sepsis [107]. Vary [108] has extensively studied PDH activity in sepsis and found that the proportion of active PDH in septic mitochondria was significantly reduced compared with controls. This would limit glucose oxidation and promote the conversion of pyruvate to lactate. In part, this may be responsible for the hyperlactataemia seen in sepsis.

Our studies

We have utilized an established rat model of endotoxaemic sepsis that is terminated after 3 h. Consistent with the findings described previously, no change in hepatic mitochondrial enzyme activities was demonstrated compared with sham-operated controls. However, electron paramagnetic resonance spectroscopy performed on liver and blood samples did show clear evidence of NO generation with progressive increases in haem nitrosyl signal intensities being detected in the liver samples and from as early as 1 h in venous blood. In addition, an increase in the g6 ferric haem signal was detected, indicative of methaemoglobinaemia, which may also be derived from NO. In addition, a change was also observed in the signal intensities from mitochondrial complex iron–sulphur centres. Whereas iron–sulphur centres observable in the reduced state gained in signal intensity, those in the oxidized state reduced in intensity, implying greater reduction of the mitochondrial electron-transport chain. We have also performed preliminary studies on patients in septic shock, taking muscle biopsies and blood samples soon after admission to intensive care, and then repeating these samples at 5 day intervals until either death or discharge from the unit. Early findings indicate a depression of mitochondrial enzyme activity, predominantly in complexes I (NADH ubiquinone reductase) and IV

(cytochrome oxidase), which increased as the patient's clinical condition improved. Electron paramagnetic resonance spectra of the muscle also detected haem nitrosyl complexes, indicating the presence of excess NO; again, these signals declined as the patient recovered. In addition, increased signals due to reduced iron–sulphur centres in complex I *and* oxidized iron–sulphur centres in complex II were detected, suggesting a block of respiration at complex I *in vivo*. These too normalized with clinical improvement. We explain these findings as: (i) persistent but reversible binding of NO to thiol groups on complex I; and (ii) irreversible damage due to binding of peroxynitrite to complexes I and IV, which normalizes due to synthesis of new mitochondrial electron-transfer complexes (the normal turnover and replacement of these complexes *in vivo* takes place on a similar time scale). This has been demonstrated recently as a further mode of NO-induced inhibition of mitochondrial respiration [109] and would appear to be enhanced by glutathione depletion.

We would like to thank Aruna Nathan and Ray Stidwill (Bloomsbury Institute of Intensive Care Medicine, UCL), Iain Hargreaves, Simon Heales and John Land (National Hospital for Neurology and Neurosurgery), Nathan Davies, Dimitri Svistunenko and Chris Cooper (University of Essex) for their input and collaboration on both clinical and laboratory projects and this chapter.

References

1. Singer, M. (1998) J. Antimicrob. Chemother. **41**, (Suppl. A), 103–112
2. Bone, R.C., Balk, R.A., Cerra, F.B. et al. (1992) Am. Coll. Chest Physicians/Soc. Crit. Care Med. Consensus Conference Committee. Chest. **101**, 1644–1655
3. Salvo, I., de Cian, W., Musicco, M. et al. (1995) Intens. Care Med. **21** (Suppl. 2), 244–249
4. Death investigation – United States (1987) Morb. Mortal Weekly Report (MMWR) **39**, 31–34
5. Fagan, E.A. and Singer, M. (1995) Postgrad. Med. J. **71**, 71–78
6. Vallance, P. and Moncada, S. (1993) New Horizons **1**, 77–86
7. Westendorp, R.G., Langermans, J.A., Huizinga, T.W. et al. (1997) Lancet **349**, 170–173
8. Roy, S., McGuire, W., Mascie-Taylor, C.G. et al. (1997) J. Infect. Dis. **176**, 530–532
9. Stuber, F., Petersen, M., Bokelmann, F. and Schade, U. (1996) Crit. Care Med. **24**, 381–384
10. Zimmerman, J.E., Knaus, W.A., Wagner, D.P., Sun, X., Hakim, R.B. and Nystrom, P.O. (1996) Crit. Care Med. **24**, 1633–1641
11. Bone, R.C. (1996) Ann. Intern. Med. **125**, 680–687
12. Docke, W.D., Randow, F., Syrbe, U. et al. (1997) Nat. Med. **3**, 678–681
13. The Veterans Administration Systemic Sepsis Cooperative Study Group (1987) N. Engl. J. Med. **317**, 659–665
14. Wakabayashi, Y., Kamijou, Y., Soma, K. and Ohwada, T. (1996) Br. J. Surg. **83**, 393–394
15. Fourrier, F., Jourdain, M., Tournois, A., Caron, C., Goudemand, J. and Chopin, C. (1995) Intens. Care Med. **21** (Suppl. 2), S264–S268
16. Lam, C., Tyml, K., Martin, C. and Sibbald, W.J. (1994) J. Clin. Invest. **94**, 2077–2083
17. Fink, M. (1997) Acta Anaesthesiol. Scand. (Suppl.) **110**, 87–95
18. Gutierrez, G. and Wulf, M.E. (1996) Intens. Care Med. **22**, 6–16
19. Hotchkiss, R.S. and Karl, I.E. (1992) J. Am. Med. Assoc. **267**, 1503–1510

20. Kreymann, G., Grosser, S., Buggisch, P., Gottschall, C., Matthaei, S. and Greten, H. (1993) Crit. Care Med. **21**, 1012–1019
21. Singer, M., Millar, C., Stidwill, R. and Unwin, R. (1996) Intens. Care Med. **22**, 324–328
22. Stidwill, R.P., Rosser, D.M. and Singer, M. (1998) Intens. Care Med. **24**, 1209–1216
23. Rosser, D.M., Stidwill, R.P., Jacobson, D. and Singer, M. (1995) J. Appl. Physiol. **79**, 1878–1882
24. Rosser, D.M., Stidwill, R.P., Jacobson, D. and Singer, M. (1996) Am. J. Physiol. **271**, H891–H895
25. VanderMeer, T.J., Wang, H. and Fink, M.P. (1995) Crit. Care Med. **23**, 1217–1226
26. Boekstegers, P., Weidenhofer, S., Pilz, G. and Werdan, K. (1991) Infection **19**, 317–323
27. Levy, E., Slusser, R.J. and Ruebner, B.H. (1968) Am. J. Pathol. **52**, 477–502
28. Raffa, J. and Trunkey, D.D. (1978) J. Trauma **18**, 617–622
29. Manny, J., Livni, N., Schiller, M., Guttman, A., Boss, J. and Rabinovici, N. (1980) Isr. J. Med. Sci. **16**, 153–161
30. Kilpatrick, S.L., Erecinska, M. and Silver, I.A. (1981) Circ. Shock **8**, 585–600
31. Fry, D.E., Kaelin, C.R., Giammara, B.L. and Rink, R.D. (1981) Adv. Shock Res. **6**, 45–54
32. Connell, R.S., Harrison, M.W., Mela, R.L. et al. (1988) Progr. Clin. Biol. Res. **264**, 325–332
33. Gotloib, L., Shostak, A., Galdi, P., Jaichenko, J. and Fudin, R. (1992) Circ. Shock **36**, 45–56
34. Hauptmann, S., Klosterhalfen, B., Weis, J., Mittermayer, C. and Kirkpatrick, C.J. (1994) Virchows Arch. **424**, 653–659
35. Welty, W.K., Simonson, S.G., Huang, Y.C., Fracica, P.J., Patterson, J.W. and Piantadosi, C.A. (1996) Shock **5**, 378–384
36. Farrar, W.J., Corwin, L.M. and Kent, T.H. (1965) J. Bacteriol. **90**, 1365–1372
37. Harris, R.A., Harris, D.L. and Green, D.E. (1968) Arch. Biochem. Biophys. **128**, 219–230
38. Moss, G.S., Erve, P.P. and Schumer, W. (1969) Surg. Forum **20**, 24–25
39. Schumer, W., Erve, P., Kapica, S.K. and Moss, G.S. (1970) J. Surg. Res. **10**, 609–612
40. Mela, L., Bacalzo, L.J. and Miller, L.D. (1971) Am. J. Physiol. **220**, 571–577
41. Kilpatrick, S.L. and Erecinska, M. (1983) Circ. Shock **11**, 85–99
42. Kilpatrick, S.L., Deas, J., Erecinska, M. and Silver, I.A. (1983) Circ. Shock **11**, 101–111
43. Ishiyama, S., Hiraga, K. and Tsukamoto, M. (1985) Nippon Geka Gakkai Zasshi **86**, 23–31
44. Sakaguchi, S., Ibata, H. and Yokota, K. (1989) Microbiol. Immunol. **33**, 99–110
45. Kudoh, A., Kudoh, E., Ishihara, H. and Matsuki, A. (1998) Crit. Care Med. **26**, 138–141
46. Llesuy, S., Evelson, P., Gonzalez, F.B. et al. (1994) Free Radic. Biol. Med. **16**, 445–451
47. Mela, L. (1981) Prog. Clin. Biol. Res. **62**, 15–21
48. Mela, L. and Miller, L.D. (1983) Circ. Shock **10**, 371–381
49. Tavakoli, H. and Mela, L. (1982) Infect. Immun. **38**, 536–541
50. Mela-Riker, L. and Tavakoli, H. (1984) Am. J. Emerg. Med. **2**, 2–7
51. Mela-Riker, L., Bartos, D., Vlessis, A.A., Widener, L., Muller, P. and Trunkey, D.D. (1992) Circ. Shock **36**, 83–92
52. Kopprasch, S., Horkner, U., Orlik, H., Kemmer, C. and Scheuch, D.W. (1989) Biomed. Biochim. Acta **48**, 653–659
53. Schaefer, C.F., Biber, B., Lerner, M.R., Jobsis-VanderVliet, F.F. and Fagraeus, L. (1991) J. Surg. Res. **51**, 382–391
54. Schaefer, C.F., Lerner, M.R. and Biber, B. (1991) Circ. Shock **33**, 17–25
55. Schaefer, C.F. and Biber, B. (1993) Circ. Shock **40**, 1–8
56. Simonson, S.G., Welty, W.K., Huang, Y.T. et al. (1994) Circ. Shock **43**, 34–43

57. Kang, Y.H., McKenna, T., Watson, L.P., Williams, R. and Holt, M. (1988) J. Histochem. Cytochem. **36**, 665–678
58. Clemens, M., Chaudry, I.H. and Baue, A.E. (1981) Adv. Shock Res. **6**, 55–64
59. Geller, E.R., Jankauskas, S. and Kirkpatrick, J. (1986) J. Surg. Res. **40**, 514–517
60. Garrison, R.N., Ratcliffe, D.J. and Fry, D.E. (1982) Adv. Shock Res. **7**, 71–76
61. Asher, E.F., Garrison, R.N., Ratcliffe, D.J. and Fry, D.E. (1983) Arch. Surg. **118**, 441–445
62. Taylor, D.E., Ghio, A.J. and Piantadosi, C.A. (1995) Arch. Biochem. Biophys. **316**, 70–76
63. Hirai, F., Aoyama, H., Ohtoshi, M., Kawashima, S., Ozawa, K. and Tobe, T. (1984) Eur. Surg. Res. **16**, 148–155
64. Ohtoshi, M., Jikko, A., Asano, M., Uchida, K., Ozawa, K. and Tobe, T. (1984) Res. Exp. Med. Berlin **184**, 209–219
65. Mizobata, Y., Prechek, D., Rounds, J.D., Robinson, V., Wilmore, D.W. and Jacobs, D.O. (1995) J. Surg. Res. **59**, 165–173
66. Dawson, K.L., Geller, E.R. and Kirkpatrick, J.R. (1988) Arch. Surg. **123**, 241–244
67. Poderoso, J.J., Fernandez, S., Carreras, M.C. et al. (1994) Circ. Shock **44**, 175–182
68. Tanaka, J., Kono, Y., Shimahara, Y. et al. (1982) Adv. Shock Res. **7**, 77–90
69. Kantrow. S.P., Taylor, D.E., Carraway, M.S. and Piantadosi, C.A. (1997) Arch. Biochem. Biophys. **345**, 278–288
70. Shiomi, M., Wakabayashi, Y., Sano, T. et al. (1998) Hepatology **27**, 108–115
71. Richter, C., Schweizer, M., Cossarizza, A. and Franceschi, C. (1996) FEBS Lett. **378**, 107–110
72. Galley, H.F., Davies, M.J. and Webster, N.R. (1996) Crit. Care Med. **24**, 1649–1653
73. Richter, C., Gogvadze, V., Laffranchi, R. et al. (1995) Biochim. Biophys. Acta **1271**, 67–74
74. Jin, M.B., Shimahara, Y., Yamaguchi, T. et al. (1995) J. Surg. Res. **58**, 509–515
75. Kurose, I., Miura, S., Higuchi, H. et al. (1996) Hepatology **24**, 1185–1192
76. Geng, Y., Hansson, G.K. and Holme, E. (1992) Circ. Res. **71**, 1268–1276
77. Crouser, E.D. and Dorinsky, P.M. (1996) Clin. Chest Med. **17**, 249–261
78. Sayeed, M.M., Zhu, M. and Maitra, S.R. (1989) Magnesium **8**, 179–189
79. Sakaguchi, S., Abe, H. and Sakaguchi, O. (1984) Microbiol. Immunol. **28**, 517–527
80. Deaciuc, I.V. and Spitzer, J.A. (1987) Cell Calcium **8**, 365–376
81. Kasden, S.E. and Kirkpatrick, J.R. (1990) Am. Surg. **56**, 707–714
82. Richter, C., Gogvadze, V., Schlapbach, R., Schweizer, M. and Schlegel, J. (1994) Biochem. Biophys. Res. Commun. **205**, 1143–1150
83. Schweizer, M. and Richter, C. (1996) Biochemistry **35**, 4524–4528
84. Ghafourifar, P. and Richter, C. (1997) FEBS Lett. **418**, 291–296
85. Brown, G.C. (1997) Mol. Cell. Biochem. **174**, 189–192
86. Schweizer, M. and Richter, C. (1994) Biochem. Biophys. Res. Commun. **204**, 169–175
87. Takehara, Y., Kanno, T., Yoshioka, T., Inoue, M. and Utsumi, K. (1995) Arch. Biochem. Biophys. **323**, 27–32
88. Borutaite, V. and Brown, G.C. (1996) Biochem. J. **315**, 295–299
89. Poderoso, J.J., Carreras, M.C., Lisdero, C., Riobo, N., Schopfer, F. and Boveris, A. (1996) Arch. Biochem. Biophys. **328**, 85–92
90. Stadler, J., Curran, R.D., Ochoa, J.B. et al. (1991) Arch. Surg. **126**, 186–191
91. Stadler, J., Billiar, T.R., Curran, R.D., Stuehr, D.J., Ochoa, J.B. and Simmons, R.L. (1991) Am. J. Physiol. **260**, C910–C916
92. Heales, S.J., Bolanos, J.P., Land, J.M. and Clark, J.B. (1994) Brain Res. **668**, 243–245

93. Cleeter, M.W., Cooper, J.M., Darley-Usmar, V., Moncada, S. and Schapira, A.H. (1994) FEBS Lett. **345**, 50–54
94. Bolanos, J.P., Peuchen, S., Heales, S.J., Land, J.M. and Clark, J.B. (1994) J. Neurochem. **63**, 910–916
95. Brown, G.C. (1995) FEBS Lett. **369**, 136–139
96. Lizasoain, I., Moro, M.A., Knowles, R.G., Darley-Usmar, V. and Moncada, S. (1996) Biochem. J. **314**, 877–880
97. Cassina, A. and Radi, R. (1996) Arch. Biochem. Biophys. **328**, 309–316
98. Castro, L., Rodriguez, M. and Radi, R. (1994) J. Biol. Chem. **269**, 29409–29415
99. Radi, R., Rodriguez, M., Castro, L. and Telleri, R. (1994) Arch. Biochem. Biophys. **308**, 89–95
100. Bolanos, J.P., Heales, S.J., Land, J.M. and Clark, J.B. (1995) J. Neurochem. **64**, 1965–1972
101. Kaneda, K., Makita, K., Yokoyama, K., Toyooka, H. and Amaha, K. (1998) Acta Anaesthesiol. Scand. **42**, 399–405
102. Kaneda, K., Yoshioka, Y., Makita, K., Toyooka, H. and Amaha, K. (1997) Crit. Care Med. **25**, 1019–1029
103. Bolanos, J.P., Heales, S.J., Peuchen, S., Barker, J.E., Land, J.M. and Clark, J.B. (1996) Free Radic. Biol. Med. **21**, 995–1001
104. Nishikawa, M., Sato, E.F., Kashiba, M., Kuroki, T., Utsumi, K. and Inoue, M. (1998) Hepatology **27**, 422–426
105. Jones, C.G. and Titheradge, M.A. (1993) Biochem. J. **289**, 169–172
106. de Vasconcelos, P.R., Kettlewell, M.G. and Williamson, D.H. (1987) Clin. Sci. **72**, 683–691
107. Yamamoto, T. (1993) Surg. Today **23**, 137–143
108. Vary, T.C. (1991) Am. J. Physiol. **260**, E669–E674
109. Clementi, E., Brown, G.C., Feelisch, M. and Moncada, S. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 7631–7636