

Recent Progress in Pharmacological Research of Antioxidants in Pathological Conditions: Cardiovascular Health

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Abstract: Antioxidants are essential, and are involved in several important biological processes such as immunity, protection against tissue damage, reproduction, growth and development. Antioxidants preserve adequate function of cells against homeostatic disturbances such as those caused by septic shock, aging and, in general, processes involving oxidative stress. Each year, many scientific articles are published describing the pharmacological and biological properties of antioxidants. This review article compiles recent findings on these properties, focusing mainly on the anti-inflammatory properties of antioxidants in different pathological areas, such as cardiovascular damage and sepsis. In relation to this process, this review focuses on the involvement of reactive oxygen and nitrogen species. Finally, the protective role of antioxidants against homeostatic disturbances such as those caused by endotoxin toxicity and cardiovascular damage, their potential clinical use, and the effects on the redox state of immune cells are discussed.

Keywords: Antioxidant, cardiovascular dysfunction, endotoxin, flavonoids, immune system, ischemia, mitochondria, *N*-acetyl-cysteine, NF- κ B, nitric oxide, patent, peroxynitrite, reactive oxygen species, septic shock, vitamin.

INTRODUCTION

The presence of free radicals in biological materials was discovered about 50 years ago [1]. Today, there is a large body of evidence indicating that patients in hospital intensive care units (ICUs) are exposed to excessive free radicals from drugs and other substances that alter cellular reduction-oxidation (redox) balance, and disrupt normal biological functions [2-3]. However, low levels of free radicals are also vital for many cell signaling events and are essential for proper cell function.

Excess free radicals can result from a variety of conditions such as tissue damage and hypoxia (limiting oxygen levels), overexposure to environmental factors (tobacco smoke, ultraviolet radiation, and pollutants), a lack of antioxidants, or destruction of free radical scavengers. When the production of damaging free radicals exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs. The resultant cellular injury caused by oxidative stress has been linked to over 200 clinical disorders, many of which are seen in ICU patients units [4].

FREE RADICALS AND ANTIOXIDANTS: AN OVERVIEW

A free radical can be described as any atom or a group of atoms or molecules in which there is at least one unpaired electron in the outermost shell [5]. These free radicals are

very reactive with adjacent molecules such as lipids, proteins, and carbohydrates and can cause cellular damage [6]. Paradoxically, free radicals can also be produced by many cells as a protective mechanism, for example neutrophils produce free radicals to attack and destroy pathogens, while the liver uses free radicals for detoxification [7]. However, the presence of free radicals within the body can also have a significant role in the development and progression of many disease processes for example heart disease, hypertension, cerebrovascular accidents, and diabetic complications.

Any free radical involving O_2 is referred to as a reactive oxygen species (ROS) [8]. Normal cellular metabolism involves the production of ROS, and in humans, superoxide (O_2^-) is the most commonly produced free radical. Phagocytic cells such as macrophages and neutrophils are prominent sources of O_2^- . During an inflammatory response, these cells generate free radicals that attack invading pathogens such as bacteria and, because of this, the production of O_2^- by activated phagocytic cells in response to inflammation is one of the most studied free radical-producing systems [5].

O_2^- produced from a one-electron reduction of O_2 can undergo either spontaneous or enzyme-catalyzed dismutation to hydrogen peroxide (H_2O_2). H_2O_2 , although not technically considered an oxygen free radical, is a member of the ROS family and may selectively participate in free radical generation. The majority of the H_2O_2 is broken down to O_2 and water by the antioxidant enzyme catalase. In addition to catalase, glutathione peroxidase can also break down H_2O_2 and also any peroxides that form on lipids within the body [5]. When O_2^- reacts with nitric oxide (NO), the toxic product peroxynitrite ($ONOO^-$) is formed. Although it is true that

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most ROS originate from O_2^- generated as a by-product of oxidative phosphorylation (mitochondrial respiration), they differ in their mechanism of production, necessary cofactors, diffusion range, hydrophobicity, biological targets, detoxification pathways and breakdown products. O_2^- is membrane impermeant and its damaging reactions largely involve disassembly of iron-sulphur clusters in proteins. In fact, studies in rat liver lysates have shown that H_2O_2 or O_2^- alone lacked reactivity toward iron regulatory protein-1 (IRP-1), but a combined action of the two species induced reversible inactivation of IRP-1. Such an effect was attributed to direct interactions of O_2^- and H_2O_2 with a preformed pool of IRP-1, resulting in reversible modifications of -SH residues; in fact, its action would be limited to removing only iron atoms, an effect sufficient to abolish enzyme activity.

The hydroxyl radical ($\cdot OH$) is the most reactive of the free radical molecules [1]. $\cdot OH$ damages cell membranes and lipoproteins by a process termed lipid peroxidation. In fact, lipid peroxidation can be defined as the process whereby free radicals “steal” electrons from the lipids in our cell membranes, resulting in cell damage and increased production of ROS. This process takes place in 3 stages:

1. **Initiation:** In a peroxide-free lipid system, the initiation of a peroxidation sequence refers to the attack of an ROS (with sufficient reactivity) able to abstract a hydrogen (H) atom from a methylene group ($-CH_2-$), as these hydrogens have very high mobility. This attack easily generates free radicals from polyunsaturated fatty acids. $\cdot OH$ is the most efficient ROS for this attack, whereas O_2^- is insufficiently reactive.
2. **Propagation:** A peroxy radical is able to abstract H from another lipid molecule (adjacent fatty acid), especially in the presence of metals such as copper or iron, thus causing an autocatalytic chain reaction. The peroxy radical combines with H to give a lipid hydroperoxide (or peroxide). This reaction characterizes the propagation stage.
3. **Termination:** formation of a hydroperoxide.

Lipid peroxidative damage to lipids in low-density lipoprotein (LDL) plays an important role in atherosclerosis [9].

To protect against oxidative damage, organisms have developed a variety of antioxidant defenses that include proteins, compounds such as vitamins, and specialized antioxidant enzymes. Antioxidants can be described as substances capable of counteracting the damaging effects of oxidation in body tissues. Antioxidants are divided in general into two classes based on mechanisms of action:

1. chain-breaking antioxidants, such as Vitamin E and beta-carotene, “break the chain” of free radical formation by donating an electron to stabilize an existing free radical. In general, they act by reacting with peroxy radicals.
2. preventive antioxidants are enzymes that scavenge initiating radicals before they start an oxidation chain.

Chain-breaking antioxidants are found in the blood and the fluids of the extracellular space, where preventive antioxidant enzymes are absent or present in very small quantities [8]. These small-molecule antioxidants include both water and lipid-soluble varieties. The lipid-soluble

antioxidants are located in the cellular membranes and lipoproteins, whereas the water-soluble antioxidants are present in the aqueous environments, such as fluids inside cells and in the blood.

Preventative antioxidant enzymes inside the cell are an important defense against free radicals. The main enzymatic “scavengers” responsible for the prevention of ROS formation and oxidation are superoxide dismutase (SOD), catalase, and glutathione peroxidase. SOD is found in virtually every oxygen-based organism, and its major function is to catalyze the dismutation of O_2^- to H_2O_2 . This reaction is generally considered to be the body's primary antioxidant defense because it prevents further generation of free radicals. In humans, the highest levels of SOD are found in the liver, adrenal gland, kidney, and spleen [10].

Catalase and glutathione peroxidase both work to detoxify O_2^- -reactive radicals by catalyzing the formation of H_2O_2 derived from O_2^- . The liver, kidney, and red blood cells possess high levels of catalase, which helps to detoxify chemicals in the body. The water-soluble tripeptide-thiol glutathione also plays an important role in a variety of detoxification processes. Glutathione is found in millimolar concentrations in the cell cytosol and other aqueous phases, and readily interacts with free radicals, especially the hydroxyl radical, by donating a hydrogen atom. This reaction provides protection by neutralizing reactive hydroxyl radicals that are thought to be a major source of free radical pathology, including cancer [11]. Glutathione is also an essential co-factor for antioxidant enzymes.

Below, the data is summarized regarding some of the most frequently antioxidants in pathologies which implicate free radicals production such as sepsis and cardiovascular dysfunction. These pathogenesis generally involve impairment in endothelial function.

SEPSIS AND SIGNALING PATHWAYS

Serious infections trigger systemic inflammatory response and can result in sepsis. It is believed that sepsis and therefore septic shock are due to the inappropriate increase in the innate immune response *via* circulating and tissue inflammatory cells, such as monocytes/macrophages and neutrophils. These cells normally exist in a non-activated state but are rapidly activated in response to bacteria, their products or inflammatory mediators, to become highly active phagocytes. In doing so, they can contribute to the development of injuries by producing mediators such as cytokines and ROS. In fact, sepsis induces a dysfunction in immune cells [12-14].

As most infections occur primarily in the tissue and not in the blood stream, extravasation of leukocytes is essential to bring inflammatory cells and foreign pathogens into contact. This requires both a chemotactic gradient and coordinated up-regulation of endothelial and inflammatory cell adhesion molecule expression (ICAM). Leukocytes have only a finite life-span at the inflammation site. Neutrophils rapidly undergo apoptosis to be cleared by inflammatory macrophages, which themselves emigrate from the inflamed site during the resolution phase. Thus, a successful inflammatory event requires not only appropriate activation of cells and mediators with subsequent phagocytosis and

removal of the exciting stimulus, but also a consequent elimination of the inflammatory cells and debris to allow the tissues to reform a normal architecture and function.

LIPOPOLYSACCHARIDE (LPS) SIGNALING

The molecular mechanisms by which LPS induces gene activation, and hence inflammatory mediator expression, have been reviewed recently [15]. LPS of Gram-negative organisms induces macrophages to secrete cytokines, which in turn activate T, and B cells to upregulate the adaptive immune responses. Toll-like receptor 4 (TLR4) is the LPS receptor and its stimulation induces nuclear factor κ B (NF- κ B) activation. The activation of NF- κ B involves phosphorylation and degradation of I κ B, an inhibitor of NF- κ B, which allows the translocation of NF- κ B heterodimers to the nucleus to bring about its action. The NF- κ B/I κ B system exerts transcriptional regulation on proinflammatory genes encoded for various adhesion molecules and cytokines. Most of these genes have functional NF- κ B binding elements in their promoter regions. Activation of NF- κ B leads to the induction of NF- κ B binding elements in their promoter regions. Activation of NF- κ B leads to the induction of NF- κ B dependent effector genes such as TNF, IL-1, chemokines, adhesion molecules and also ROS production. All these biologically active molecules/factors produce modifications in blood flow, and aggregation of neutrophils, and platelets. This results in damaged endothelium and also coagulation abnormalities often seen in patients with sepsis and septic shock. Therefore, NF- κ B is reported to be an O₂ sensor in LPS-induced endotoxemia. A schematic representation of the LPS/NF- κ B pathway is illustrated in (Fig. 1).

Accordingly, there is a need to improve our understanding of I κ B degradation and for the identification of modulators of this degradation process for use in treating diseases associated with activation of NF- κ B. Some patent compounds satisfy these needs and further provide other related advantages by using modulating agents that comprise a recognition domain for E3 ubiquitin ligase, a necessary factor for ubiquitin-controlled I κ B degradation [16,17].

In addition, some patents have been developed in order to counteract the effect of other important mediator in sepsis, TNF. For example, [18] shows that anti-TNF antibodies, fragments and regions thereof, which are specific for human TNF, are useful for *in vivo* diagnosis and therapy of a number of TNF-mediated pathologies and conditions. Further, polynucleotides coding for murine and chimeric antibodies, methods of producing the antibody, methods of use of the anti-TNF antibody, or fragment, region or derivative thereof, in immunoassays and immunotherapeutic approaches are described.

FREE RADICALS AND ANTIOXIDANTS IN SEPSIS

The pathogenetic sequence of reactions mediated by endotoxin (LPS) leading to the development of sepsis involves ROS [12-13,19]. Among ROS, \cdot OH either singly or in combination with ONOO \cdot , leads to tissue damage often observed during septic injury. Inactivation of these damaging radicals by antioxidants or NO inhibitor(s) may be helpful

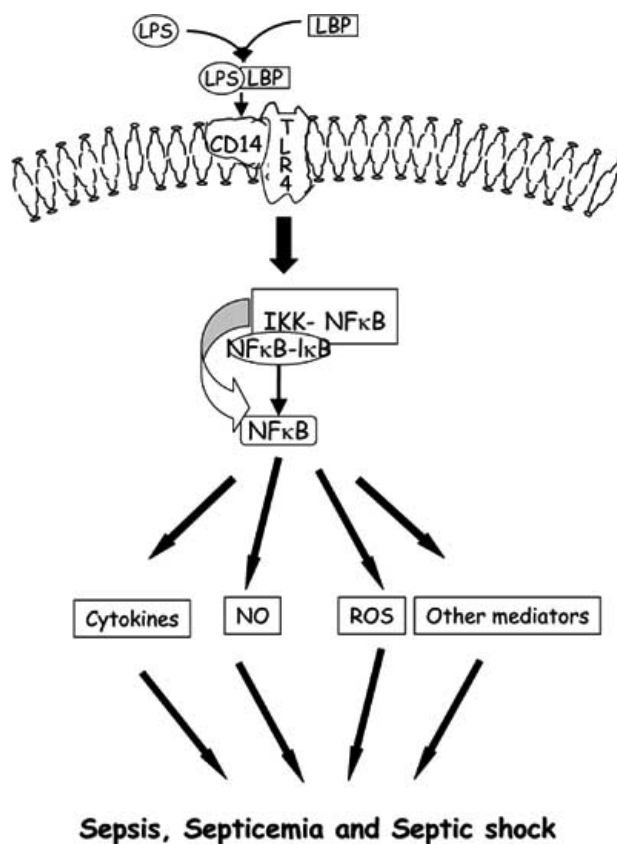


Fig. (1). Schematic diagram of LPS-induced signalling pathway of the host inflammatory response in monocytes, macrophages and neutrophils. For a detailed explanation of this figure see text. NF- κ B, nuclear factor κ B; LPS, lipopolysaccharide; LBP, LPS-binding protein; CD14, CD14 surface receptor; TLR4, Toll like receptor 4; IKK-NF- κ B, I κ B kinase-NF κ B pathway; NF- κ B-I κ B, NF- κ B-inhibitory protein I κ B; complex MAPK, mitogen-activated protein kinase pathway.

for protecting sepsis mediated derangements, but the application of these agents as drugs in humans has not been fully successful.

The sources of ROS during sepsis are:

- (1) the mitochondrial respiratory chain.
- (2) the metabolic cascade of arachidonic acid.
- (3) the protease-mediated enzyme xanthine oxidase.
- (4) granulocytes and other phagocytes activated by complement, bacteria, endotoxin, lysosomal enzymes, etc.
- (5) Other oxidases mainly NADPH oxidase.

Under normal physiological conditions, the majority of ROS are formed during cellular respiration and by activated phagocytic cells, including neutrophils, involved in the inflammatory response. ROS have physiologically essential roles in mitochondrial respiration, prostaglandin production pathways and host defense [20]. The electron reduction of O₂ occurs in the mitochondrial electron transport system of all aerobically respiring cells. The enzyme catalyzing this

reaction (cytochrome c oxidase or Complex IV) contains the transition metals iron and copper in its active site. These ions can be paramagnetic and contain stable unpaired electrons. By using the unpaired electrons in these transition metals to control the O₂ reactions, mitochondria prevent the unwanted release of ROS.

In sepsis, there are several potential sources of ROS, including the mitochondrial respiratory electron transport chain, xanthine oxidase activation as a result of ischemia and reperfusion, the respiratory burst associated with immune cell activation, arachidonic acid metabolism and NADPH oxidase. In fact, activated immune cells produce O₂⁻ as a cytotoxic agent as part of the respiratory burst *via* the action of membrane-bound NADPH oxidase on O₂.

The increase of ROS after LPS challenge has been demonstrated in different models of septic shock in peritoneal macrophages and lymphocytes [12-13,19]. This disturbance in the balance between pro-oxidants (ROS) and antioxidants in favor of the former is characteristic of oxidative stress in immune cells in response to endotoxin. In this context, a typical behavior of these cells under an oxidative stress situation implies changes in different immune functions such as an increase in adherence and phagocytosis and a decrease in chemotaxis [12-15,19].

Neutrophils play a crucial role in the primary immune defense against infectious agents. They have several well-established functions, which include phagocytosis and the production of ROS. However, in addition to the microbicidal activity, neutrophils can also damage the host by disrupting tissue integrity and function. In fact, neutrophils are activated and increased in number during sepsis as a means to control infection, but may, also be destructive to the tissue. Because of this dual role, targeting therapies for sepsis may involve inhibiting or enhancing neutrophil function.

Neutrophils and macrophages also produce NO, which can react with O₂ to produce peroxynitrite (ONOO⁻), itself a powerful oxidant, which may decompose to form HO[•]. Under ischemic conditions followed by subsequent reperfusion, the enzyme xanthine oxidase catalyzes the formation of uric acid with the co-production of O₂⁻, whose release results in the recruitment and activation of neutrophils and their adherence to endothelial cells.

During oxidative stress, damage mediated by ROS can occur. Oxidation of DNA and proteins takes place, along with membrane damage due to lipid peroxidation, leading to alterations in membrane permeability, modification of protein structure and functional changes [21].

ANTIOXIDANT DEFENSES

Antioxidants are central to the redox balance in the human body. They do not act in isolation, but synergistically with other classes of molecules. Primary antioxidants prevent oxygen radical formation, by either removing free radical precursors or by inhibiting catalysis, e.g. the enzymes glutathione peroxidase and catalase. Secondary antioxidants react with ROS which have already been formed, either to remove or inhibit them, e.g. vitamins C and E. Endogenous

antioxidant defenses exist in a number of locations, namely intracellularly, on the cell membrane and extracellularly [5].

The immune system is highly reliant on accurate cell-cell communication for optimal function, and any damage to the signaling systems involved will result in an impaired immune responsiveness. Oxidant-mediated tissue injury is a particular hazard to the immune system, since phagocyte cells produce ROS as part of the defense against infection. Therefore, adequate amounts of neutralizing antioxidants are required to prevent damage to the immune cells themselves. For example, vitamin A deficiency can affect the function of different cells in the immune system. Different studies have reported defects in phagocytic activity (defect in chemotaxis, adhesion and the ability to generate ROS in neutrophils) and impairment of T and B cell function. In general, improvement of immune function and increased resistance to infection is observed in vitamin A-deficient hosts after supplementation [22].

Several patents have described antioxidant treatment in order to improve immune function by diet before or during oxidative stress situations such as:

U.S. Pat. No. 4,981,844 [23] discloses a method of improving the immune response in patients comprising the ingestion of a diet that provides 20-60 kilo calories per kg of patient body weight and wherein 20-80% of the calories are derived from linoleic acid. The Alexander *et al.* patent also teaches the consumption of from 100-1000 IU per day of vitamin E.

U.S. Pat. No. 5,556,644 [24] discloses a multi-nutrient nutritional supplement designed to be effective in increasing immunity and decreasing the instances and severity of infection among the elderly. This patent specifically teaches the consumption of a nutritional supplement having recited levels of various vitamins and minerals. The patent more specifically teaches the consumption of the nutritional supplement by the elderly to improve their immunological status.

U.S. Pat. No. 5,444,054 [25] discloses a nutritional product for patients suffering from ulcerative colitis or inflammation of the colon. The nutritional product utilizes an oil blend containing specified fatty acids and a source of indigestible carbohydrate. The indigestible carbohydrate is disclosed as being metabolized to short-chain fatty acids by microorganisms present in the human colon.

U.S. Pat. No. 5,223,285 [26] discloses a liquid nutritional product that contains a specific lipid blend for pulmonary patients. This patent discloses that the lipid should have a particular ratio of n-6 to n-3 fatty acids. Further, this reference describes a nutritional product containing quantities of nutrients having antioxidative properties *in vivo*. Examples of such antioxidative nutrients include - carotene, vitamin E, vitamin C, selenium and taurine.

U.S. Pat. No. 4,871,768 [27] describes a dietary supplement that contains a structured glyceride comprising n-3 fatty acids and medium chain fatty acids. This patent describes synthetic triglycerides or structured lipids that provide a high energy fat source and fatty acids that assist in fighting infection. This patent also describes a method of minimizing the effects of infection and minimizing the

effects of subsequent infection by administering a diet containing 10-80% by weight of an oil-based fraction comprising glycerol, fatty acids and combinations thereof, wherein 50-90% of the fatty acids are caprylic acid, capric acid or mixtures thereof and 10-50% by weight of n-3 fatty acids. This reference teaches that the dietary supplement will not prevent the onset of infections, however, it will promote survival of infected patients. This patent fails to suggest that stress-induced down regulation of the immune system can be lessened by a nutritional composition that comprises (1) a structured glyceride; and (2) an antioxidant system comprising of at least vitamin E, vitamin C, selenium and β -carotene. Other patents have included similar compounds in order to treat diabetes or other pathologies [28].

U.S. Pat. No. 4,607,052 [29] describes triglycerides wherein specific polyunsaturated acyl fragments are present at the sn-2 position of the glycerol molecule. The structured lipids of Mendy *et al.* are described as being useful for the treatment of lipid digestion problems, metabolic diseases, nutritional deficiencies, hypertension and in conditions where immune modulation is desired. There is neither teaching nor suggestion in the Mendy *et al.* patent that a structured glyceride, when combined with a specific antioxidant system, would be effective in reducing the immunosuppression typically seen in animal models subjected to stress.

De Michele *et al.* [30] describes a patent directed to the discovery of immunonutritional products that are useful in reducing the immunological system suppression that results from stress. The stress may be in the form of physical exertion, mental exhaustion, disease states and the like. In one embodiment, the invention relates to a nutritional composition comprising a structured glyceride component and an antioxidant system. This nutritional composition has been shown to be highly effective in reducing immune system downregulation or dysregulation as a result of stress.

As previously described, the SOD enzymes are a family of metalloenzymes which rapidly promote the conversion of O_2^- to H_2O_2 . Three forms of SOD are recognized to be important: copper-zinc SOD (cytoplasmic-located), manganese SOD (mitochondrial-located) and extracellular SOD (extracellular matrix-located). Catalase and glutathione peroxidase, a selenium containing enzyme which requires the presence of reduced GSH for its action, both catalyze the conversion of H_2O_2 to H_2O . GSH also has direct antioxidant activity, through donation of hydrogen ions, to repair damaged DNA. Oxidative stress and modulation on GSH/GSSG (GSSG=oxidized GSH) levels also up-regulate gene expression of several other antioxidant proteins, such as manganese SOD, glutathione peroxidase, thioredoxin (Trx) and metallothionein.

The hydrophobic lipid interior of membranes require a different spectrum of antioxidants. Fat-soluble vitamin E (α -tocopherol) is the most important antioxidant in this environment. β -carotene, lycopene and co-enzyme Q have also been implicated as membrane antioxidants. Lipid-soluble antioxidants are important in preventing membrane polyunsaturated fatty acids from undergoing lipid peroxidation, which leads to loss of membrane integrity.

ROS may also be present in the extracellular compartment, especially as a result of neutrophil activation. The plasma and red cell components of blood both act as antioxidants; red cells have a copper-zinc SOD-dependent pathway for the inactivation of O_2^- , and catalase and glutathione peroxidase for dealing with H_2O_2 . A number of metal-binding plasma proteins function as valuable antioxidants in addition to their transport roles, including apotransferrin, lactoferrin and caeruloplasmin. Albumin is also effective *via* its oxidizable thiol group, which permits radical scavenging, and the binding of reactive transition metal ions. A number of important smaller molecules are present in the plasma, which act as secondary antioxidants. These include vitamin E, vitamin C, uric acid and bilirubin. Vitamin C interacts with O_2^- to form dehydroascorbic acid. Vitamin C may also reduce Fe^{3+} to Fe^{2+} , which can then be involved in iron-catalyzed hydroxyl generation, thereby implicating vitamin C as both pro-oxidant and an antioxidant. Vitamin C exerts anti-inflammatory effects in human and animal studies. Vitamin E is an important nutrient for maintaining the immune system, and plays a role in the differentiation of immature T cells in the thymus.

Many groups have investigated the possibilities of quenching ROS production in different pathologies which involve oxidative stress. In this regard, there are several approximations to modulate arachidonic acid metabolism. For example, the compounds heterocyclo-alkylsulfonyle pyrazoles and sulfonyl heteroaryl triazoles have demonstrated anti-inflammatory/analgesic properties [31]. Both compounds are reported to control cyclooxygenase (COX) in different pathogenic situations.

Other approximations to control free radical production, include compounds such as hydroxyguanidines which control the enzyme xanthine oxidase in different pathogenic situations that include sepsis as well as ischemic diseases, hypoxia or arrhythmias [32].

In reference to another important source of free radicals, NADPH oxidase, there are several patents which have demonstrated excellent results to modulate this enzyme such as *o*-methoxycatechol (apocynin) [33], as well as in another method [34], another patent has demonstrated that compositions derived from low molecular weight GTP-binding proteins (LMWG), mastaparan, GAP proteins, and related peptides can regulate the oxidative burst [35]. This method contemplates compositions useful in inhibiting activation of NADPH oxidase or in promoting GDP/GTP exchange and therefore inhibits O_2^- production in phagocytic cells, reducing the severity of inflammation and inflammatory disorders.

EFFECTS OF NITRIC OXIDE

NO is synthesized from L-arginine by different isoenzymes of (NOS) [36], and is implicated in a wide range of disease processes, exerting both detrimental and beneficial effects at the cellular and vascular levels. To date, three main isoforms of NOS are known: neuronal NOS (NOS-1 or nNOS), inducible NOS (NOS-2 or iNOS), and endothelial NOS (NOS-3 or eNOS).

NO has been shown to play a key role in the pathogenesis of septic shock [37]. Hyperproduction of NO induces

excessive vasodilation, changes in vascular permeability, and inhibition of noradrenergic nerve transmission, all characteristics of human septic shock.

The recognition of NO production by activated macrophages as part of the inflammatory process was an important milestone for assessing both the biological production of NO and the phenomenon of induction of NOS activity [38]. The observation has been extended to neutrophils [39], lymphocytes [40], and other cell types. The role of NO in the pathophysiology of endotoxic shock was advanced by Thiemermann and Vane [41], who observed that administration of the specific NOS inhibitor *N*-methyl-L-arginine (L-NMMA) decreased the severe hypotension produced by administration of LPS. Other groups simultaneously reported similar results indicating that endotoxin increases NO production [42,43] and prompted the idea that pharmacological inhibition of NOS may be useful in the treatment of inflammation and septic shock [44]. However, clinical trials using L-NMMA failed to show a beneficial effect in septic shock patients [45]. The major limitation for the use of NOS inhibitors in clinical studies is the development of pulmonary hypertension as a side effect of NOS blockade, which can be alleviated by the use of inhaled NO. However, several compounds which modulate NO synthesis have been patented in recent years, such as various inflammatory mediators that have been implicated in the induction and activation of iNOS, particularly IFN γ , TNF α , IL-1 β , and platelet-activating factor (PAF) alone or synergistically [46,47]. In addition to the activation of iNOS, cytokines and endotoxin may increase NO release by increasing arginine availability through the opening of the specific Ca^{2+} channels and the expression of the cationic amino acid transporter (CAT), or by increasing tetrahydrobiopterin levels, a key cofactor in NO synthesis. Several experimental studies have demonstrated a decrease in NOS activity resulting in an impairment in endothelial-dependent relaxation during endotoxemia and experimental sepsis [48], possibly as the result of a cytokine- or hypoxia-induced shortened half-life of NOS mRNA [49], or of altered calcium mobilization [50]. Other investigators have shown increased endothelial NO release immediately after endotoxin administration either directly [51] or indirectly as suggested by the lack of effect of a specific iNOS inhibitor on the initial hypotension after endotoxin administration.

NO exerts *in vitro* toxic effects including nuclear damage, protein and membrane phospholipid alterations, and the inhibition of mitochondrial respiration in several cell types. Mitochondrial impairment could also be considered as an adaptive phenomenon, decreasing cellular metabolism when the energy supply is limited. The relevance of mitochondrial impairment is, however, questionable, as administration of the NO donor SIN-1 in a canine model of endotoxic shock increased O_2 extraction capabilities [52]. The toxicity of NO itself may be enhanced by the formation of ONOO $^-$ from the reaction of NO with O_2^- [53]. Therefore, the multiple organ failure syndrome (MOFS) that often accompanies severe sepsis may be related to the cellular effects of excess NO or ONOO $^-$. In contrast, NO may protect cells from oxidative damage by scavenging ROS [54].

NO has effects in vascular tone. During sepsis and endotoxemia, convincing data suggest a pivotal role for NO in the endotoxin-induced [51] vasodilation and vascular hyporeactivity to vasoconstrictors. Both pharmacological inhibition and iNOS gene deficiency are associated with a loss of endotoxin-induced vasodilation [55]. The increased NO release has also been implicated in the diminished response to vasoconstrictors [56].

Some patents have been designed in order to modulate the levels of NO and pro-inflammatory cytokines, such as described by Singh *et al.* [57]. This invention first provides a method for suppressing the induction of iNOS and/or proinflammatory cytokines in a cell comprising contacting said cell with an effective amount of at least one induction suppressor and/or inhibitor of iNOS. Preferred cells throughout the various embodiments of the invention are lymphocytes, macrophages, endothelial cells, astrocytes, mesangial cells, myocytes, Kuffer cells, epithelial cells, microglia, oligodendrocytes and neurons. Proinflammatory cytokines that are preferred include TNF- α , IL-1 β , IL-2, IL-6, IL-8 and IFN γ . Inhibition of NO cytotoxicity includes inhibition of iNOS activity, production of iNOS protein, production or translation of iNOS mRNA, and inhibition of LPS- or cytokine-induced NF- κ B activation in a cell. In preferred aspects of the invention, the induction suppressor and/or inhibitor of iNOS and/or proinflammatory cytokines may be selected from the group including, but not limited to, lovastatin, mevastatin, FPT inhibitor II, forskolin, rolipram, phenylacetate (NaPA), N-acetyl cysteine (NAC), pyroldine dithiocarbamate (PDTC), 4-phenylbutyrate (4PBA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), theophylline, papaverine, cAMP, 8-bromo-cAMP, (S)-cAMP, and salts, analogs, or derivatives thereof.

Other patents have described amidino compounds useful as NOS inhibitors in inflammation disorders [47]. Apart from this, the following individual publications disclose compounds that inhibit NO synthesis and preferentially inhibit the iNOS [58-64].

INVOLVEMENT OF NITROGEN SPECIES

ROS and reactive nitrogen species (RNS) play an important role in inflammatory processes as mediators of injury. RNS is a collective name that includes NO, ONOO $^-$, nitrogen dioxide radical (NO $_2^{\cdot}$), other oxides of nitrogen and products arising when NO reacts with O_2^- , RO $^{\cdot}$ and RO $_2$. NO reacts rapidly with ferrous iron, and at physiological concentrations, NO also binds to soluble guanylate cyclase and to another hemoprotein, cytochrome c oxidase (Complex IV), the terminal enzyme of the mitochondrial respiratory chain. NO can therefore control cellular functions *via* the reversible inhibition of respiration [65]. There are a number of reactive NO species, such as N $_2$ O $_3$ and ONOO $^-$ that can also alter critical cellular components.

During the first hours after injury, iNOS-mediated NO production is upregulated, producing a burst of NO that far exceeds basal levels. This overabundance of NO produces significant cellular injury *via* several mechanisms. First NO may directly promote overwhelming peripheral vasodilation, resulting in vascular decomposition; second, NO may upregulate the transcription NF- κ B initiating an inflamma-

tory signaling pathway that, in turn, triggers numerous inflammatory cytokines [66]. NO also interacts with the O_2^- to yield ONOO $^-$, a highly reactive compound that exacerbates the injury produced by either O_2^- alone or NO alone [67]. These investigators have proposed that the ONOO $^-$ generation which occurs during fluid resuscitation in the injured subject produces cellular death by enhancing DNA single strand breakage, activating the nuclear enzyme polyADP ribose synthetase (PARS), which in turn leads to cellular energy depletion and cellular necrosis [68]. The detrimental effects of ONOO $^-$ in shock and resuscitation have been attributed to oxidation of sulfhydryl groups, the nitration of tyrosine, tryptophane, and guanine, as well as inhibition of the membrane sodium-potassium adenosine triphosphatase. In addition, PARS activation depletes NAD and thus alters electron transport, ATP synthesis, and glycolysis; more recently, studies have shown that PARS activates the caspase cascade, which results in DNA fragmentation and cellular apoptosis [66,69].

The activation of monocytes, macrophages and endothelial cells by LPS results in the expression of iNOS, and consequently increases the transformation of L-arginine to NO, which can combine with O_2^- to form ONOO $^-$, causing tissue injury during shock, inflammation and ischemia-reperfusion [70]. NO also stimulates H_2O_2 and O_2^- production by mitochondria [71], possibly by inhibiting COX, thus increasing leakage of electrons from the respiratory chain. H_2O_2 , in turn, participates in the upregulation of iNOS expression *via* NF κ B activation [72]. Cultured astrocytes and macrophages expressing iNOS produce up to 1 μ M NO, and so it is possible that catalase in these cells could be inhibited. Indeed, iNOS expression in activated astrocytes leads to increased H_2O_2 levels [73], as well as inhibition of cellular respiration at the level of COX [74]. ONOO $^-$ has also been shown to stimulate H_2O_2 production by isolated mitochondria [75]. Paradoxically, NO can also decrease ROS-produced damage that occurs at physiological levels of NO [76]. The high reactivity of NO with radicals might be beneficial *in vivo*, for example, by scavenging peroxyl radicals and inhibiting peroxidation. Additionally, it has been suggested that ONOO $^-$ may also be a signal transmitter and can mediate vasorelaxation, similarly to NO [77]. Apart from ONOO $^-$, other RNS such as nitrogen oxides and nitryl chloride are believed to cause several different pathophysiological events, including inflammation [78].

Inflammatory reactions play an important role in endotoxin-induced tissue injury. The latter is mediated by adhesion and migration of leukocytes through the endothelium, generation of ROS and RNS [79], and release of several proinflammatory cytokines by monocytes/macrophages. Local generation of RNS contributes to tissue injury. Recent studies have demonstrated that activation of the nuclear enzyme poly(ADP-ribose) polymerase-1 by RNS-mediated DNA damage is an important pathway of tissue injury in conditions associated with oxidative stress [80]. Increased formation of RNS in response to endotoxin challenge is organ specific [81].

Some authors have described different methods to avoid the harmful effects of ONOO $^-$. For example Szabo *et al.* [82] have shown that mercaptoethylguanidine and guanidine

inhibitors of NOS react with ONOO $^-$ and protect against ONOO $^-$ -induced oxidative damage.

CARDIOVASCULAR DYSFUNCTION

There is mounting evidence for involvement of oxidative stress in the mechanism of cardiovascular disease (stroke, congestive heart failure, hypertension and coronary artery disease). Among the most numerous causes of mortality is cardiac arrest. In relation to the heart, a principal deleterious influence is arrhythmia, resulting from toxic action on membranes that are concerned with cardiac conduction.

Impaired myocardial contractile function is a well-documented feature in both experimental and human septic shock [83] that greatly contributes to the mortality associated with this pathological condition [84]. Several hypotheses have been proposed to explain endotoxin-induced cardiovascular failure, including microvascular dysfunction, the presence of activated leukocytes, and the effects of various circulating and/or locally produced proinflammatory cytokines, such as TNF and IL-1 [85]. Most of the deleterious effects of proinflammatory cytokine release have been attributed to the production of large amounts of NO, which may exert detrimental effects on the myocardium in animal models, isolated hearts, and isolated cardiac myocytes [86]. Deleterious effects of NO have been mainly reported in biological conditions in which ROS generation is also enhanced [87]. The relevance of these biological events in mediating myocardial dysfunction is further illustrated by the finding that antioxidant therapy [79] and inhibition of NO synthase activity can improve endotoxin and cytokine-induced contractile dysfunction [79,88].

In the cardiovascular system, endothelium, which separates the vascular wall from the circulation and the blood components, plays a key role, because it regulates vascular homeostasis by elaborating a variety of factors that act locally in the blood vessel wall and lumen, such as NO, prostacyclin and endothelin. Endothelium, in fact plays important roles in maintaining a normal vascular tone and blood fluidity, reduced platelet activity and leukocyte adhesion, and limiting vascular inflammatory reactions.

In sepsis, NO may exert direct and indirect effects on cardiac function. Sustained generation of NO occurs in systemic inflammatory reactions, such as septic shock with involvement in circulatory failure. In fact, myocardial iNOS activity has been reported in response to endotoxin and cytokines and inversely correlated with myocardial performance [89].

Recently, several studies have added to the confusion surrounding the role of NO by demonstrating no effect of NO or NOS inhibition on the myocardium or on α -adrenergic responsiveness [90]. Nevertheless, in most studies, low-to-moderate doses of iNOS inhibitors restore myocardial contractility in hearts exposed to proinflammatory cytokines, whereas at higher doses, the effects are reversed. This finding may indicate that small amounts of NO produced by iNOS may be necessary to maintain contractility [90] and can be cardio-protective in experimental sepsis [91].

Systemic sepsis is associated with cardiovascular dysfunction through its effects on the myocardium, endothelium and vascular smooth muscle [92]. Moreover, during septic shock, ventricular function is altered despite normal or increased cardiac output.

The number of endogenously produced inflammatory mediators which can potentially contribute to septic myocardial depression is extensive. The list includes arachidonic acid metabolites, PAF, histamine, and endorphins. Some evidence also suggests that ROS may increase the left ventricular diameter and decrease left ventricular contractility [93].

The most proposed mechanism implicated in the pathogenesis of endothelial dysfunctions is related to an increased production of ROS. This would deplete the bioavailability of NO and exacerbate local oxidative stress by directly reacting with NO to form ONOO⁻, which in turn, would further sustain an oxidative injury to the endothelium.

In human neutrophils, ONOO⁻ triggers the down-regulation of L-selectin expression, and upregulation of CD11/CD18 expression [94]. These effects are likely to be mediated by the ability of ONOO⁻ to trigger nuclear factor NF- κ B activation [95]. The development of ONOO⁻ neutralizers has provided a more direct approach to assess the role of ONOO⁻ in organ injury in a variety of inflammation states [82]. For example, mercaptoethylguanidine (MEG), a ONOO⁻ scavenger and iNOS inhibitor [82] and 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrinato iron (III) (FeTPPS), which catalyzes the isomerization of ONOO⁻ to nitrate anion [96], may decrease the generation of highly reactive intermediates such as nitrogen dioxide and OH

radicals. Besides, ONOO⁻ neutralizers may attenuate inflammatory processes associated with ischemia reperfusion [97], and infection [98]. Future investigations are necessary to see the effects in endotoxin-induced cardiovascular inflammation.

ROLE OF OXIDATIVE STRESS IN HEART FAILURE

The pathological mechanism of heart failure is complex and involves the activation of numerous secondary pathways (cytokines, oxidative stress and nitrosative stress) (Fig. 2), which leads to: (i) abnormalities in various signaling processes, cardiac receptors and Ca²⁺ homeostasis; (ii) contractile protein desensitization; and (iii) endothelial dysfunction.

Experimental and clinical studies demonstrate increased production of ROS in the pathogenesis of acute heart failure. In fact, malondialdehyde (a marker of lipid peroxidation), is increased in patients with ischemic cardiomyopathy.

The generation of ROS in the myocardium is triggered by ischemia and reperfusion and by exposure of the heart to inflammatory cytokines. Source of ROS in failing myocardium includes, among others, xanthine and NADPH oxidoreductases, the mitochondrial electron transport chain and activated neutrophils [99].

The generation of ONOO⁻ has been demonstrated in acute heart failure. In fact, the pathogenesis of endothelial dysfunction associated with acute heart failure involves enhanced oxidative stress from various local sources. A potential additional source of ROS is iNOS, which, paradoxically, can produce O₂⁻.

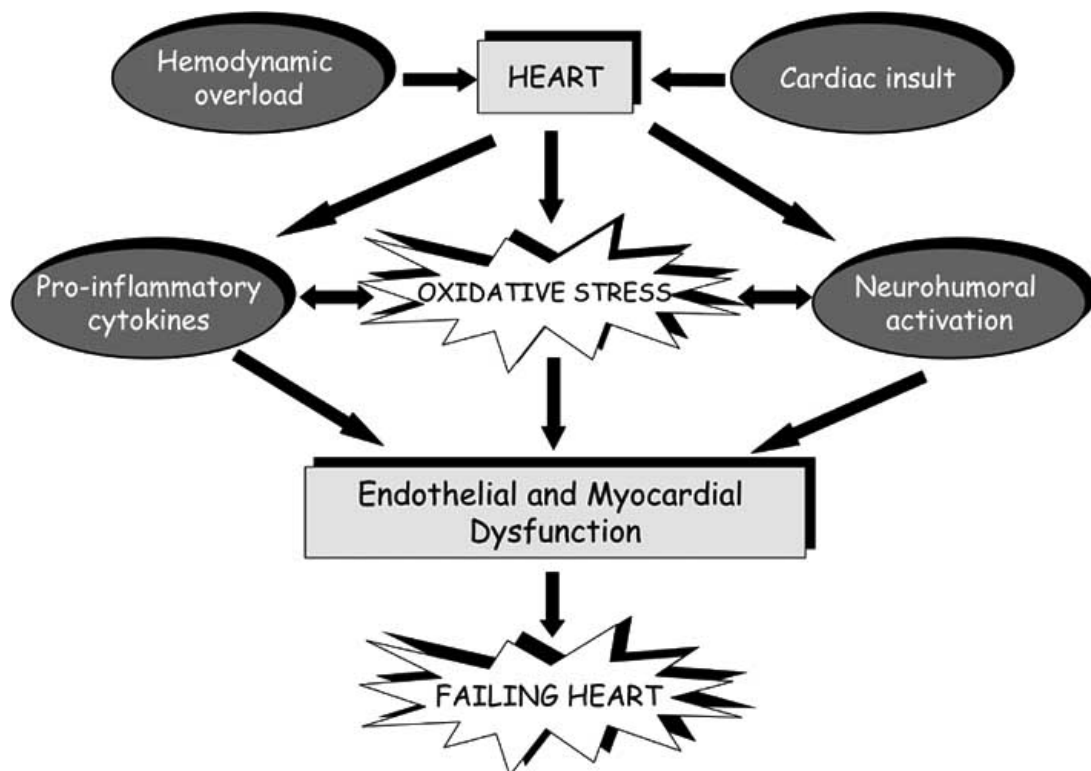


Fig. (2). Progression of heart failure and the role of oxidative and nitrosative stress.

There are several mechanisms of ONOO⁻-induced cardiac and vascular dysfunction (Fig. 3). For example, ONOO⁻-induced tyrosine nitration can lead to dysfunctional nitrated proteins, whereas ONOO⁻-induced oxidation of sulfhydryl groups can inhibit important enzymes in the mitochondrial respiratory chain. Furthermore, ONOO⁻-modified cellular proteins are subject to accelerated degradation via the proteasome and ONOO⁻ inhibits a variety of ion pumps [100]. ONOO⁻ also potently oxidizes various molecules including BH₄, a crucial cofactor of NOS, to quinoid 5,6-dihydropterin. The generation of ONOO⁻ also decreases the availability of NO for G-protein stimulation and vasodilation, thus further contributing to endothelial dysfunction.

In addition, ONOO⁻ can inhibit SOD, glutaredoxin and other antioxidant molecules and systems, which leads to positive feedback cycles of intracellular oxidant generation and oxidative injury [100]. ONOO⁻ can also activate matrix metalloproteinases (MMPs) [101] and the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [102]. ONOO⁻ also triggers the expression of P-selectin, ICAM-1 and Mac-1, and mediates the cytokine-induced expression of IL-8 in human leukocytes via enhancement of NF-κB activation [100], thereby promoting proinflammatory responses.

Some patents have been designed in order to modulate cardiovascular damage, by supplementing with antioxidants. For example, Zeng *et al.* (2004) [103], describe an invention with antioxidant composition comprising the constituents of

-thioctacid and vitamin E, which prevents cardiovascular and cerebrovascular diseases. Other patents describe inventions which include an antioxidant cocktail designated to treat and prevent heart disease and stroke [104-106].

Besides heart failure, ROS and vascular oxidative stress have been involved in hypertension and atherogenesis. Compelling experimental evidence indicates that ROS play an important pathophysiological role in the development in atherogenesis and therefore in hypertension. This is due, in large, to oxidative stress and decreased NO bioavailability in the vasculature and to ROS-mediated cardiovascular remodeling.

Atherosclerosis, a chronic inflammatory disease of the arterial wall, is a major cause of morbidity and mortality from cardiovascular disease in much of the world's population. For this reason, we would like to mention some important patents in the treatment of these pathologies. For example, Morawalla *et al.* [107] describes the medicinal properties of *Terminalia arjuna* in alleviation of various health related disorders, showing cardio-protective principles. In other patent, De Simone [108] describes an antioxidant combination composition to protect from oxidative stress in cardiovascular damage. The composition includes: L-carnitine inner salt, acetyl L-carnitine inner salt, -lipoic acid, coenzyme Q10, vitamin E and selenomethionine.

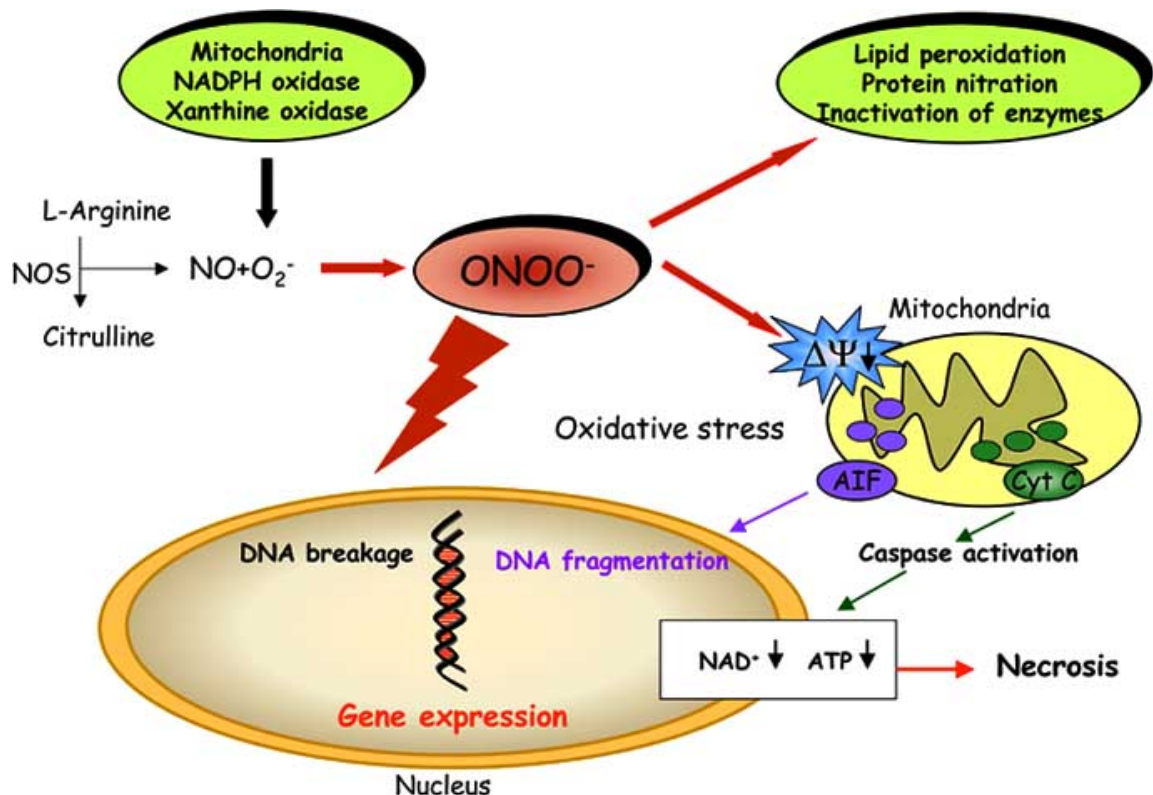


Fig (3). Role of oxidative stress in oxidant-induced cellular dysfunction and necrosis. Nitric oxide (NO) and superoxide (O₂⁻), induce cell damage *via* lipid peroxidation, inactivation of enzymes by oxidation and nitration. Peroxynitrite (ONOO⁻) also acts on mitochondria decreasing the membrane potential (ΔΨ), triggering the release of pro-apoptotic factors such as cytochrome c (Cyt c) and apoptosis-inducing factor (AIF). These factors mediate caspase-dependent and caspase-independent apoptotic death pathways, respectively. Moreover, peroxynitrite, causes strand breaks in DNA.

ANTIOXIDANT PROTECTION IN SEPSIS

It has been reported that sepsis increases markers of oxidative stress in different animal models and in humans. This effect is correlated with an imbalance of antioxidant amount [109]. The clear evidence for oxidative stress in sepsis and the link with inflammatory gene expression has provided a foundation for intervention to either reduce oxidative stress or inhibit transcriptional activation. For this reason, the protective role of antioxidants against endotoxin toxicity and their potential clinical use is discussed.

Endogenous and synthetic antioxidants, such as iron-chelating agents, *N*-acetyl-L-cysteine (NAC), apocynin, probucol, and edaravone, are useful in preventing cardiovascular injury and diseases. Endogenous antioxidants in the myocardium include vitamins C and E, cysteine and the enzymes, SOD, catalase and glutathione peroxidase. In cases where events such as ischemia and hypoxia have compromised the cardiac cell's natural defense mechanisms, synthetic antioxidants such as NAC, a thiol containing compound, have proved successful in protection against oxidative stress on reperfusion.

Secondary plant metabolites containing flavonoids and coumaric acid derivatives from spinach are effective against oxidative stress in the rat heart [79]. Targets for antioxidant therapy include NADPH oxidase, a source of O_2^- and peroxides.

GSH comprises more than 90% of the nonprotein low molecular weight reduced thiols in cells and is one of the most important intracellular mechanisms protecting normal cells from oxidative injury. GSH linked redox homeostasis controls the levels of ROS that are constantly formed during cellular metabolism. It is recognized that the antioxidant GSH plays a very important role in several biological processes, and is implicated in the modulation of immune function and inflammatory response [110].

One of the treatments to enhance the GSH pool is NAC supplementation, which, of course, also has direct antioxidant activity [111]. NAC has been used safely to treat acetaminophen overdose for two decades [112]. NAC shows an *in vitro* and *in vivo* stimulatory effect on the phagocytic process of macrophages, and controls the functions of lymphocytes [113-116]. The regulation of redox state by NAC in immune cells and in animals with endotoxic shock [117], can lead to decreased NF- κ B activation [118], TNF-release and increased survival [119].

In the clinical setting, NAC alone or in combination with other antioxidants has been shown to have variable results. An early acute study, in which NAC was administered in conjunction with vitamin C and α -tocopherol in patients with septic shock, did not measure NF- κ B activation, and neither total antioxidant capacity nor lipid peroxidation was changed [120].

Victor *et al.* [117] demonstrated that NAC treatment in animals with endotoxic shock decreased ROS, TNF, MDA levels, the GSSG/GSH ratio and iNOS levels, and increased the antioxidant defenses in peritoneal macrophages and lymphocytes. Moreover, NAC treatment prevented the

activation of NF- κ B, improved the redox state of these immune cells and increased survival.

Byrne *et al.* [121] have demonstrated the therapeutic antioxidant effects of glutamine and NAC composition. Compositions comprising glutamine in combination with other ingredients such as NAC are disclosed. Such compositions can be administered to treat patients with certain disease states. In fact, glutamine and other amino acids have been given to patients with immune-compromised states, both alone and in combination with an immunopotentiator such as levitinan, OK-432, or sizofiran [122]. Vitamin C is a powerful electron donor, reacting with both O_2^- and OH. Vitamin C plays an important role in the defence against oxidative damage especially in leukocytes. The main role of vitamin C in the organism is linked to its function as a reductor, but it also participates in the modulation of complex biochemical pathways which are an essential part of the normal metabolism of immune cells. This antioxidant also inhibits the activation of the nuclear transcription factor NF- κ B produced by endotoxin [123], which could result in a decrease of TNF. These results suggest that early administration of vitamin C may be a valuable adjunct treatment in sepsis. Although several investigations revealed no association between vitamin C intake and lower risk of coronary disease, high doses of this supplement are reported to improve vascular function in some cases.

Cardiovascular disease is the leading cause of death in the United States and many other countries. Nutritional factors are widely recognized as playing a role in preventing, delaying the onset of and/or slowing the progression of atherosclerosis and coronary heart disease. Attempts have been made in the prior art to design multivitamin supplements specifically for heart health. For example, U.S. Pat. No. 5,770,215 [124] discloses, a multivitamin composition containing various vitamins, minerals, and acetylsalicylic acid. Boulos *et al.* [125] have recently patented a multivitamin/multimineral optimized for cardiovascular health containing increased levels of Vitamin E and Folic Acid, low levels of iron, as well as containing mixed carotenoids.

There have been several studies which have reported low circulating vitamin E (α -tocopherol) levels in sepsis. Vitamin E has been shown to have several additional biologically important effects apart from its role as an antioxidant. These include the inhibition of arachidonic acid oxidative metabolism and the inhibition of protein kinase C (PKC) activity. A steady decrease in circulating α -tocopherol levels in plasma was reported in a pig septic shock model in both survivor and non-survivor animals. This was accompanied by a simultaneous increase of markers of oxidative injury in the animals which subsequently died [126]. Vitamin E has been shown to be protective in reducing the effects of oxidative stress in sepsis in a number of animal studies [127]. Taken together, these studies reveal vitamin E to be a potent immunomodulator [128] agent that can interrupt the activation of inflammatory cells at the level of signal transduction.

One strategy for preventing oxyradical-induced damage is to inhibit the formation of oxyradicals such as O_2^- . Iron

chelators, such as desferrioxamine (also called deferoxamine or Desferol) and others, inhibit iron ion-dependent OH generation and thus act as inhibitors of free radical formation [129]. Amino-steroid-based antioxidants such as the 21-aminosteroids termed "lazaroids" (e.g., U74006F) have also been proposed as inhibitors of ROS formation. Desferrioxamine, allopurinol, and other pyrazolopyrimidines such as oxypurinol, have also been tested for preventing oxyradical formation in a myocardial stunning model system and following hemorrhagic and endotoxic shock [130]. However, each of these compounds has notable drawbacks for therapeutic usage. For example, deferoxamine is not an ideal iron chelator and its cellular penetration is quite limited.

Another strategy for preventing oxyradical-induced damage is to catalytically remove oxyradicals such as superoxide once they have been formed. SOD and catalase have been extensively explored, with some success, as protective agents when added to reperfusates in many types of experiments or when added pre-ischemia [131]. The availability of recombinant SOD has allowed more extensive evaluation of the effect of administering SOD in the treatment or prevention of various medical conditions including reperfusion injury of the brain and spinal cord [132], endotoxemia [133], and myocardial infarction [134], and for osteoarthritis and intestinal ischemia [135]. SOD also has been reported to have positive effects in treating systemic lupus erythematosus, Crohn's disease, gastric ulcers, oxygen toxicity, burns patients, renal failure attendant to transplantation, and herpes simplex infection.

An alternative strategy for preventing oxyradical-induced damage is to scavenge oxyradicals such as O_2^- once these have been formed, typically by employing small molecule scavengers which act stoichiometrically rather than catalytically. Congeners of GSH have been used in various animal models to attenuate oxyradical injury. For example, *N*-2-mercaptopropionylglycine has been found to confer protective effects in a canine model of myocardial ischemia and reperfusion [136] and NAC ("Mucomyst") has been used to treat endotoxin toxicity in sheep [137]. Dimethyl thiourea (DMTU) and butyl-phenylnitron (BPN) are believed to scavenge OH, and have been shown to reduce ischemia-reperfusion injury in rat myocardium and in rabbits [138]. Mannitol has also been used as a free radical scavenger to reduce organ injury during reoxygenation [139]. Thus, application of inhibitors of oxyradical formation and/or enzymes that remove O_2^- and H_2O_2 and/or small molecule oxyradical scavengers have all shown promise for preventing reoxygenation damage present in a variety of ischemic pathological states and for treating or preventing various disease states associated with free radicals. However, each of these categories contains several drawbacks. For example, inhibitors of oxyradical formation typically chelate transition metals which are used in essential enzymatic processes in normal physiology and respiration; moreover, even at very high doses, these inhibitors do not completely prevent oxyradical formation. SOD and catalase are large polypeptides which are expensive to manufacture, do not penetrate cells or the blood-brain barrier, and generally require parenteral routes of administration. Free radical

scavengers act stoichiometrically and are thus easily depleted and must be administered in high dosages to be effective.

The complex formed between the chelator desferrioxamine and manganese has SOD activity and has shown some activity in biological models, but the instability of the metal ligand complex apparently precludes its pharmaceutical use. Porphyrin-manganese complexes have been shown to protect bacteria from paraquat toxicity and to promote the aerobic survival of SOD-deficient *E. coli* mutants.

Based on the foregoing studies, it is clear that a need exists for antioxidant agents which are efficient at removing dangerous oxyradicals, particularly O_2^- and H_2O_2 , and which are inexpensive to manufacture, stable, and possess advantageous pharmacokinetic properties, such as the ability to cross the blood-brain barrier and penetrate tissues. Such versatile antioxidants would find use as pharmaceuticals, chemoprotectants, and possibly as dietary supplements.

In fact, for example Malfroy-Camine *et al.* [140] describes synthetic catalytic free radical scavengers useful as antioxidants for prevention and therapy of disease. In this patent, they provide antioxidant salen metal complexes, compositions of such antioxidant salen-metal complexes having O_2^- activity, catalase activity, and/or peroxidase activity, compositions of salen-metal complexes in a form suitable for pharmaceutical administration to treat or prevent a disease associated with cell or tissue damage produced by free radicals such as O_2^- , and cosmetic and free radical quenching formulations of salen metal compounds.

Therefore, many groups of researchers have focused their attention on the biological activity of antioxidants and its active principles. Many scientific articles are published every year in different international journals related to the pharmacological properties of new antioxidants in order to counteract the effect of ROS in different pathologies. For example, Rao *et al.* have synthesized and used antioxidants such as (-)-mesquitol from natural sources (*Dichrostachys cinerea*), as well as (-)-Olivil from the plant *Stereospermum personatum*. Both compounds have demonstrated antioxidant activity [141,142]. Other studies have implicated mitochondria-targeted antioxidants which have also been shown to modulate the role of mitochondrial ROS production in redox signaling pathways [143].

CURRENT AND FUTURE DEVELOPMENTS

In an approach to design more optimally effective antioxidant regimes by targeting cells rather than normal systemic route, Michael Murphy *et al.* have recently embarked on a program of work to deliver antioxidant moieties (for example vitamin E and ubiquinone) specifically to the mitochondrion, the major site of oxidative stress [144]. By using a lipophilic cationic group as a carrier, to facilitate targeting to the highly charged mitochondrion, Murphy *et al.* have shown that in principle, this method is very effective at combating oxidative stress in a variety of *in vitro* scenarios such as NF B activation [145], Friedreich Ataxia complications [146], and stress-related telomere shortening [147]. The first *in vivo* demonstration (proof-of-principle) of mitochondrially-targeted antioxidants in the area of cardiac ischemia reperfusion was recently reported [148]. These successes now open the doorway for novel treatments for

clinical conditions in which oxidative stress plays a major role [149].

In this regard, the patent WO9926582A2 [150] relates to antioxidants having a lipophilic cationic group and to uses of these antioxidants, for example, as pharmaceuticals. This can be to prevent the elevated mitochondrial oxidative stress associated with particular diseases, or diseases associated with mitochondrial DNA mutations. Accordingly, they have the potential to be used as an adjunct to cell transplant therapies for e.g. neurodegenerative diseases, as a means to increase survival rates of the implanted cells. In addition, these compounds could be used as prophylactics to protect organs during transplantation, or ameliorate the ischemia-reperfusion injury that occurs during surgery. The compounds of the invention could also be used to reduce cell damage following stroke and heart attack. As these therapies use the selectivity of mitochondria, one can foresee that a major advantage over current antioxidant therapies is that the antioxidant is taken to the cellular compartment under the greatest oxidative stress. It is thought that this should increase the efficacy of the therapy.

In conclusion, antioxidants are involved in several important biological processes such as immunity, protection against tissue damage, reproduction, growth and development. Antioxidants preserve adequate function of cells against homeostatic disturbances such as processes involving oxidative stress such as septic shock and cardiovascular dysfunction. Many groups of researchers have focused their attention on the biological activity of antioxidants and its active principles. The protective role of antioxidants against homeostatic disturbances such as those caused by endotoxin toxicity and cardiovascular damage have been patented, their potential clinical use and the effects on the redox state of have been discussed.

Although there are a large number of patents which describe beneficial effects of antioxidant treatment in several oxidative stress disorders, in general, the results obtained so far are slightly disappointing. From our point of view, the use of novel targeted antioxidants, such as those described by Murphy *et al.* appears to be an exciting new leap into the use of antioxidant therapy.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

REFERENCES

- [1] Drogue W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002; 82: 47-95.
- [2] Kehrer JP. Free radicals as mediators of tissue injury and disease. *Crit Rev Toxicol* 1993; 23: 21-48.

- [3] Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol* 1999; 39: 67-101.
- [4] Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol* 2002; 30: 620-50.
- [5] Gutteridge JM, Mitchell J. Redox imbalance in the critically ill. *Br Med Bull* 1999; 55: 49-75.
- [6] Kuhn MA. Oxygen free radicals and antioxidants. *Am J Nurs* 2003; 103: 58-62.
- [7] Lunec J, Holloway KA, Cooke MS, Faux S, Griffiths HR, Evans MD. Urinary 8-oxo-2'-deoxyguanosine: redox regulation of DNA repair *in vivo*?. *Free Radic Biol Med* 2002; 33: 875-85.
- [8] McDermott JH. Antioxidant nutrients: current dietary recommendations and research update. *J Am Pharm Assoc* 2000; 40: 785-99.
- [9] Halliwell B. Oxidation of low-density lipoproteins: questions of initiation, propagation, and the effects of antioxidants. *Am J Clin Nutr* 1995; 61:670-7.
- [10] Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic Res* 1996; 25: 57-74.
- [11] Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and ageing. *Free Radic Biol Med* 2000; 29: 222-30.
- [12] Victor VM, Miñano M, Guayerbas N, Del Rio M, Medina S, De la Fuente M. Effects of endotoxin shock in several functions of murine peritoneal macrophages. *Mol Cell Biochem* 1998; 189: 25-31.
- [13] Victor VM, De la Fuente M. Comparative study of peritoneal macrophages functions in mice receiving lethal and non-lethal doses of LPS. *J Endotox Res* 2000; 6: 235-241.
- [14] Victor VM, De la Fuente M. Changes in the superoxide production and other macrophage functions could be related with the mortality of mice with endotoxin-induced oxidative stress. *Physiol Res* 2003; 52: 101-110.
- [15] Victor VM, Rocha M, De la Fuente M. Immune cells: free radicals and antioxidants in sepsis. *Int Immunopharmacol* 2004; 4: 327-47.
- *[16] Ben-Neriah, Y., Alkalay-Snir, I., Hatzubai, A. *et al.*: US6905836 (2005).
- [17] Alkalay-Snir, I., Ben-Neriah, Y., Ciechanover, A., Manning, A., Mercurio, F., Yaron, A.: US5932425 (1999).
- [18] Le, J., Vilcek, J., Dadbona, P., Ghayeb, J., Knight, D., Siegel, S.: US6835823 (2004).
- [19] Victor VM, De la Fuente M. Immune cells redox state from mice with endotoxin-induced oxidative stress. Involvement of NF- κ B. *Free Radic Res* 2003; 37: 19-27.
- [20] Webster NR, Nunn JF. Molecular structure of free radicals and their importance in biological reactions. *Brit J Anaesth* 1988; 60: 98-108.
- [21] Zimmerman JJ. Defining the role of oxyradicals in the pathogenesis of sepsis. *Crit Care Med* 1995; 23: 616-7.
- [22] Meydani SN, Erickson KL. Nutrients as regulators of immune function: Introduction. *FASEB J* 2001; 15: 2555.
- [23] Alexander, J.W., Peck, M.D.: US4981844 (1991).
- [24] Chandra, R.K.: US5556644 (1996).
- [25] Garleb, K.A., Demichele, S.J.: US5444054 (1995).
- [26] DeMichele, S.J.; Gregory, T.J.: US5223285 (1993).
- [27] Bistrrian, B.R., Babayan, V.K., Blackburn, G.L., Mascioli, E.A.: US4871768 (1989).
- [28] Katz, D.P., Komorowski, J.R.: US6809115 (2004).
- [29] Mendy, F., Barthelemy, P.: US4607052 (1986).
- [30] De Michele, S.J., McEwen, J.W., Wood, S.M.: US6444700 (2002).
- *[31] Sakya, S.M., Rast, B.: US6900230 (2005).
- [32] Wikberg, J., Prusis, P., Dambrova, M., Uhlen, S.: US6599943 (2004).
- [33] Gehlsen, K.R.: US6350785 (2002).
- [34] Hellstrand, K., Hermodsson, S., Gehlsen, K.R.: US6730692 (2004).
- [35] Bokoch, G.M., Curnutte, J.T.: US6184203 (2001).
- [36] Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994; 298: 249-58.
- [37] Vincent JL, Zhang H, Szabo C, Preiser JC. Effects of nitric oxide in septic shock. *Am J Respir Crit Care Med* 2000; 161: 1781-5.

- [38] Forman HJ, Torres M. Redox signaling in macrophages. *Mol Aspects Med* 2001; 22: 189-216.
- [39] Carreras MC, Pargament GA, Catz S, Poderoso JJ, Boveris A. Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. *FEBS Lett* 1994; 341: 65-8.
- [40] Valdez LB, Boveris A. Nitric oxide and superoxide radical production in human mononuclear leukocytes. *Antioxid Redox Signal* 2001; 3: 505-13.
- [41] Thiemermann C, Vane JR. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharide in the rat *in vivo*. *Eur J Pharmacol* 1990; 182: 591-5.
- [42] Kilbourn RG, Jubran A, Gross SS, *et al.* Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem Biophys Res Commun* 1990; 172: 1132-8.
- [43] Gray GA, Julou-Schaeffer G, Oury K, Fleming I, Parrat JR, Stoclet JC. An L-arginine-derived factor mediates endothelin-induced vascular hyposensitivity to calcium. *Eur J Pharmacol* 1990; 191: 89-92.
- [44] Hobbs AJ, Higgs A, Moncada S. Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu Rev Pharmacol Toxicol* 1999; 39: 191-220.
- [45] Ruetten H, Thiemermann C. Nitric oxide and septic shock. In: Ignarro L (Ed). *Nitric oxide: biology and pathobiology*. San Diego: Academic Press, 2000; p. 747-758.
- [46] Arnaiz, D.O., Baldwin, J.J., Davey, D.D. *et al.*: US6887865 (2005).
- [47] Webber, R.K., Durley, R.C., Awasthi, A.K. *et al.*: US6914158 (2005).
- [48] Umans JG, Wylam ME, Samsel RW, Edwards J, Schumacker PT. Effects of endotoxin *in vivo* on endothelial and smooth-muscle function in rabbit and rat aorta. *Am Rev Respir Dis* 1993; 148: 1638-45.
- [49] Liao JK, Zulueta JJ, Yu FS, Peng HB, Cote CG, Hassoun PM. Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. *J Clin Invest* 1995; 96: 2661-6.
- [50] Graier WF, Myers PR, Rubin LJ, Adams HR, Parker JL. *Escherichia coli* endotoxin inhibits agonist-mediated cytosolic Ca²⁺ mobilization and nitric oxide biosynthesis in cultured endothelial cells. *Circ Res* 1994; 75: 659-68.
- [51] Salvemini DR, Korb R, Anggard E, Vane J. Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by *Escherichia coli* lipopolysaccharide. *Proc Natl Acad Sci USA* 1990; 87: 2593-7.
- [52] Zhang H, Rogiers P, Smail N, *et al.* Effects of nitric oxide on blood flow distribution and oxygen extraction capabilities during endotoxic shock. *J Appl Physiol* 1997; 83: 1164-73.
- [53] Szabo C, Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL. Endothelial dysfunction in a rat model of endotoxic shock: Importance of the activation of poly (ADP-ribose) synthetase by peroxynitrite. *J Clin Invest* 1997; 100: 723-35.
- [54] Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J, Mitchell JB. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci USA* 1993; 90: 9813-7.
- [55] Gunnelt CA, Chu Y, Heistad DD, Loihl A, Faraci FM. Vascular effects of LPS in mice deficient in expression of the gene for inducible nitric oxide synthase. *Am J Physiol* 1998; 275: H416-21.
- [56] Hollenberg SM, Cunnion RE, Zimmerberg J. Nitric oxide synthase inhibition reverses arteriolar hyporesponsiveness to catecholamines in septic rats. *Am J Physiol* 1993; 264: H660-3.
- [57] Singh, I.: US6511800 (2003).
- [58] Griffith, O.W.: US5132453 (1991).
- [59] Hallinan, E.A., Hansen, D.W., Tsymbalov, S.: US5684008 (1994).
- [60] Moore, W.M., Hallinan, E.A.: US5830917 (1998).
- [61] Hallinan, A.E., Hansen, D.W., Donald, W., Tsymbalov, S.: US5854251 (1998).
- [62] Beams, R.M., Hodson, H.F., Palmer, R.M.J.: US5863931 (1999).
- [63] Webber, R.K., Tjoeng, F.S., Manning, R.E.: US5945408 (1999).
- [64] Gapud, R.E., Hagen, T.J., Hallinan, A.E. *et al.*: US5981511 (1999).
- [65] Clementi E, Brown GC, Foxwell N, Moncada S. On the mechanism by which vascular endothelial cells regulate their oxygen consumption. *Proc Natl Acad Sci USA* 1999; 96: 1559-62.
- [66] Szabo C. Role of nitric oxide in endotoxic shock. An overview of recent advances. *Ann NY Acad Sci* 1998; 851:422-5.
- [67] Szabo C, Billiar T. Novel roles of nitric oxide in hemorrhagic shock. *Shock* 1999; 12:1-9.
- [68] Szabo C, Oshima H. DNA damage induced by peroxynitrite: subsequent Biological effects. *Nitric Oxide* 1997; 1: 373-85.
- [69] Virag L, Scott G, Cuzzocrea S, Marmer D, Salzman AL, Szabo C. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly (ADP-ribose) synthetase (PARS) activation. *Immunology* 1998; 94:345-55.
- [70] Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta* 2001; 1504: 46-57.
- [71] Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch Biochem Biophys* 1996; 328: 85-92.
- [72] Han YJ, Kwon YG, Chung HT, *et al.* Antioxidant enzymes suppress nitric oxide production through the inhibition of NF-kappa B activation: role of H₂O₂ and nitric oxide in inducible nitric oxide synthase expression in macrophages. *Nitric Oxide* 2001; 5: 504-13.
- [73] Bolanos JP, Peuchen S, Heales SJ, Land JM, Clark JB. Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J Neurochem* 1994; 63: 910-6.
- [74] Brown GC. Reversible binding and inhibition of catalase by nitric oxide. *Eur J Biochem* 1995; 232: 188-91.
- [75] Radi R, Rodriguez M, Castro L, Telleri R. Inhibition of mitochondrial electron transport by peroxynitrite. *Arch Biophys Biochem* 1994; 308: 89-95.
- [76] Wink DA, Miranda KM, Espey MG, *et al.* Mechanisms of the antioxidant effects of nitric oxide. *Antiox Redox Signal* 2001; 3: 203-13.
- [77] Graves JE, Lewis SJ, Kooy NW. Peroxynitrite-mediated vasorelaxation: evidence against the formation of circulating S-nitrothiols. *Am J Physiol* 1998; 274: H1001-8.
- [78] Masuda M, Nishino H, Ohshima H. Formation of 8-nitrosoguanosine in cellular RNA as a biomarker of exposure to reactive nitrogen species. *Chem Biol Interact* 2002; 139: 187-97.
- [79] Ben-Shaul V, Lomnitski L, Nyska A, Zurovsky Y, Bergman M, Grossman S. The effect of natural antioxidants, NAO and apocynin, on oxidative stress in the rat heart following LPS challenge. *Toxicol Lett* 2001; 123: 1-10.
- [80] Liaudet L, Pacher P, Mabley JG, *et al.* Activation of poly(ADP-ribose) polymerase-1 is a central mechanism of lipopolysaccharide-induced acute lung inflammation. *Am J Respir Crit Care Med* 2002; 165: 373-7.
- [81] Kozlov AV, Szalay L, Umar F, *et al.* EPR analysis reveals three tissues responding to endotoxin by increased formation of reactive oxygen and nitrogen species. *Free Radic Biol Med* 2003; 34: 1555-62.
- [82] Szabo C, Ferrer-Sueta G, Zingarelli B, Southan GJ, Salzman AL, Radi R. Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. *J Biol Chem* 1997; 272: 9030-6.
- [83] Natanson C, Eichenholz PW, Danner RL, *et al.* Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *J Exp Med* 1989; 169: 823-32.
- [84] Kumar A, Haery C, Parrillo JE. Myocardial dysfunction in septic shock. *Crit Care Clin* 2000; 16: 251-87.
- [85] Grocott-Mason RM, Shah AM. Cardiac dysfunction in sepsis: new theories and clinical implications. *Intensive Care Med* 1998; 24: 286-95.
- [86] Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL. Negative inotropic effects of cytokines on the heart mediated by nitric oxide. *Science* 1992; 257: 387-9.
- [87] Joe EK, Schussheim AE, Longrois D, *et al.* Regulation of cardiac myocyte contractile function by inducible nitric oxide synthase (iNOS): mechanisms of contractile depression by nitric oxide. *J Mol Cell Cardiol* 1998; 30: 303-15.

- [88] Lancel S, Tissier S, Mordon S, *et al.* Peroxynitrite decomposition catalysts prevent myocardial dysfunction and inflammation in endotoxemic rats. *J Mol Cell Cardiol* 2004; 43: 2348-58.
- [89] Flesch M, Kilter H, Cremers B, *et al.* Effects of endotoxin on human myocardial contractility involvement of nitric oxide and peroxynitrite. *J Am Coll Cardiol* 1999; 33: 1062-70.
- [90] Panas D, Khadour FH, Szabo C, Schulz R. Proinflammatory cytokines depress cardiac efficiency by a nitric oxide-dependent mechanism. *Am J Physiol* 1998; 275:H1016-23.
- [91] Price S, Mitchell JA, Anning PB, Evans TW. Type II nitric oxide synthase is cardio-protective in experimental sepsis. *Eur J Pharmacol* 2003; 472: 111-8.
- [92] Parrillo JE. Pathogenetic mechanisms of septic shock. *N Engl J Med* 1993; 328: 1471-7.
- [93] Goode HF, Cowley HC, Walker BE, Howdle PD, Webster NR. Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. *Crit Care Med* 1995; 23: 646-51.
- [94] Zouki C, Zhang SL, Chan JS, Filep JG. Peroxynitrite induces integrin-dependent adhesion of human neutrophils to endothelial cells via activation of the Raf-1/MEK/Erk pathway. *FASEB J* 2001; 15: 25-7.
- [95] Lush CW, Cepinskas G, Kvietys PR. Regulation of intestinal nuclear factor-kappa B activity and E-selectin expression during sepsis: a role for peroxynitrite. *Gastroenterology* 2003; 124: 118-28.
- [96] Salvemini D, Wang ZQ, Stern MK, Currie MG, Misko TP. Peroxynitrite decomposition catalysts: therapeutics for peroxynitrite-mediated pathology. *Proc Natl Acad Sci USA* 1998; 95: 2659-63.
- [97] Cuzzocrea S, Misko TP, Costantino G, *et al.* Beneficial effects of peroxynitrite decomposition catalysts in a rat model of splachnic artery occlusion and reperfusion. *FASEB J* 2000; 14: 1061-72.
- [98] Zingarelli B, Cuzzocrea S, Szabo C, Salzman AL. Mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, reduces trinitrobenzene sulfonic acid-induced colonic damage in rats. *J Pharmacol Exp Ther* 1998; 287: 1048-55.
- [99] Pacher P, Schulz R, Liaudet L, Szabo C. Nitrosative stress and pharmacological modulation of heart failure. *TRENDS in Pharmacol Sci* 2005; 26: 302-10.
- [100] Szabo C. Multiple pathways of peroxynitrite cytotoxicity. *Toxicol Lett* 2003; 140-141: 105-12.
- [101] Okamoto T, Akaike T, Sawa T, Miyamoto Y, van der Vliet A, Maeda H. Activation of matrix metalloproteinases by peroxynitrite-induced protein S-glutathiolation via disulfide S-oxide formation. *J Biol Chem* 2001; 276: 29596-602.
- [102] Virag L, Szabo C. The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. *Pharmacol Rev* 2002; 54: 375-429.
- *[103] Zeng, F., Wang, Y., Wang, L.: CN1524447A (2004).
- *[104] Gorsek, W.F.: US6551629 (2003).
- *[105] Hsia, H.S., Fan, D.: US6620440 (2003).
- *[106] Wechter, W.J.: US6908943 (2005).
- *[107] Patell-Villo, M., Vyas, D.: WO05016361A1 (2005).
- *[108] De Simone, C.: US6923960 (2005).
- [109] Poon BY, Raharjo E, Patel KD, Tavener S, Kubes P. Complexity of inducible nitric oxide synthase: cellular source determines benefit versus toxicity. *Circulation* 2003; 108: 1107-12.
- [110] De Flora S. Mechanisms of inhibitors of mutagenesis and carcinogenesis. *Mutat Res* 1998; 402: 151-8.
- [111] Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med* 1989; 6: 593-7.
- [112] Vale JA, Proudfoot AT. Paracetamol (acetaminophen) poisoning. *Lancet* 1995; 346: 547-52.
- [113] Victor VM, Guayerbas N Garrote D, Del Rio M, De la Fuente M. Modulation of murine macrophage function by N-acetylcysteine in a model of endotoxemic shock. *BioFactors* 1999; 10:347-57.
- [114] De la Fuente M, Victor VM. Anti-oxidants as modulators of immune function. *Immunol Cell Biol* 2000; 78: 49-54.
- [115] Victor VM, De la Fuente M. N-acetylcysteine improves *in vitro* the function of macrophages from mice with endotoxin-induced oxidative stress. *Free Radic Res* 2002; 36: 33-45.
- [116] De la Fuente M, Victor VM. Ascorbic acid and N-acetylcysteine improve *in vitro* the function of lymphocytes from mice with endotoxin-induced oxidative stress. *Free Radic Res* 2001; 35:73-84.
- [117] Victor VM, Rocha M, De la Fuente M. N-acetylcysteine protects mice from lethal endotoxemia by regulating the redox state of immune cells. *Free Radic Res* 2003; 37: 919-29.
- [118] Blackwell TS, Blackwell TR, Holden EP, Christman BW, Christman JW. *In vivo* antioxidant treatment suppresses nuclear factor-kB activation and neutrophilic lung inflammation. *J Immunol* 1996; 157: 1630-7.
- [119] Zhang H, Spapen H, Nguyen DN, Benlabel M, Buurman WA, Vincent JL. Protective effects of N-acetyl-L-cysteine in endotoxemia. *Am J Physiol* 1994; 266: H1746-54.
- [120] Galley HF, Howdle PD, Walker BE, Webster NR. The effects of intravenous antioxidants in patients with septic shock. *Free Radic Biol Med* 1997; 23: 768-74.
- [121] Byrne, T.A., Somerville, J.A.: US6797729 (2004).
- [122] Aoki, T., Miyakoshi, H.: US5491150 (1996).
- [123] Staal FJ, Roederer M, Raju PA, Anderson MT, Ela SW, Herzenberg LA. Antioxidants inhibit stimulation of HIV transcription. *AIDS Res Hum Retroviruses* 1993; 9:299-306.
- [124] Moshayed, E.P.: US5770215 (1998).
- *[125] Boulous, A., Desai, J., Martin, N., Stillman, R., Udwin, M.: US6914073 (2005).
- [126] Basu S, Eriksson M. Vitamin E in relation to lipid peroxidation in experimental septic shock. *Prostaglandins Leuk Essent Fatty Acids* 2000; 62: 195-9.
- [127] Goode HF, Webster NR. Free radicals and antioxidants in sepsis. *Crit Care Med* 1993; 21: 1770-6.
- [128] Bulger EM, Maier RV. An argument for vitamin E supplementation in the management of systemic inflammatory response syndrome. *Shock* 2003; 19: 99-103.
- [129] Van der Kraaij AM, van Eijk HG, Koster JF. Prevention of postischemic cardiac injury by the orally active iron chelator 1,2-dimethyl-3-hydroxy-4-pyridone (L1) and the antioxidant (+)-cyanidanol-3. *Circulation* 1989; 80: 158-64.
- [130] Bolli R, Jeroudi MO, Patel BS, *et al.* Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circ Res* 1989; 65: 607-22.
- [131] Gutteridge JM, Mitchell J. Redox imbalance in the critically ill. *Br Med Bull* 1999; 55: 49-75.
- [132] Uyama O, Shiratsuki N, Matsuyama T, *et al.* Protective effects of superoxide dismutase on acute reperfusion injury of gerbil brain. *Free Radic Biol Med* 1990; 8: 265-8.
- [133] Schneider J, Friderichs E, Heintze K, Flohe L. Effects of recombinant human superoxide dismutase on increased lung vascular permeability and respiratory disorder in endotoxemic rats. *Circ Shock* 1990; 30: 97-106.
- [134] Patel BS, Jeroudi MO, O'Neill PG, Roberts R, Bolli R. Effect of human recombinant superoxide dismutase on canine myocardial infarction. *Am J Physiol* 1990; 258: H369-80.
- [135] Vohra K, Rosenfeld W, Singh I, Anderson V. Ischemic injury to newborn rabbit ileum: protective role of human superoxide dismutase. *J Pediatr Surg* 1989; 24: 893-8.
- [136] Mitsos SE, Askew TE, Fantone JC, *et al.* Protective effects of N-2-mercaptopyrionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals. *Circulation* 1986; 73: 1077-86.
- [137] Bernard GR, Lucht WD, Niedermeyer ME, Snapper JR, Ogletree ML, Brigham KL. Effect of N-acetylcysteine on the pulmonary response to endotoxin in the awake sheep and upon *in vitro* granulocyte function. *J Clin Invest* 1984; 73: 1772-84.
- [138] Vander Heide RS, Sobotka PA, Ganote CE. Effects in the free radical scavenger DMTU and mannitol on the oxygen paradox in perfused rat hearts. *J Mol Cell Cardiol* 1987; 19: 615-25.
- [139] Fox RB. Prevention of granulocyte-mediated oxidant lung injury in rats by a hydroxyl radical scavenger, dimethylthiourea. *J Clin Invest* 1984; 74: 1456-64.
- [140] Malfroy-Camine, B., Doctrow, S.R.: US6900198 (2005).
- *[141] Rao, J.M., Tiwari A.K., Kumar, U.S., Yadav, J.S., Raghavan, K.V.: US6592911 (2003).
- *[142] Rao, J.M., Rao, R.J., Tiwari A.K., Yadav, J.S., Raghavan, K.V.: US6781002 (2004).

- [143] San Juan-Pla A, Cervera AM, Apostolova N, Garcia-Bou R, Victor VM, Murphy MP, McCreath KJ. A targeted antioxidant reveals the importance of mitochondrial reactive oxygen species in the hypoxic signaling of HIF-alpha. *FEBS Letters* 2005; 579: 2669-74.
- [144] Kelso GF, Porteous CM, Coulter CV, *et al.* Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem* 2001; 276: 4588-96.
- [145] Hughes G, Murphy MP, Ledgerwood EC. Mitochondrial reactive oxygen species regulate the temporal activation of nuclear factor kappaB to modulate tumour necrosis factor-induced apoptosis: evidence from mitochondria-targeted antioxidants. *Biochem J* 2005; 389: 83-9.

- [146] Jauslin ML, Meier T, Smith RA, Murphy MP. Mitochondria-targeted antioxidants protect Friedreich Ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants. *FASEB J* 2003; 17: 1972-4.
- [147] Saretzki G, Murphy MP, von Zglinicki T, Mito, Q. Counteracts telomere shortening and elongates lifespan of fibroblasts under mild oxidative stress. *Aging Cell* 2003; 2: 141-3.
- [148] Adlam VJ, Harrison JC, Porteous CM, *et al.* Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. *FASEB J* 2005; 19: 1088-95.
- [149] Victor VM, Rocha M, Esplugues JV, De la Fuente M. Role of free radicals in sepsis: antioxidants therapy. *Curr Pharm Des* 2005; 11: 3141-58.
- *[150] Murphy, M.P., Smith R.: WO9926582A2 (1999).