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Superoxide, NO, peroxynitrite and PARP in circulatory shock and inflammation

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1. ABSTRACT

Oxidative stress results from an oxidant/antioxidant imbalance, an excess of oxidants and/or a depletion of antioxidants. A vast amount of circumstantial evidence implicates oxygen-derived free radicals (especially, superoxide and hydroxyl radical) and high energy oxidants (such as peroxynitrite) as mediators of tissue injury associated with circulatory shock and inflammation. Reactive oxygen species (ROS) (e.g., superoxide, peroxynitrite, hydroxyl radical and hydrogen peroxide) are all potential reactants capable of initiating DNA single strand breakage, with subsequent activation of the nuclear enzyme poly (ADP ribose) synthetase (PARS), leading to eventual severe energy depletion of the cells, and necrotic-type cell death. Moreover, Poly (ADP-ribosyl)ation is regulated by the synthesizing enzyme poly (ADP-ribose) polymerase-1 (PARP-1) and the degrading enzyme poly (ADP-ribose) glycohydrolase (PARG). Here we review the roles of ROS, PARP-1 and PARG in circulatory shock and inflammation as well as the beneficial effect of the *in vivo* treatment with novel pharmacological tools (e.g. peroxynitrite decomposition catalysts, selective superoxide dismutase mimetics (SODm), PARP-1 and PARG inhibitors.

2. INTRODUCTION

Inflammation occurs as a defensive response to invasion of the host by foreign material, often of microbial nature. This response is normally a localized protective response that at the microscopic level involves a complex series of events including dilatation of arterioles, venules, and capillaries with increased vascular permeability, exudation of fluids including plasma proteins, and leukocyte migration into the inflammatory area. The role of several mediators such as histamine, serotonin, bradykinin, prostaglandins, and, more recently, cytokines and nitric oxide has been evaluated, and a contribution for- each one of these mediators has been proposed.

The delayed phase of the inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals and oxidants, such as H_2O_2, $O_2^{-\cdot}$ and $HO^{\cdot}$, as well as to the release of other neutrophil-derived mediators (1, 2)

Some of the most destructive free radicals generated in organisms derive from oxygen (O_2, dioxygen) and are continually generated within cells of aerobic organisms. Thus the molecule most critical for sustaining

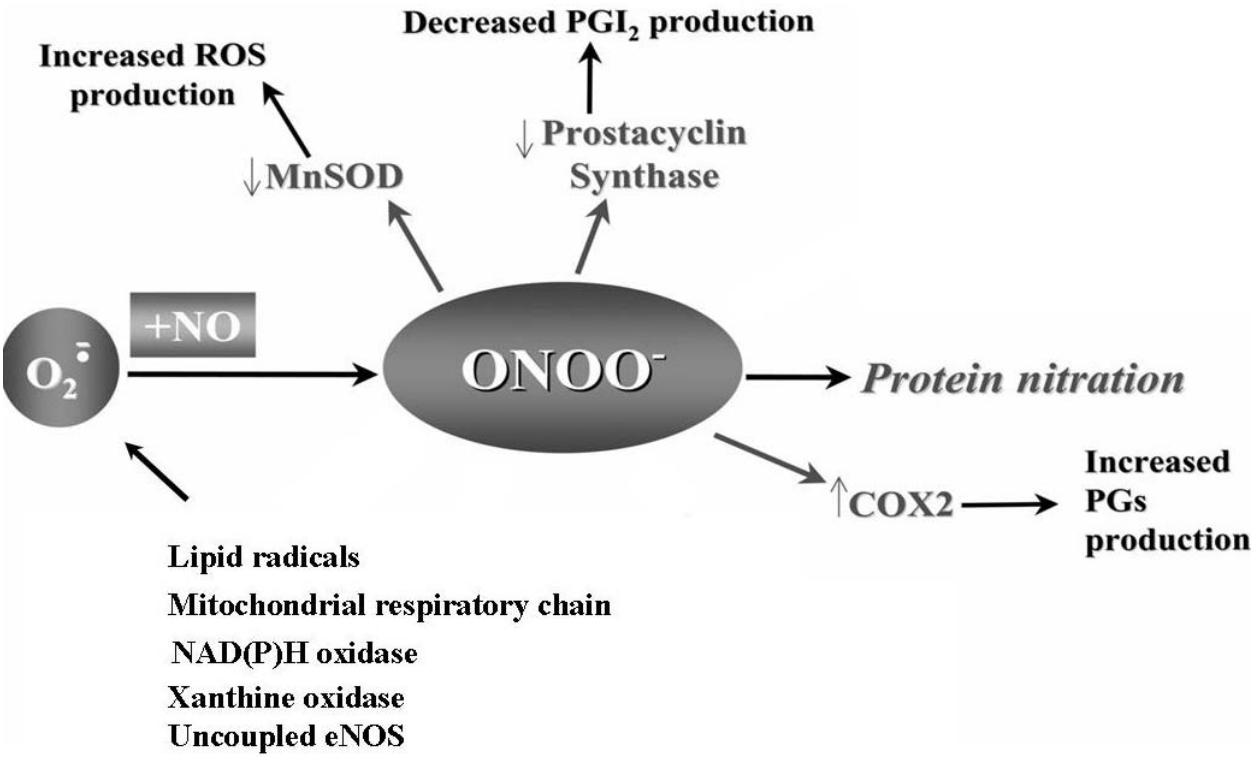

Figure 1. Impact of reactive oxygen species (ROS) generation. Excessive production of superoxide can lead to inflammation through various pathways including generation of destruction of beneficial nitric oxide (NO) and simultaneous generation of cytotoxic and pro-inflammatory peroxynitrite (ONOO-).

life may also damage cells to the point where most organs and organisms fail.

The best known reactive species generated from O_2 include the superoxide anion radical (dioxide or O_2^-), the hydroxyl radical (OH) and the peroxynitrite anion (ONOO-). Because of their high reactivity, these radicals can be devastatingly toxic to other molecules and can cause cellular dysfunction and sometimes cells death. There are molecular defences against free radicals. Organisms developed an antioxidative defence system. It consists of a system of direct free radical scavengers, metal chelators and enzymes that metabolise the radicals to non-harmful products.

Free radical damage is a component of several diseases condition, a vast amount of evidence implicates oxygen derived free radicals (especially superoxide and hydroxyl radical) and high-energy oxidant (such as peroxynitrite) as mediators of inflammation, shock, and ischemia/reperfusion injury.

Superoxide anions increase neutrophil adhesion and infiltration and generate potent chemotactic mediators such as leukotriene B_4 (3-6). While hydrogen peroxide does not possess an unpaired electron and, therefore, is not a free radical *per se*, it is usually classified as a reactive oxygen intermediate or species. Hydrogen peroxide can diffuse through membranes and has a half-life much longer than that of superoxide. Hydrogen peroxide has several fates intracellularly. It can be metabolized by one or two anti-oxidative enzymes, i.e. glutathione peroxidase or catalase, and, in the presence of the transition metals Fe^{2+} or Cu^{1+}, it is decomposed to hydroxyl radicals via the Fenton reaction (7). Although hydroxyl radicals are reactive and highly toxic, their role (s) in disease states is not known (Figure 1).

In some cases, these newly formed radicals can be toxic and, in fact, may initiate other damaging free radical reactions. An example of this type of chain reaction is lipid peroxidation, where the lipid peroxyl radical, once produced, abstracts a hydrogen atom from a neighouring polyunsaturated fatty acid to continue the process, converting itself into a lipid peroxide. The end result of extensive lipid peroxidation is cell death. A radical can also interact with another radical to form a stable molecule. This is what happens when superoxide reacts with nitric oxide to form peroxynitrite (8). While the biological activity and decomposition of peroxynitrite is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of nitric oxide and superoxide, carbon dioxide levels and other factors) it is becoming clear that in most cases peroxynitrite is pro-inflammatory (Figure 2).

3. BACKGROUND

Superoxide is formed from various sources, including normal cellular respiration, activated

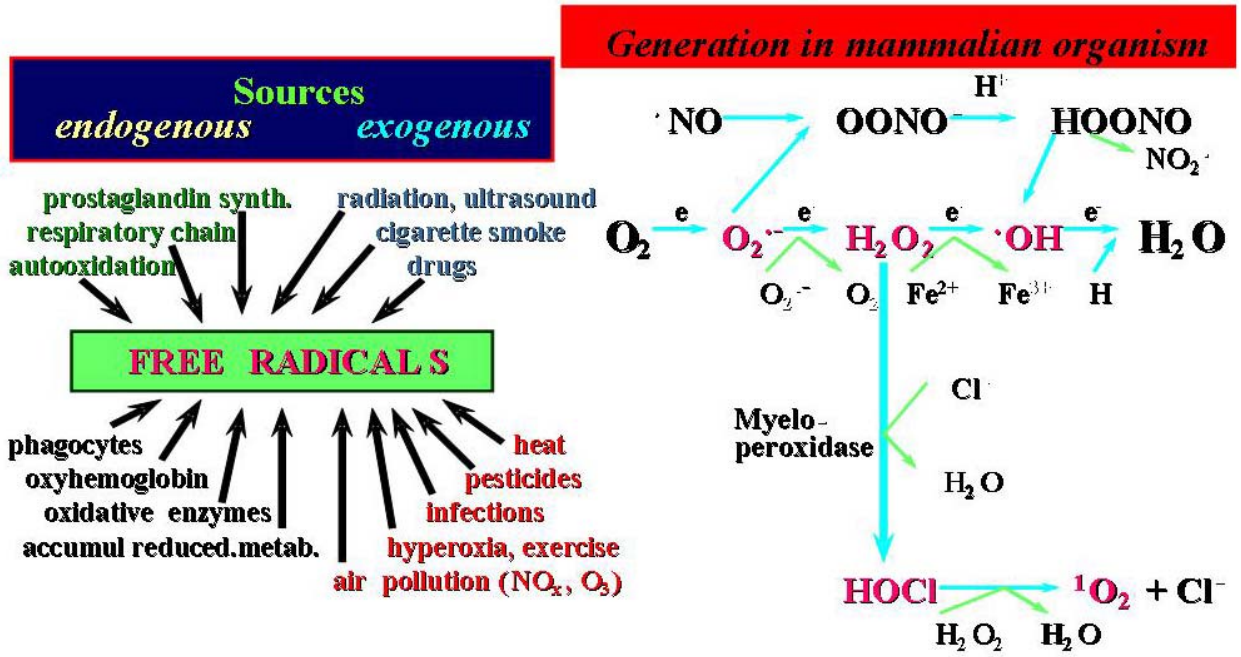

Figure 2. Endogenous and exogenous ROS sources.

polymorphonuclear leukocytes, endothelial cells, and mitochondrial electron flux (9, 10). Superoxide generation by phagocytic NADPH oxidase is known to be important toward bacterial killing. In host defense, free radicals serve as microbicidal and parasiticidal agents that are sensitive to oxidative damage (11, 12) but they also contribute to the pathogenesis of a wide array of diseases (13) and may facilitate or even promote replication of parasites, depending on the cell and virus involved. In acute and chronic inflammation, the production of superoxide is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defence system to remove it. The consequence of this imbalance results in superoxide mediated damage. The list of patho-physiological conditions associated with the production of superoxide continues to expand. The most exciting realization is that there appears to be a commonality to the tissue injury observed in various disease states; namely, superoxide produces tissue injury (and associated inflammation) in most tissues in similar ways. Tissue injury and inflammation form the basis of many disease pathologies: ischemia and reperfusion injuries, radiation injury, hyperoxic lung damage, asthma, atherosclerosis .

The microvasculature is the critical interface for oxygen and energy delivery to the tissues. Thus, any damage to or obstruction of the microvasculature may have potentially harmful consequences. One of the major injuries to arteries is an impairment of Ca^{2+}-regulating mechanisms. Recently reactive oxygen species formation during shock and ischemia and reperfusion and have been linked to multiple effects on Ca^{2+} signaling in both the endothelium and smooth muscle. Therefore, it is likely that the signals affected by reactive oxygen species are important mediators of arterial injury. The evidence supporting a role of ROS in arterial dysfunction is complicated by differences in the type of species examined and variations in experimental protocols.

Overproduction of superoxide in vascular cells creates both an imbalance in nitric oxide-signaling and changes in several intracellular signaling pathways. Superoxide is generated by vascular cells through multiple mechanisms. An important source of extracellular superoxide is the oxidation of xanthine by xanthine oxidase, which binds to glycosaminoglycan sites in the arterial wall (14). Another source of superoxide in the vasculature is specific NAD (P)H oxidases, which is the primary source for smooth muscle–derived reactive oxygen species (15). Evidence also exists for the formation of superoxide by nitric oxide synthase and cyclooxygenase in vascular cells when their normal substrate is deficient. Arachidonic acid metabolism mediated by Ca^{2+} and cyclooxygenase has been shown to produce superoxide dependent effects on arterial contractions, suggesting an additional role for cyclooxygenase in superoxide production (16). Thus, oxidants appear to play a major role in the pathogenesis of the endothelial dysfunction (17).

Endothelial cells represent both a source and a possible target of oxidants released in the vasculature (18). At the same time, oxidants are known to activate nuclear transcription factors (19).

In rat mesenteric venules, free radical scavengers decrease leukocyte–endothelial cell adhesion and albumin leakage after arterial occlusion and reperfusion (20). In addition, superoxide dismutase enhances survival of ischemic tissue in an ischemic hindlimb model (21); and transgenic mice overexpressing copper/zinc superoxide

dismutase (CuZn-SOD) exhibit less accumulation of leukocytes and less no-reflow than wild-type mice after superior mesenteric artery occlusion and reperfusion (22). In patients who sustain an acute myocardial infarction, plasma superoxide dismutase activity increases after successful reperfusion of the infarcted vessel and predicts improvement in left-ventricular function (23).

Vascular oxidative stress has been implicated in a wide spectrum of cardiovascular and pulmonary diseases, which have an enormous impact on health in industrial countries. Oxidative stress is often initiated and propagated by overproduction of O_2 and H_2O_2, and their conversion to potent oxidants, such as the hydroxyl radical, hypochlorous acid and peroxynitrate. Therefore, interception and degradation (detoxification) of O_2 and H_2O_2 appear to represent important therapeutic goals. However, the results of diverse animal and clinical studies suggest that SOD (a family of enzymes which convert O_2 to H_2O_2) and catalase (an enzyme which converts H_2O_2 to water) afford modest, if any, protection against vascular oxidative stress. The lack of a more impressive benefit may be explained in part by inadequate delivery of these enzymes to their sites of therapeutic action, such as an ischemic zone or focus of inflammation.

Endothelial cells help to regulate levels of vasoactive agents (nitric oxide, angiotensin, bradykinin, prostacyclin, thrombin, etc.), as well as control blood pressure, vascular permeability, coagulation, fibrinolysis, leukocyte adhesion and migration into tissues.

Endothelial disorders or injury compromise these functions. Thus, oxidative stress compromises normal endothelial properties such as vasorelaxation and inhibition of thrombosis by inactivation of NO. Oxidative stress to the endothelium can be initiated and/or propagated by oxidants generated in the cellular milieu (plasma, other vascular cells, extracellular matrix, adjacent alveolar compartment), released by activated leukocytes or generated by endothelial cells themselves under pathological conditions. Endothelium can generate ROS under pathological conditions for a more prolonged period of time than leukocytes, albeit at a lower rate and amplitude. Ultimately, when ROS generation exceeds endothelial antioxidant defense mechanisms, endothelial cells may undergo apoptotic or necrotic death leading to vascular injury (24).

Inflammation leads to oxidative vascular injury in diverse pathological settings (25). However, ROS and other inflammatory mediators (e.g., cytokines, interleukins, leukotrienes and components of complement) activate endothelium and up-regulate the density of these receptors and induce exposure P- and E-selectins on endothelium. Endothelial selectins and adhesion molecules facilitate leukocyte adhesion and infiltration into sub-endothelial tissues (24).

It is now accepted that reactive oxygen species play important roles in normal physiological states and that depending on the species involved the effect could be highly varied. Nitric oxide (similarly to superoxide anion), functions in the regulation of a wide range of cell systems. As part of the normal physiological process, superoxide anion and NO function separately and interactively as second messengers. Superoxide anion and nitric oxide play an intrinsic role in the regulated ordered turnover of proteins, rather than randomly cause protein damage and their inactivation. The combination between these two radicals to give peroxynitrite has received considerable attention over the past few years since it has been suggested that peroxynitrite may represent an important mediator of cytotoxicity and cytostasis (26). Currently, little information is available regarding the "physiological" roles of peroxynitrite, while the evidence for its roles in pathophysiological conditions is expanding (27, 28). Although there are a number of experimental difficulties related to delineation of the actual role of peroxynitrite in shock and other pathophysiological conditions, theoretical considerations strongly favor the production of peroxynitrite when NO and superoxide are produced simultaneously, because the reaction of these two species is nearly diffusion controlled. In fact, the reaction of superoxide with NO is the only reaction that out competes the reaction of superoxide with superoxide dismutase (29, 30). The finding that peroxynitrite is produced during inflammation and shock is not surprising, in light of the previous evidence for the overproduction of oxygen derived free radicals. Specific peroxynitrite scavengers that could help to further elucidating the role of peroxynitrite in pathological situations are not available. Glutathione, melatonin, uric acid, a putative scavenger of peroxynitrite, are sometimes used as a probe for peroxynitrite (31-33). Therefore, the evidence implicating the role of peroxynitrite in a given pathophysiological condition can only be indirect. A simultaneous protective effect of superoxide neutralizing strategies and NO synthesis inhibition, coupled with the demonstration of peroxynitrite in the particular pathophysiological condition, can be taken as a strong indication for the role of peroxynitrite.

Free radicals-mediated reaction can cause structural alterations in DNA (e.g. nicking, base-pair mutations, rearrangements, deletions, insertions and sequence amplification). NO or, more likely, reactive products derived from it, such as NO^-_2, $ONOO^-$, N_2O_3 and HNO_2, are mutagenic agents, with the potential to produce nitration, nitrosation, and deamination reactions on DNA bases (34). The chemistry of DNA damage by several ROS has been well characterized *in vitro* (35-38), although specific information is needed about the changes produced by peroxyl (RO^-_2), alkoxyl ($RO^·$), ozone (O_3), and several of the reactive nitrogen species (RNS) (e.g., $ONOO^-$) is lacking. Different ROS affect DNA in different ways (e.g., H_2O_2 does not react with DNA bases at all (36, 39), whereas $HO^·$ generates a multiplicity of products from all four DNA bases, and this pattern seems to be a diagnostic "fingerprint" of $HO^·$ attack (39). Damage to DNA by ROS/RNS seems to occur naturally, in that low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues (40-44). ROS/RNS can also damage mitochondrial DNA, and such damage has been suggested to be important in several human diseases and in the aging process (45).

DNA damage can be repaired by the action of a series of enzymes (46). DNA damage by ROS/RNS can

cause multiple lesions, including single and double strand breaks, apurinic/apyrimidinic sites and modified pyrimidines and purines. Repair of these lesions occurs primarily by base excision repair, although nucleotide excision repair may also be involved. A repair system for the abasic apurinic/apyrimidinic sites produced by spontaneous depurination also exists. Areas of current interest include the role of poly (ADP-ribose) polymerase (PARP) in the rejoining of DNA strand breaks, including those induced by ROS (47, 48).

The aim of this review is to describe recent experimental evidence implicating superoxide, NO, and PARP as a pathophysiological modulator of acute and chronic inflammation.

4. SUPEROXIDE

Free radicals are molecules or portions thereof which possess one or more unpaired electrons in their outer orbital, a state which greatly increases their reactivity. The best known reactive species generated from oxygen include superoxide anion, hydroxyl radical and peroxynitrite.

The list of patho-physiological conditions associated with the over-production of superoxide expands every day. Much of the knowledge compiled on the role of this radical in disease has been gathered using the native superoxide dismutase enzyme and, more recently by the use of superoxide dismutase (SOD) knockout models or transgenic models that over express the various isoforms of the enzyme. Based on the concept that removal of superoxide modulates the course of inflammation, synthetic, low molecular weight mimetics of the superoxide dismutase enzymes that could overcome some of the limitations associated with the use of the native enzyme have been designed. Advances made using various superoxide dismutase mimetics led to the proposal that superoxide is an important mediator of inflammation, and to the conclusion that superoxide dismutase mimetics can be utilized as therapeutic agents in diseases of various etiologies.

Effective protection against vascular oxidative stress using administration of exogenous SOD and catalase proteins remains to be achieved. Derivatives that are characterized by prolonged circulation, specific recognition of endothelium, precise subcellular addressing and beneficial side-effects have the highest probability of serving as viable therapeutic modalities. On the other hand, transfection of endothelium with genes encoding SOD, catalase or other antioxidant proteins, i.e. antioxidant gene therapy, represents an exciting alternative.

The therapeutic efficacy of SOD itself in animals with systemic inflammation, haemorrhage or shock is controversial. The following reasons may explain the lack of effect of SOD against the tissue injury associated with local or systemic inflammation: 1) SOD metabolized $O_2^{\cdot-}$ to H_2O_2. Without efficient removal of the H_2O_2, however, H_2O_2 is converted to the highly toxic $HO^{\cdot}$ (49, 50). Indeed, SOD may function as a pro-oxidant by catalysing the conversion of H_2O_2 to $HO^{\cdot}$ (51), such as is believed to be the case in Down's syndrome. 2) Neither SOD nor $O_2^{\cdot-}$ easily cross biological membranes. Thus, an increase in the amounts of extracellular SOD does not attenuate the effects of the $O_2^{\cdot-}$ generated by intracellular sources (52). In contrast to SOD, spin trapping nitrones, such as phenyl N-*tert*-butyl nitrone (PBN), consistently improve outcome in rat models of endotoxic (53, 54) and traumatic shock (55, 56).

Recently, it has been identified a potential role of superoxide in septic shock (57, 58). It is well established that septic shock is characterized by severe hypotension and decreased perfusion to critical organ systems despite increased circulating levels of endogenous catecholamines. A secondary characteristic of this condition is the loss of vascular responses (hyporeactivity) that develops to both endogenous and, presumably, exogenously administered catecholamines. Indeed, the clinical treatment of this life-threatening condition consisting of fluid resuscitation therapy coupled with intravenous (i.v) infusions of the catecholamines dopamine and norepinephrine, is limited as a result of this hyporeactivity (59-61). In addition, sepsis, like other inflammatory conditions, results in a large increase in the production of ROS within the body (62, 63).

Firstly superoxide is a pro-inflammatory mediator. Some of the pro-inflammatory properties of superoxide pertinent to septic shock include recruitment of neutrophils at sites of inflammation, formation of chemotactic factors, DNA damage (66), initiation of lipid peroxidation, release of pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin 1β (68) via activation of nuclear factor-κB (64-68).

4.1. Superoxide dismutase

Superoxide is enzymatically reduced to hydrogen peroxide in the presence of a ubiquitously distributed enzyme, superoxide dismutase (SOD) (9). SOD enzymes are a class of oxido-reductase enzymes which in mammals contain either Cu or Mn at the active site.

There are two forms of SOD: the Mn enzyme present in mitochondria (SOD2) and the Cu/Zn enzyme present in the cytosol (SOD1) and extracellular surfaces (SOD3). The importance of SOD2 is highlighted by the findings that in contrast to SOD1 (69) and SOD3 (9), the SOD2 knockout is lethal to mice (36, 70). Thus, endogenous SOD enzymes keep superoxide under very tight control. However in many disease states, there is an imbalance between the amount of superoxide formed and the ability of SOD enzyme to remove them: this leads to superoxide-driven damage.

Superoxide dismutase (SOD) enzymes are a class of oxido-reductase enzymes which in mammals contain either Cu or Mn at the active site and catalyze the dismutation of superoxide (1), the one-electron reduction product of molecular oxygen (O2.- + Mn+1 ---> O_2 + Mn+ (Equation 1) - HO2. + Mn+ ---> H2O2 + Mn+1 (Equation 2)) where Mn is the metalloenzyme in

the reduced state and Mn+1 is the enzyme in the oxidized state to oxygen and hydrogen peroxide. The SOD enzymes have distinct genomic structure and are well compartmentalized.

SOD1 has been found in the cytoplasm, nuclear compartments and in the inter membrane space of the mitochondria (71-74). SOD2 is localized in the mitochondria matrix of the cells and SOD3 has been found in the extracellular compartments where it binds the extracellular matrix through its high affinity carboxyterminus (75-78). The mammalian ECSOD is a tetrameric glycosylated CuZnSOD (79).

The uses of the knock-out models and the genetic increase of the SOD enzymes have been crucial in the understanding the importance of the SOD antioxidant system under physiological and pathological conditions. SOD1 maps to chromosome 21 (80) a finding that was crucial in understanding the contribution of this enzyme in Down's syndrome (81, 82). Using mutant SOD1-expressing transgenic mice, several studies have pointed to a variety of functions of mutant SOD1, which has enhanced catalytic activity of the peroxynitrite-mediated tyrosine nitration, readily releases the reactive Cu ions, induces apoptotic cell death, has enhanced peroxidase activity, damages the mitochondria to release Ca2+, and forms SOD1-containing aggregates in the cytoplasm. In considering the findings of increased oxidative damage in mutant SOD1-expressing transgenic mice, it should be remembered that overexpression of mutant SOD1 may enhance oxidative stress generation from this enzyme (83). Down-regulation of SOD1 in vitro and in vivo models has been associated with neuronal death while over expression of SOD1 in transgenic mice has been associated with protection of the cerebral tissue in several pathological conditions such as ischemia or Parkinson's disaese (84-87).

SOD2 maps to chromosome 6 and the knock-out of this gene turn to be lethal (88). The deficiency of SOD2 resulted in an increased production of superoxide which in turn inhibits the respiratory chain by inactivating complex I and complex II (89). Loss or reduction of the SOD2 activity has been associated with mitochondrial vacualization and lipid peroxidation which in turn leads to neurodegeneration and heart failure. SOD2 gene have several polymorphisms which have been associated with a reduction of the enzyme activity and in turn to increased risk of sporadic motor neuron disease, non familial idiopathic cardiomyopathy, breast cancer and reduction of the tumor-suppressive effect SOD2 (90). Several efforts have been done in order to determine whether an up-regulation of SOD2 would be critical for the improvement of pathological conditions where the loss of SOD activity is known to contribute to the underlying pathology (eg ischemia and reperfusion injury). It has been shown that transgenic animals that over express SOD2 had a 35% reduction of infarct size compared to wild type animals (91). The SOD2 over expression, or induction of the enzyme activity lead to a reduction of lipid peroxidation and tyrosine nitration protecting tissues from cellular death (92).

SOD3 is the least characterised among the SOD family enzymes. It maps to chromosome 4 and part of 5 and has been shown to have high affinity for heparin. The only known mutation to date has been demonstrated to be localised to the heparin binding site (93). SOD3 has been detected in several compartments such as plasma, lymph, ascites, cerebrospinal fluid and lung (this organ has the highest concentration of this enzyme) (79). The studies performed using knock-out animals showed the role of SOD3 in focal cerebral ischemia, the implications of the loss of this enzyme activity is in impaired spatial learning (94) and increased sensitivity of the null mice to hyperoxia exposure (95). Transgenic animals with increased expression of SOD3 in particular compartments such as alveolar type II and non-ciliated bronchial epithelial cells demonstrated the critical role of this enzyme during hyperoxic pulmonary disease. Thus enhancement of the SOD3 expression and activity in the lung attenuates the hyperoxic lung injury response by attenuating neutrophil infiltration (93). The over-expression of SOD3 seems to be critical during lung injury after haemorrhage (77). Here, overexpression improves lung injury as shown by overall inhibition of neutrophil infiltration, lipid peroxidation and pulmonary oedema.

4.2. Superoxide Dismutase Mimetics

4.2.1. Manganese (III) Metalloporphyrins

Manganese-based metalloporphyrin complexes scavenge of superoxide, hydrogen peroxide, peroxynitrite and lipid peroxyl radicals (96-101). Metalloporphyrins have been shown to be protective in a wide variety of *in vitro* oxidative stress models involving the generation of superoxide, hydrogen peroxide and peroxynitrite alone or in concert. At micromolar levels, they protect cultured cells against the toxicity of superoxide generators paraquat (102) and pyocyanine (101), hydrogen peroxide generator, glucose oxidase (97) and peroxynitrite injury produced by endotoxin (99) or peroxynitrite itself (103). Metalloporphyrins such as Mn (III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) are also potent inhibitors of lipid peroxidation (96) exerting protective effect against some of the detrimental effects associated with endotoxic and haemorrhagic shock (96, 97) as well as in acute liver failure (104). Since these SOD mimetics scavenge other reactive oxygen species including peroxynitrite, the efficacy of MnTBAP in these models probably relates to its peroxynitrite-scavenging activity in addition to its superoxide-scavenging activity (102). Therefore, one general limitation of these porphyrin-based SOD mimetics, is that they react not only with superoxide but also with a wide variety of reactive oxygen species.

4.2.2. Manganese^{III} (Salen) Complexes

The Mn (III)salen complexes have been reported to be superoxide scavengers/SOD mimetics and possess catalase activity (105). The salen compounds are generally aromatic, substituted ethylenediamine metal complexes. The Mn (III)-containing salen complexes have been reported to have two key antioxidant properties – the scavenging of $O_2^{-\cdot}$ or H_2O_2 (106). Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), a novel SOD mimetic metalloporphyrin, is active, stable, nontoxic, and cell

permeable. Manganese-based metalloporphyrin complexes have been shown to possess at least four distinct antioxidant properties (96-100, 107, 108). The manganese moiety of the SOD mimetics functions in the dismutation reaction with $O_2^{-\cdot}$ by alternate reduction and oxidation changing in its valence between Mn (III) and Mn (II), much like native SODs. The catalase activity of metalloporphyrins could be attributed to their extensive conjugated ring system that undergoes reversible one-electron oxidations, much like the haem prosthetic groups of endogenous catalases and peroxidases (109). In general, metalloporphyrins with higher SOD activity possessed greater catalase activity. It is noteworthy that the catalase activity of such complexes is less than 1% that of native catalases. However, in spite of this, manganese porphyrins are still able to protect cells from a toxic amount of H_2O_2 (97). MnTBAP inhibits neuronal apoptosis in culture (110). Cerebroventricular microinjection of MnTBAP inhibited kainate-induced mitochondrial $O_2^{-\cdot}$ production, DNA oxidation and neuron loss in the hippocampus (111). The mechanism by which metalloporphyrins scavenge $ONOO^-$ is thought to involve the formation of an oxo-Mn (IV) complex that can be readily reduced to the Mn (III) oxidation state by a wide variety of endogenous antioxidants (i.e. ascorbate and glutathione) and even by $O_2^{-\cdot}$. The exact mechanism by which metalloporphyrins inhibit lipid peroxidation is not known, but is thought to be similar to that described for $ONOO^-$ scavenging.

Biological studies have been published for two of these complexes, EUK-8 (manganese *N,N'*- bis (salicyldene)ethylenediamine chloride) and EUK-134 (manganese 3 methoxy *N,N'*-bis (salicyldene)ethylenediamine chloride) in several disease models related to oxidative stress including stroke, ischemia-reperfusion injury, Parkinson's disease and experimental allergic encephalomyelitis (EAE) (105, 112-115). However, Mn (III)salen complexes, in the presence of peroxynitrite and/or hypochlorite, become oxidized to oxoMn-Salen. OxoMn-Salens are potent oxidants that rapidly oxidize NO to NO_2 and also oxidize nitrite to nitrate (116). These data support the evidence that Mn (III)salen complexes is a non selective free radicals scavenger that protect cells from oxidative stress but are not useful pharmacological tools to dissect the relative importance of superoxide in disease. Evidence is available to support that EUK-8 significantly attenuated many of the features of a porcine model of LPS-induced adult respiratory distress syndrome (ARDS) where the role of superoxide has been well described by Kinnula and co-workers (117). EUK-134 attenuates the multiple organ injury and dysfunction caused by endotoxin in the rat (115).

Furthermore, it has been shown that a small increase in myocyte oxidative stress due to partial inhibition of SOD1 leads to myocyte hypertrophy, whereas a higher level of oxidative stress caused by totally inhibition of SOD1 results in apoptosis (118). The response of cardiac myocytes to low and high levels of mechanical stretch is similar to the effect of directly increasing myocyte oxidative stress and the phenotype shift at each level of stretch is inhibited by EUK-8 (119). Recently it has been demonstrated that manganese (Salen) complexes compounds when administered to MnSOD knockout mice are able to double the life span of these animals and to inhibit the cardiomyopathies associated with the knockout phenotype (120). These results suggest that these compounds effectively enter the mitochondria, intracellular location where the majority of superoxide is generated and where endogenous MnSOD enzyme is localized, to protect against the damage elicited by the deregulation of superoxide radical.

EUK-8 has been shown to reduce arrythmias induced by a 10 min regional ischemia/reperfusion injury induced by left coronary artery ligation and release (121). Therefore, although these agents are protective in some models of diseases, the results obtained from the studies cannot be used to support the nature of the radical involved.

4.2.3. Nitroxides

The majority of catalytic antioxidants are designed with redox-active metal centres that catalyse the dismutation reaction by a mechanism that is similar to the mode of action of the active-site metals of SOD and catalase.

Macrocyclic compounds are specific scavengers for superoxide anion. They have a manganese atom (Mn) at the center of the pentaazamacrocyclic ligand-based mimetics , which is held by five coordination points and is only available for one-electron transfers (122).

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a water-soluble analogue of the spin label TEMPO. Tempol is a stable piperidine nitroxide of low molecular weight (MW: 172), which permeates biological membranes. There is now good evidence that tempol exerts beneficial effects in animal models of shock, ischemia-reperfusion injury, inflammation, hypertension, diabetes and endothelial cell dysfunction. Although there is some controversy as to whether tempol and other stable nitroxides are "SOD-mimetics" or act as stoichiometric scavengers of superoxide anions, it should not be. There is clear evidence that tempol attenuates the effects of superoxide anions *in vitro* (123-125). Tempol also reduces the formation of hydroxyl radicals (126) and attenuates the cytotoxic effects of hydrogen peroxide, which is mediated by hydroxyl radicals (127). Tempol also inhibits the peroxynitrite-mediated nitration of phenolic compounds in the presence of a large molar excess of peroxynitrite, suggesting a catalytic-like mechanism (128).

There is additional evidence that tempol reduces blood pressure and endothelial dysfunction in certain animal models of hypertension (i.e. angiotensin II-induced hypertension and associated pathologies due to activation of NADPH oxidase) and diabetes. The doses of tempol necessary to protect tissues against the injury associated with the above disorders are relatively high (ranging from ~ 10 to 100 mg/kg for i.v. bolus administration of the compound). The effects of tempol in patients with chronic (rather than acute) inflammatory conditions, will require a better understanding not only of the toxicology, but also of

the pharmacokinetics of this compound after oral administration.

An enhanced formation of ROS and/or peroxynitrite importantly contributes to the multiple organ failure in hemorrhagic shock, and tempol may represent a novel therapeutic approach for the therapy of hemorrhagic shock.

The most promising clinical target for any future use of tempol is acute myocardial infarction (or any other condition associated with ischemia and reperfusion of the heart).

4.2.4. Manganese (II) (pentaazamacrocyclic ligand)-based complexes

This dual design goal of *high stability* and *high SOD activity* was achieved utilizing a combination of computer-aided modeling studies (129) and synthesis activities and has led to the development of a novel class of highly active superoxide dismutase catalysts which are also very stable complexes. These synthetic superoxide dismutase mimetics are exemplified by the prototypical complex, M40403, derived from the 15-membered macrocyclic ligand, 1,4,7,10,13-pentaazacyclopentadecane, containing the added bis (cyclohexylpyridine) functionalities (129). M40403 is a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function, and catalytic rate of native superoxide dismutase enzymes, but with the advantage of being a much smaller molecule (MW 483 *vs* MW 30,000 for the mimetic and native enzyme, respectively) (3, 4). Another important advantage of these synthetic enzymes is that they do not possess the bell shape curve that is a common characteristic to the native SOD enzymed. At low doses the SOD enzymes are anti-inflammatory whereas at high doses they exhibit pro-inflammatory effects (130). The pro-inflammatory effects of the SOD enzyme is not well understood, but it is speculated to be due to its reaction with the dismutation product hydrogen peroxide to generate hydroxyl radicals via Fenton chemistry (49). The lack of a bell-shaped dose response curve with the SOD mimetics may be related to the selective reactivity of the SOD mimics with superoxide and the complexes' inability to react with hydrogen peroxide.

The Mn (II)-based SOD mimetics such as M40403 and M40401 are *catalytic agents*; i.e., the compounds do not involve a stoichiometric interaction with a biological target, such as a receptor, but instead enhance the rate of conversion of superoxide to molecular oxygen and hydrogen peroxide without the complex itself being consumed. The SOD mimetics have been rigorously characterized for SOD activity by stopped-flow kinetic analysis, for *in vitro* stability by kinetic and thermodynamic assays, and for *in vivo* stability by electron spin resonance and radiolabel studies using H, and C-labeled mimics. The ability of the SOD mimetics to scavenge superoxide *in vivo* has also been demonstrated by electron spin resonance studies (88, 101, 131).

An important mechanism by which superoxide dismutase mimetics attenuate inflammation is by reducing peroxynitrite formation by simply removing superoxide anion before it can react with nitric oxide. This is important since the pro-inflammatory and cytotoxic effects of peroxynitrite are numerous (132). M40403 class of SODm block nitrotyrosine staining in models of inflammation, suggesting that superoxide anion-driven peroxynitrite formation is in fact responsible for the formation of nitrotyrosine and that it's inhibition could account for the anti-inflammatory effects of SOD mimetics. This in fact was the first evidence to show *in vivo* a superoxide-dependent nitration, since the M40403 class of SOD mimetics do not react with nitric oxide or peroxynitrite. A similar pattern of immunoreactivity for nitrotyrosine is observed in a lung model of pleurisy (115, 131, 133, 134).

M40403 and M40401 are stable *in vivo* for extended periods and are amphoteric with log P values of ~-0.3-0.4 (slightly hydrophilic). The recently developed M40401 (the *S,S*-dimethyl substituted derivative of the M40403 biscyclohexylpyridyl class of mimetic) actually possesses a higher catalytic activity at pH=7.4 than the native Mn SOD enzyme (122). All three agents (M40403 and M40401, SC 55850) have a catalytic rate greater than the native MnSOD enzyme at pH=6.5, for example.

In light of the critical roles of superoxide in disease, these new *selective*, potent and stable synthetic enzymes of superoxide dismutase, as represented by M40403, have broad potential both as pharmacological tools to dissect the role of superoxide in disease models where other such relevant biological oxidants may be present and be expected to play a role.

Several research groups have shown over the last several years that superoxide dismutase mimetics are anti-inflammatory (3, 4), and protective in models of septic shock and ischemia-reperfusion injury (13, 66, 135, 136). Superoxide contributes in a precise pattern of intracellular protein tyrosine nitration (137).

M40403 or M404041 by removing superoxide inhibit cytokines production at least in part through protection and preservation of endogenous catecholamines. Furthermore, the production of superoxide by activated neutrophils and macrophages is also negatively modulated by catecholamines through β-adrenergic receptor activation underlining the potential immunoregulatory effects of catecholamines (138, 139). This mechanism is potentially relevant to sepsis and multi organ failure.

Selective SOD mimetics should be considered as a therapeutic means to attenuate nitric oxide-driven inflammatory responses. In addition, superoxide by interacting with nitric oxide destroys the biological activity of this mediator (140), attenuating important anti-inflammatory and tissue protective properties of nitric oxide; namely, its maintenance of blood vessel tone, platelet reactivity, cytoprotective effects in numerous organs (including heart, intestine, and kidney) (141, 142) and release of anti-inflammatory and cytoprotective

prostacyclin (via activation of the constitutive cyclooxygenase enzyme) (2). Therefore, removal of superoxide protects nitric oxide and reduces the formation of the cytotoxic peroxynitrite.

Removal of superoxide inhibits the infiltration of neutrophils at sites of inflammation as shown by experiments performed in transgenic mice that over-express the human CuZnSOD enzyme (85) and by use of SOD mimetics such as SC-55858 and M40403 (3, 4, 143). This correlates well with an attenuation of lipid peroxidation and overall attenuation of acute and chronic inflammation. A possible mechanism by which SOD mimetics attenuate neutrophil infiltration is by down-regulating adhesion molecules such as ICAM-1 and P-selectin. The release of a variety of pro-inflammatory cytokines is also regulated by superoxide. Thus, SOD mimetics inhibit a number of inflammatory cytokines, including tumor necrosis factor α, interleukin-1β and interleukin-6 (TNF-α, IL-1β and IL-6 respectively) as shown in models of acute and chronic inflammation (134). The mechanism (s) through which superoxide regulate cytokines is under evaluation in various research groups. Recent data demonstrates that superoxide anions can directly release TNFα from macrophages (144). Interestingly, the anti-inflammatory cytokine IL-10 is not affected (4). Thus, removal of superoxide impacts the inflammatory cascade through at least 3 major pathways: 1) inhibition of peroxynitrite formation and sparing of nitric oxide, 2) inhibition of neutrophils infiltration at the site of inflammation, and 3) inhibition of pro-inflammatory cytokine release. It is possible that another mechanism which may reveal itself as being important through which superoxide might modulate the inflammatory response is through the deactivation of catecholamines (noradrenaline and adrenaline). Once deactivated, catecholamines are no longer able to exert their effect. Deactivation of catecholamines accounts for the hyporeactivity and hypotension associated with shock contributing therefore to the development of both the vascular abnormalities with this disease (57). In addition to their pivotal role in transducing the sequential cardiovascular and hemodynamic crisis following severe gram-negative bacteremic sepsis, endogenous catecholamines are instrumental in controlling the expression of cytokines. M40403 by removing superoxide inhibit cytokines production at least in part through protection and preservation of endogenous catecholamines. Furthermore, the production of superoxide by activated neutrophils and macrophages is also negatively modulated by catecholamines through β-adrenergic receptor activation, underlining the potential immunoregulatory effects of catecholamines (138, 139).

It has been shown that transgenic animals that over express SOD2 had a 35% reduction of infarct size compared to wild type animals (91).

Some important pro-inflammatory roles for superoxide include: endothelial cell damage and increased microvascular permeability (145), recruitment of neutrophils at sites of inflammation, auto-catalytic destruction of neurotransmitters and hormones such as norepinephrine and epinephrine respectively, lipid peroxidation and oxidation, DNA damage, activation of poly-ADP-ribose polymerase (PARP), inactivation of nitric oxide and formation of peroxynitrite (the reaction product of nitric oxide and superoxide) (3, 8, 57, 99, 140, 146). Peroxynitrite nitrates endogenous superoxide dismutase, the enzyme that keeps superoxide under tight control. Once nitrated, MnSOD and/or CuZn SOD lose their enzymatic activity, an event favoring the accumulation of superoxide and superoxide-driven damage (89, 147, 148). This is supported by evidence which shows that nitration of this enzyme is closely linked to those disease states driven by overt production of superoxide, for example, ischemia and reperfusion, organ transplantation, shock and inflammation, neurodegeneration such as Alzheimer disease, amyotrophic lateral sclerosis or AIDS dementia complex (149, 150).

4.3 Superoxide dismutase: preclinical and clinical experience

Protective and beneficial roles of superoxide dismutase have been demonstrated in a broad range of diseases, both preclinically and clinically. For example, preclinical studies have revealed that superoxide dismutase enzymes have a protective effect in animal models of ischemia-reperfusion injury (including heart, liver, kidneys, brain), transplant-induced reperfusion injury, inflammation, Parkinson's disease, cancer, AIDS, asthma, chronic obstructive pulmonary diseases and respiratory syncytial virus infections (18, 77, 85, 149, 151-164).

When tested in humans in various clinical trials, Orgotein® (bovine CuZnSOD) showed promising results in acute and chronic conditions associated with inflammation, including rheumatoid arthritis and osteoarthritis as well as side effects (acute and chronic) associated with chemotherapy and radiation therapy (165-170). Interestingly, the first clinical pilot studies with the native enzyme were done as early as 1970's in rheumatoid arthritis (RA) and osteoarthritis (OA) with preliminary results demonstrating efficacy. Further studies then showed that Orgotein®, when given by intrarticular injection, attenuates signs and symptoms (inflammation and pain) of RA and OA (165, 166). Interestingly, in these studies Orgotein® led to some 60% decrease in the consumption of analgesics. Furthermore, Orgotein® was found to be effective when given by intraarticular injection in patients with temporomandibular joint (TMJ) dysfunction who failed to respond to standard therapy (171) and was found to reduce pain in patients with duodenal ulcer pain (172). Possibly the most compelling data for the efficacy of SOD in human disease comes from a large body of data gathered since the early 1980's showing protective effects of Orgotein® (given by intramuscular injection) against acute and chronic side effects associated with chemotherapy and radiation therapy (168, 169, 173). Of major importance is that Orgotein® is able to reverse reverse fibrosis once it is established (174, 175). Other clinical settings in which SOD (whether of recombinant or bovine origin) was used include patients with Crohn's, various forms of periarticular inflammation, and Peyronie's disease (176).

Thus, in clinical trials, the use of the native enzyme supported the concept that removal of superoxide had a beneficial outcome. The main problem was the non-human origin of the enzyme: bovine. This inevitably gave rise to a variety of immunological problems, which eventually led to its removal from the market, except in Spain where it is still clinically used to prevent radiation-induced side effects.

Based on the concept that removal of superoxide modulates the course of inflammation, several studies have pursued the concept of designing synthetic, low molecular weight mimetics of the superoxide dismutase enzymes, which could overcome some of the limitations associated with Orgotein. This allows the synthetic superoxide dismutase mimetics to serve as pharmaceutical candidates in a variety of diseases in which the native superoxide dismutase enzyme was found to be effective.

4.4 Superoxide dismutase mimetics and hydrogen peroxide-driven toxicity

SOD and catalase are metalloproteins that employ efficient dismutation reactions in their mechanisms to detoxify ROS. A dismutation reaction involves a series of one- or two-electron transfers where the electrons are accepted from one $O_2^{-\cdot}$ or H_2O_2 and then donated to another (177). These efficient reactions do not require reducing equivalents and thus do not require energy from the cell to operate. The over expression of these enzymes in cell culture and in whole animals has provided protection against the deleterious effects of a wide range of oxidative stress paradigms. The main limitations of these natural products are their large size, which limits cell permeability, short circulating half-life, antigenicity and expense. An increasing number of low-molecular-weight SOD mimetics have been developed to overcome some of these limitations.

Hydrogen peroxide is itself not a radical but actually quite an inert oxidant whose cellular toxicity is probably in the 100 μM to mM range. The hydrogen peroxide's toxicity is likely due to the generation of reduced iron (Fe (III) is the oxidation state of iron in iron storage sites) which as Fe (II) reacts with hydrogen peroxide (Fenton reaction) undergoing homolytic cleavage to generate Fe (III) (OH) and hydroxyl radical. Iron (III) must first be reduced to "free" soluble Fe (II) for this reaction to occur, and one of the best reductants available in inflammatory, or reperfusion disease states is superoxide and it has been shown to be an excellent kinetically competent reductant of Fe (III) in iron stage sites liberating Fe (II) (178). Thus, superoxide becomes (not hydrogen peroxide) the culprit leading to generation of conditions favourable for Fenton chemistry to be initiated. Oxidation reactions involving hydrogen atom abstraction from a biological target molecule such as a catecholamine, DNA, RNA, and an allylic CH of a fatty acid, steroids, are free radical chain reactions and produce at least one hydrogen peroxide per oxidation in fact these are all free radical chain reactions that in the presence of oxygen will yield many molecules of hydrogen peroxide with one initiation from superoxide. When superoxide is dismuted, the stoichiometry is such that 2 superoxides and two protons generate as the net reaction one oxygen molecule and one hydrogen peroxide; thus, in effect each mole of superoxide now leads to ½ a mole of hydrogen peroxide. To date there is no evidence that *in vivo* Fenton reaction occurs (179, 180). In fact, Koppenol and colleagues have indicated that no iron complex has been identified *in vivo* that participates in the Fenton reaction (179). Thus, the use of SODm does not lead to a toxic condition by generating more hydrogen peroxide. Futhermore, when these molecules have been tested in the *in vitro* study of neutrophil-mediated injury of human aortic endothelial cells, no toxic effect has been observed (143). Several data support that hydrogen peroxide toxicity is not an issue when efficient and selective superoxide dismutation is achieved. Futhermore, the chronic treatment *in vivo*, with M40403 (10 days in a model of arthritis) did not exert any toxic effect (4).

All forms of SOD convert superoxide into H_2O_2 , which, in the presence of transient metals (iron or copper), forms a strong oxidant, the hydroxyl anion. Therefore, in the absence of an adequate detoxification of H_2O_2, SOD may aggravate oxidative stress (181). Catalase is a heme-containing tetrameric protein (*M* =240 kDa) that safely degrades H_2O_2 to water and oxygen. Thus, the tandem activity of SOD and catalase would be ideal for antioxidant therapies.

The discouraging results of animal and clinical studies can be attributed, at least in part, to unfavorable pharmacokinetic profiles and inadequate delivery of SOD and catalase.

The protective effect of the intravascular administration of SOD and catalase has been studied extensively in animal models of sepsis induced by systemic or local administration of endotoxin or cytokines. In these models, endothelium is a primary target for ROS released by activated leukocytes. Intravenous infusion of CuZnSOD attenuated vascular injury in animals injected with endotoxin and cytokines (182, 183).

Preliminary results from a limited number of clinical trials indicate that human subjects tolerate subcutaneous and systemic administration of 0.25 mg/kg SOD (184). However, in most animal studies, protective effects of SOD and catalase required multiple or prolonged intravenous infusions of much higher doses (10–150 mg/kg) than have been tested in humans (185-187).

5. NITRIC OXIDE

Nitric oxide (NO.) is a colorless gas with good solubility in water. NO is one of the simplest odd electron species. The half-life of NO in water is considerably longer (~3 sec) than would be expected for other free radicals. This is, in part, due to the reluctance of NO to dimerize and the thirdorder kinetics of its reactions with oxygen. However, NO reacts rapidly with superoxide anion to form peroxynitrite, a relatively stable product. In the gas phase, NO reacts with oxygen to form nitrogen dioxide (NO2'). In the aqueous phase, however, nitrite is produced. *In vivo*,

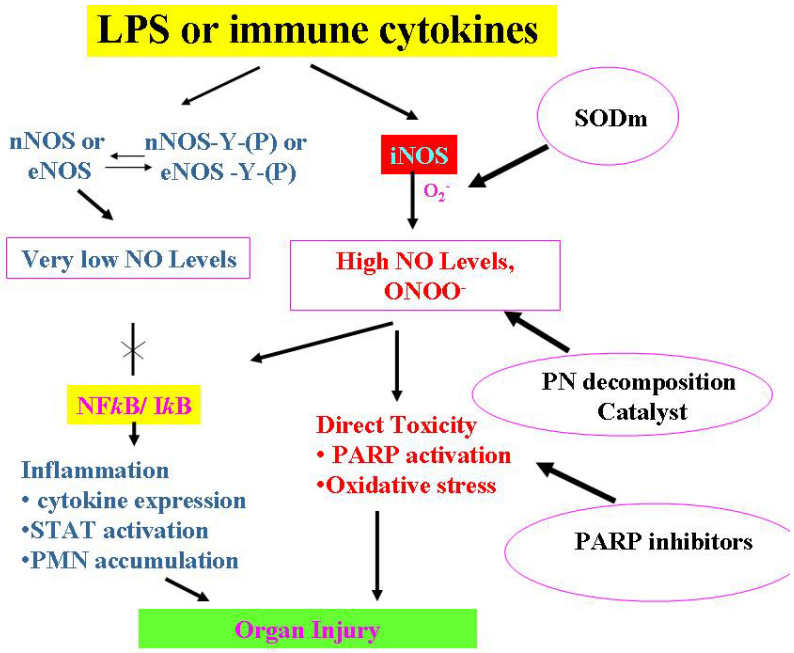

Figure 3. After inflammatory stimuli, a rapid shift of the equilibrium towards phosphorylated nNOS or eNOS occur which results in the abrupt fall in intracellular NO levels (i.e. very low NO levels). This situation can favor the activation of inducible NOS (iNOS) and the production of high levels of NO. The NO so generated combines with superoxide, forming peroxynitrite. Peroxynitrite exerts direct cellular toxicity through oxidative and nitrosative damage and PARP activation. NO, and perhaps peroxynitrite, activate inflammatory cascades through the activation of NF-κB, enhancing inflammation manifested by augmented cytokine expression, STAT3 activation, and tissue neutrophil accumulation. Collectively, these actions result in organ damage and dysfunction. In this schema, PN decomposition catalyst, SOD mimic, or PARP inhibitor each may prevent organ dysfunction and improve outcome.

nitrite reacts with various biologically active species (e.g., oxyhemoglobin), and the stable end-product that can be measured in the plasma is nitrate (188, 189). NO can react with thiols to form S-nitrosothiols, such as S-nitrosocysteine and Snitrosoglutathione. Some S-nitrosothiols, such as protein thiols, may have a significant stability (189-191). It is possible that NO circulates in the plasma as an S-nitroso adduct of serum albumin (191). While being a critical signalling messenger involved in the regulation of a vast array of physiologic functions, NO also has the ability to turn into a major cytotoxic effector involved in a number of pathophysiologic conditions and in the pathogenesis of a growing list of human diseases (192, 193). The proper effects of NO have often, and abusively, been assimilated to those of a family of NO-derived molecules, collectively termed reactive nitrogen species (RNS), which all possess their unique biochemical characteristics (194), thus creating serious confusion. NO as a free radical, is a highly reactive molecule, with a very short lifetime. Although the free radical nature of NO constitutes the chemical basis of its biological activity, its reactivity is relatively weak, and, basically, NO interacts only with transition metals, oxygen, and other free radicals (195). This low reactivity, combined to a high lipophilicity, confers to NO the potential to diffuse away from its point of origin, and thereby to carry out its function as a messenger molecule (196). The direct effects of NO prevail in conditions of low NO production and mainly support protective and signalling functions, which are consistent with the chemical biology of NO encountered under normal, physiologic conditions (196). In contrast, indirect effects will rather occur under high and sustained flux of NO as noted under pathophysiologic circumstances, and will essentially result in toxic consequences, which include oxidation, nitrosation (adjunction of NO+) and nitration (adjunction of NO2+) reactions (196, 197). The ability of NO to cause cytostasis and cytotoxicity in tumor cells and certain pathogens resulted in its initial perception as a beneficial mechanism to the host. Furthermore, the finding that NO derived from the endothelial nitric oxide synthase (eNOS) could block the adhesion of activated neutrophils suggested a beneficial role for NO in ischemia/reperfusion injury. However, the finding that the massive expression of the inducible nitric oxide synthase (iNOS) in conditions such as sepsis was associated with hypotension led to a change in this perception. Rapidly, data were published which implicated the aberrant expression of iNOS in numerous inflammatory conditions (e.g., inflammatory bowel disease, Crohn's disease, Alzheimer's disease, and hemorrhagic shock). This perception was strengthened by the demonstration that NO could lead to apoptosis of certain cell types during inflammatory diseases (Figure 3).

More recently, however, NO has been "re-invented" as a suppressor of inflammation. Nitric oxide was found to suppress the proliferation of lymphocytes and

cause their apoptosis. Simultaneously, NO was described to inhibit apoptosis of various cell types, notably hepatocytes, through mechanisms involving the nitrosative suppression of caspases.

In comparison to constitutive NOS isozymes, inducible NOS (iNOS) is thought to mediate the vast majority of pathophysiological effects attributed to NO and consequently this isoform is believed to be of fundamental importance to inflammatory process.

Under physiologic conditions, unlike eNOS and nNOS, iNOS is not expressed constitutively in mammalian cells, but rather is induced by pro-inflammatory stimuli such as bacterial lipopolysaccharide (LPS), or the cytokines TNF-α, IL-1β or interferon-γ (IFN-γ), individually, or in combination (198, 199). Therefore, once expressed iNOS continues to synthesize NO in large amounts for a prolonged period of time (190). iNOS activity is regulated by protein expression rather than functional modulation (200).

5.1. NOS inhibitors

Agents known to interfere with NF-κB activity seem to modulate the induction of iNOS. Many antioxidants including pyrrolidine-dithiocarbamate and diethyldithiocarbamate inhibit iNOS expression in cultured cells, in addition to non-selective protease inhibitors. Several other distinct classes of agents have been demonstrated to prevent expression of iNOS via inhibition of the NF-κB transduction system (201, 202). Glucocorticoids such as dexamethasone interfere with iNOS expression in many cell types (203); moreover, thrombin, macrophage deactivation factor, tumor growth factor b (TGF-b), platelet-derived growth factor (PDGF), IL-4, IL-8 and IL-10 inhibit iNOS induction. Induction of iNOS is associated with the induction of arginosuccinate synthetase, which may supply iNOS with its substrate from intracellular sources by turning on the “recycling” of L-arginine from L-citrulline (204). In some cell types, the same stimuli that induce iNOS also upregulate the membrane transport system for L-arginine, thereby supplementing intracellular L-arginine from extracellular sources. Some NOS inhibitors (e.g. NG-monomethyl-Larginine) also inhibit the Y+ cationic transporters system responsible for L-arginine and other cationic amino acid transport into the cells, whereas others (e.g. nitro-L-arginine) do not affect it .

Induction of iNOS may have either toxic or protective effects. Factors that appear to dictate the consequences of iNOS expression include the type of insult, the tissue type, the level and duration of NOS expression, and probably the redox status of the tissue. Much attention has focused on the toxic effects of iNOS. For example, induction of iNOS in endothelial cells produces endothelial injury (205). Induction of iNOS has been shown to inhibit cellular respiration in macrophages and vascular smooth muscle cells; these processes can lead to cell dysfunction and cell death. Such processes, when occurring within vascular smooth muscle cells, play a key role in the same cell where the activation of iNOS, (51) in turn, can act as NO donors, activating guanylyl cyclase (206, 207).

There are a large number of NOS inhibitors described in the literature and in use as pharmacological tools. Of these the most widely used have been L-NMMA, L-NNA and its methyl ester pro-drug (N°-vitro-L-arginine methyl ester, `L-NAME') and aminoguanidine.

Regarding the selectivity of NOS inhibitor there are many misleading statements in the literature. Several *in vivo* pharmacological effects of NOS inhibition have been associated with the functioning of one or other of the three isoforms, and effects or lack of them on these is sometimes used to infer isoform selectivity of action.

Frequently a misunderstanding is the definition of an inhibitor as selective for, e.g. iNOS versus eNOS, and then ignoring its non-selectivity for nNOS or completely distinct enzyme targets. An interesting example is aminoguanidine that is a partial selectivity for iNOS versus eNOS (208), the selectivity over nNOS is minimal. For this reason aminoguanidine should not be being described as a selective inhibitor. In the more subtle instances of agents which have selectivity versus only one NOS isoform, this needs to be made explicit when describing and using them. Furthermore, other difficulty that arises in assessing efficacy and selectivity of NOS inhibitors, and of comparing such data from different groups in the literature, is the frequent finding of time-dependent inhibition.

Inhibitors of NOS have been described which interact with the NOS enzymes in a variety of ways: different sites, as well as differing time- and substrate-dependence, and mechanism of inhibition.

In the search for selective nNOS inhibitors, some amino acids have been described, which are partially selective for nNOS versus eNOS and iNOS. For example, Sethyl- and S-methyl-L-thiocitrulline, and vinyl-LNIO all show time dependent inhibition of nNOS with significant selectivity versus isolated eNOS and iNOS enzymes (209-211). However, S-ethyl- and S-methyl-L-thiocitrutline appeared less selective in intact rat tissues and *in vivo*, raising questions over their usefulness as pharmacological tools. The non-amino acid ARL 17477 has been reported to be both a selective nNOS inhibitor *in vitro* and effective *in vivo* in animal models of brain damage in stroke (212, 213). The finding that peroxynitrite can be formed by the combination of superoxide with NO produced by ecNOS in pathophysiological conditions, such as the early phases of shock and reperfusion injury, has important practical applications, because it challenges the conventional wisdom, which proclaims that small amounts of NO are beneficial, while large amounts of NO are toxic. Based on these recent findings, one can propose that: (a) selective inhibition of iNOS does not always or necessarily prevent all NO-related cytotoxicity, and (b) in some circumstances (e.g., early phase of ischemia-reperfusion), inhibition of constitutively produced NO can be beneficial. Nevertheless, because of the numerous side effects of constitutive NO synthesis inhibition, it is difficult to

envision how such intervention may result in a clinically useful therapeutic approach.

Selective pharmacological inhibition of iNOS in inflammation is expected to exert its therapeutic benefit, without disturbing the physiological functions of ecNOS (such as inhibition of platelet and white cell adhesion, maintenance of vasodilator tone, etc.). This approach would be beneficial because it would inhibit self-amplifying circles of inflammation in the early phase of inflammatory process, and it would inhibit one important final common mediator of inflammation. Another therapeutic option would be to use scavengers that remove excess NO, superoxide, and/or peroxynitrite. This could preserve the essential basal levels of NO and suppress NO-mediated toxicity. Additional approaches may target delayed effectors of NO or/and peroxynitrite cytotoxicity. Because of massive synergism and redundancy in the pathways of inflammation associated with acute and chronic inflammation, it is likely that combined or appropriate sequential targeting of the above listed pathways will be more effective than targeting a single pathway.

Overt production of nitric oxide accounts at least in part for endotoxin induced hyporeactivity and hypotension (214). However whereas inhibitors of the inducible form of nitric oxide synthase such as aminoguanidine and N-iminoethyl-L-lysine attenuate hypotension they do not improve mortality (215, 216). In addition, results from iNOS knockout mice have been controversial, with some reporting reduced hypotension in shock models while others reporting no effects or detrimental ones (217, 218).

6. PEROXYNITRITE

The relative contribution of peroxynitrite in septic shock is not known with firmness since selective inhibitors of this reactive oxygen species have not been used. Nevertheless, peroxynitrite does deactivate catecholamines, is present in endotoxin shock and has been implicated in the pathophysiology of shock (219-222). Although chemical considerations favor the production *in vivo* of peroxynitrite, the actual demonstration of the presence or production of peroxynitrite in pathophysiological conditions is far from been straightforward.

The finding that peroxynitrite is produced during zymosan or LPS shock is not surprising, in light of the previous evidence for the overproduction of oxygen derived free radicals. Nitrotyrosine formation was initially proposed as a relatively specific means for detecting the "footprint" of peroxynitrite (131). Recent evidence, however, indicates that certain other reactions can also induce tyrosine nitration; for example, the reaction of nitrite with hypochlorous acid, and the reaction of myeloperoxidase and certain other peroxidases with hydrogen peroxide can lead to the formation of nitrotyrosine (223, 224). The exact contribution of peroxynitrite in septic shock awaits preclinical evaluation of agents which remove peroxynitrite such as the peroxynitrite decomposition catalysts (103). Some of these are anti-inflammatory and cytoprotective and do protect against endotoxin-induced intestinal damage (3). Therefore, the evidence implicating the role of peroxynitrite in a given pathophysiological condition can only be indirect. A simultaneous protective effect of superoxide neutralizing strategies and NO synthesis inhibition, coupled with the demonstration of peroxynitrite in the particular pathophysiological condition, can be taken as a strong indication for the role of peroxynitrite. However, it is likely that additional interactions of oxygen- and nitrogen-derived free radicals also contribute to the inflammatory cell injury.

Studies with the peroxidase-deficient mice reinforce previous discussions that a number of pathways apparently contribute to the nitration of tyrosine residues in proteins *in vivo* independently, or even simultaneously (225, 226).

6.1. Peroxynitrite decomposition catalyst

Peroxynitrite is cytotoxic via a number of mechanisms including initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of membrane sodium channels, modifications of oxidative protein and reduction in antioxidant enzymes (26, 227, 228).

The peroxynitrite anion is formed and can be prepared via by a number of pathways, particularly through various oxidations of nitrogen oxides and photolysis and radiolysis of solid nitrate salts. Formation of peroxynitrate by the combination of $NO^{\cdot}$ and $O_2^{\cdot-}$ radicals is quite favourable, as is the combination of $NO_2^{\cdot}$ and $HO^{\cdot}$ ($k_{obs} = 4.5 \times 10^9 M^{-1} s^{-1}$) (229, 230).

Peroxynitrite is stable in alkaline solution, but the conjugate acid ($pK_a = 6.80$) (230) is colorless and unstable, and isomerises rapidly to nitrate, which is considerably more stable ($\Delta H° = 40$ kcal/mole) (231).

Peroxynitrite and its conjugate acid are strong oxidants, capable of effecting one- and two-electron reactions akin to those of $HO^{\cdot}$, nitrogen dioxide ($NO_2^{\cdot}$) and nitrosoniun cation (NO_2^{+}). Oxidations of thiols, sulfides, transition metal complexes, deoxyribose halide ions, ascorbate, phenols and other aromatics by peroxynitrite have been described (8, 131, 231-238).

Pathologies driven by the formation of peroxynitrite are amenable to pharmacological intervention at the level of the reactant (nitric oxide, superoxide anions) or the product ($ONOO^-$). Recently it has been identified a novel class of anti-inflammatory agents: PN decompostion catalysts.

Such strategies must aim to decrease either the flux or the intrinsic lifetime of the peroxynitrite. Three particular approaches would accomplish such purposes: 1) blockage of peroxynitrite formation by limiting the availability of $NO^{\cdot}$ and $O_2^{\cdot-}$, either through inhibition of NOS or acceleration of superoxide dismutation; 2) competitive stoichiometric trapping of peroxynitrite; or 3) catalysis of peroxynitrite decomposition to benign products

(e.g., isomerization to nitrate). All three approaches afford possibilities for pharmacological intervention. Identification of a highly active catalytic peroxynitrite isomerase would facilitate destruction of many equivalents of peroxynitrite formed over an extended time interval from a single, substoichiometric drug dose. The possibility of such catalysis is real, and has now been demonstrated by highly stable molecules *in vitro*.

The initial experimental demonstration of peroxynitrite isomerization catalysis reported that addition of iron porphyrin complexes $(H_2O)Fe^{III}$ (L) (L= 5,10,15,20-*tetrakis*- (N-methyl-4'pyridyl)porphyrinato, TMPyP; 5,10, 15,20-*tetrakis*- (2',4',6'-trimethyl-3',5'-disulfonatophenyl)porphyrinato, TMPS) to solutions measureably reduced the lifetime of peroxynitrite under physiologically relevant conditions (pH 7.4, 37 °C). The product ion distribution shifted markedly towards innocuous nitrate at the expense of nitrite. For this reason, the search for other redox-active complexes that will accomplish catalysis of the peroxynitrite isomerization to nitrate continues.

Yields of oxidized catalyst intermediate and observed peroxynitrite lifetimes were nonlinearly dependent on peroxynitrite concentration; higher loadings saturated the catalyst in the oxidized state and raised the lifetime of the peroxynitrite. Addition of antioxidants, such as ascorbate, competitively quenches the intermediate and accelerates catalysis. Peroxynitrite decomposition becomes more efficient simply by increasing the concentration of either the complex or antioxidant, and harmful oxidations become less competitive. The synthetic porphyrin complexes also have an advantage over the endogenous enzymes in higher reactivity with antioxidants, which permits reductase activity to compete with the isomerization reaction and further decrease peroxynitrite lifetimes.

The role of peroxynitrite in cardiac dysfunction was recently investigated using peroxynitrite decomposition catalysts (239). In particular, cholesterol-enriched diet-induced hyperlipidemia leads to an increase in cardiac ONOO- formation and a decrease in the bioavailability of NO which contributes to the deterioration of cardiac performance and may lead to further cardiac pathologies.

Using a novel peroxynitrite decomposition catalyst, FP15, has been investigated the role of peroxynitrite in the pathogenesis of ischemia/reperfusion injury. The treatment with FP15 reduces myocardial infaret size and reactive hyperemia, infiammation and diabetes (240). Peroxynitrite is implicated in the pathogenesis of many chronic pathological conditions including arthritis, colitis and diabetes. FP15 treatment (3-10 mg/kg/d) dose dependently and reduced the incidence and severity of diabetes mellitus in rats subjected to multiple low doses of streptozotocin, as well as in nonobese mice developing spontaneous autoimmune diabetes (241).

It has reported that certain water-soluble iron (III) porphyrins are highly active and that they function by catalyzing the isomerization of $ONOO^-$ almost exclusively to nitrate. Catalysis is proposed to proceed via an oxo-Fe (IV) intermediate generated from the metal-promoted cleavage of the O-O bond. Subsequent recombination with NO_2 regenerates the Fe (III) state and produces nitrate. These catalysts thus dramatically increase the rate of $ONOO^-$ isomerization, preempting the formation of oxidizing radical species and generating the harmless nitrate anion (242).

A ferric porphyrin complexes catalytically isomerizes peroxynitrite to nitrate both *in vivo* and *in vitro*. They provide cytoprotection against peroxynitrite (EC50 = ~5 mM). Does not complex with nitric oxide and exhibits minimal SOD mimetic activity. Significant protective effects were observed with the selective peroxynitrite scavenger or decomposition catalyst, 5,10,15,20-tetrakis (2,4,6-trimethyl-3,5-sulfonatophenyi)porphyrinato iron III (FeTPPS). Base on these experimental evidence, direct pharmacological intervention with unique peroxynitrite decomposition catalysts specifically designed to decompose peroxynitrite to innocuous nitrate represents a novel strategy to tackle a wide range of disease states that are potentially govemed and driven by the overt production of this cytotoxic molecule (243, 244).

Peroxynitrite decomposition catalysts (PDCs) such as 5,10,15,20-tetrakis (*N*-methyl-4'-pyridyl)porphyrinato iron (III) (FeTMPyP) and 5,10,15,20-tetrakis (4-sulfonatophenyl)porphyrinato iron (III) (FeTPPS) were cytoprotective in focal cerebral ischemia demonstrating that the catalytic shunting of peroxynitrite to an innocuous form, *i.e.* nitrate, may have therapeutic utility in reducing tissue damage during inflammation (242).

The ability to intercept and decompose peroxynitrite may represent a novel and critical point of therapeutic intervention in diseases associated with the overproduction of NO and superoxide. These findings are consistent with the hypothesis that peroxynitrite may be ultimately responsible for the pathophysiology previously attributed to NO alone. The chemical synthesis and proposed mechanism of action of such compounds have recently been described as well as their efficacy in *in vivo* models of inflammation (242).

7. PARP

Poly (ADP) synthetase (PARS) (also known as PARP or poly (ADP-ribose) transferase) is a protein modifying and nucleotide-polymerizing enzyme that is present abundantly in the nucleus (245). Poly (ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme present in eukaryotes. PARP-1 is a 116-kDa protein consisting of three main domains: the N-terminal DNAbinding domain containing two zinc fingers, the automodification domain, and the C-terminal catalytic domain (246, 247).

The physiological function of PARS and poly (ADPribosylation) is still under much debate. From studies using pharmacological inhibitors of PARP, poly (ADPribosylation) has been suggested to regulate gene

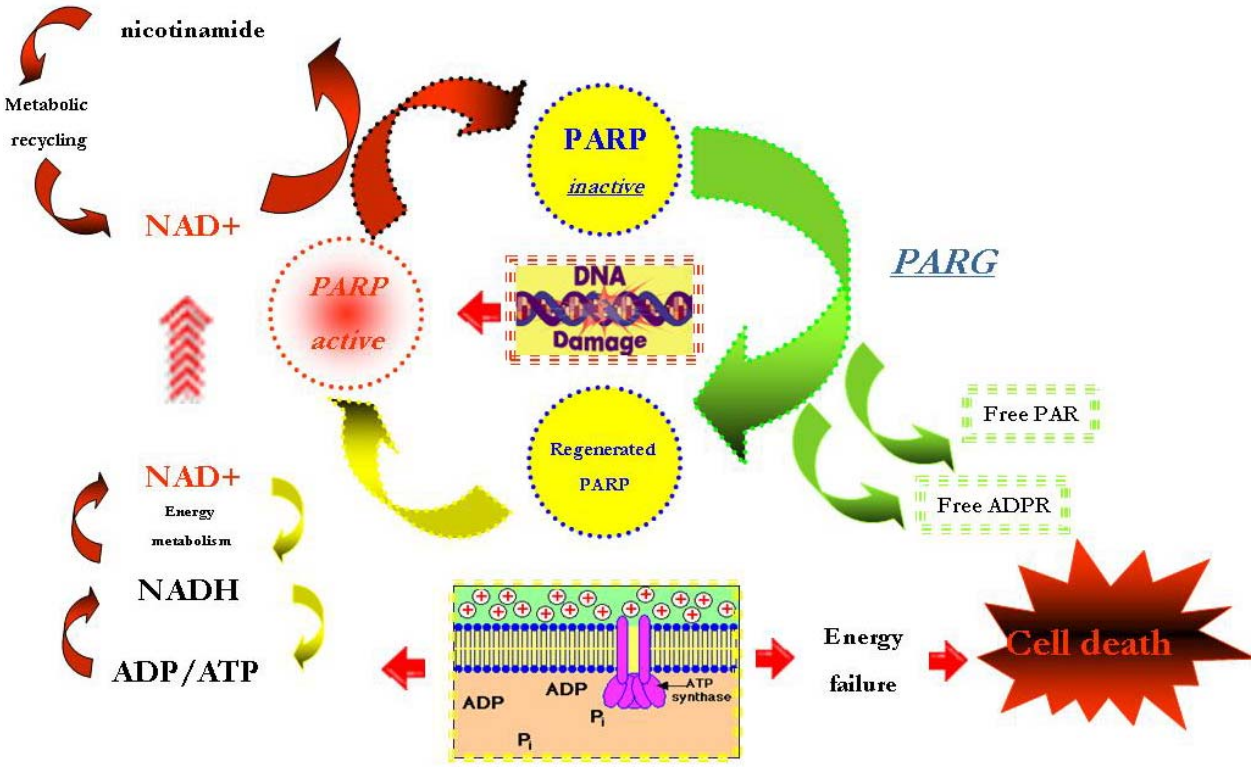

Figure 4. Poly (ADP-ribose) metabolism in response to DNA damage.

expression and gene amplification, cellular differentiation and malignant transformation, cellular division and DNA replication, as well as apoptotic cell death (245, 248). However, recent studies using cells from PARP (-/-) mice have failed to demonstrate a role for PARS in the process of apoptosis induced by various apoptotic signals, such as the Fas ligand or dexamethasone (249, 250).

The obligatory trigger of PARS activation is the nicks and breaks in the DNA strand, which can be induced by a variety of environmental stimuli and free radical (or oxidant) attacks. Reactive oxygen species and peroxynitrite cause strand breaks in DNA, which in turn results in the activation of the ubiquitous, chromatin-bound enzyme poly (adenosine 5'-diphosphate ribose) polymerase (PARP). Activation of PARP catalyses the transfer of ADP-ribose moieties from NAD^+ to various nuclear proteins including histones and PARP (automodification domain) itself (Figure 4). Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins (poly ADP-ribose chains or PAR chains) and results in a substantial depletion of intracellular NAD^+ and subsequently ATP, which may ultimately cause cell death. In the 1980s, Berger and Okamoto have observed this rapid depletion of NAD+ due to PARS activation, leading to cellular ATP depletion, and functional alterations of the cell, with eventual necrotic-type cell death: this process has been termed "the PARS suicide hypotesis" (Figure 4). Research into the "suicidal" role of PARS gained new momentum in the mid-1990s because of the observations *in vitro* that peroxynitrite can trigger DNA single-strand breakage and PARS activation (251, 252). $NO^{\cdot}$ and peroxynitrite can also inhibit mitochondrial respiration and exert other cytotoxic effects on their own. Thus, it is likely that a synergistic relationship exists between the PARS-mediated pathways and PARS-independent pathways of cellular metabolic suppression. Furthermore, the observations that $NO^{\cdot}$ and peroxynitrite are important mediators of the cellular damage in various forms of inflammation suggest that the PARS-related suicide pathway might play a role in various pathophysiological conditions *in vivo*.

During the last years, numerous experimental studies have clearly demonstrated the beneficial effects of PARP inhibition in cell cultures through rodent models and more recently in pre-clinical large animal models of acute and chronic inflammation.

Numerous studies have clearly demonstrate that PARP becomes rapidly activated in various pathophysiological conditions, and its activation is prolonged and sustained. For example, direct detection of poly (ADP-ribose) polymer accumulation has demonstrated the activation of PARP in stroke induced by middle cerebral artery occlusion and reperfusion and in the heart after myocardial infarction and heart transplantation (253-255). Similarly, PARP activation has been demonstrated in the gut, heart, and lung in hemorrhagic and septic shock, in the lung from mice subjected to a model of acute respiratory distress

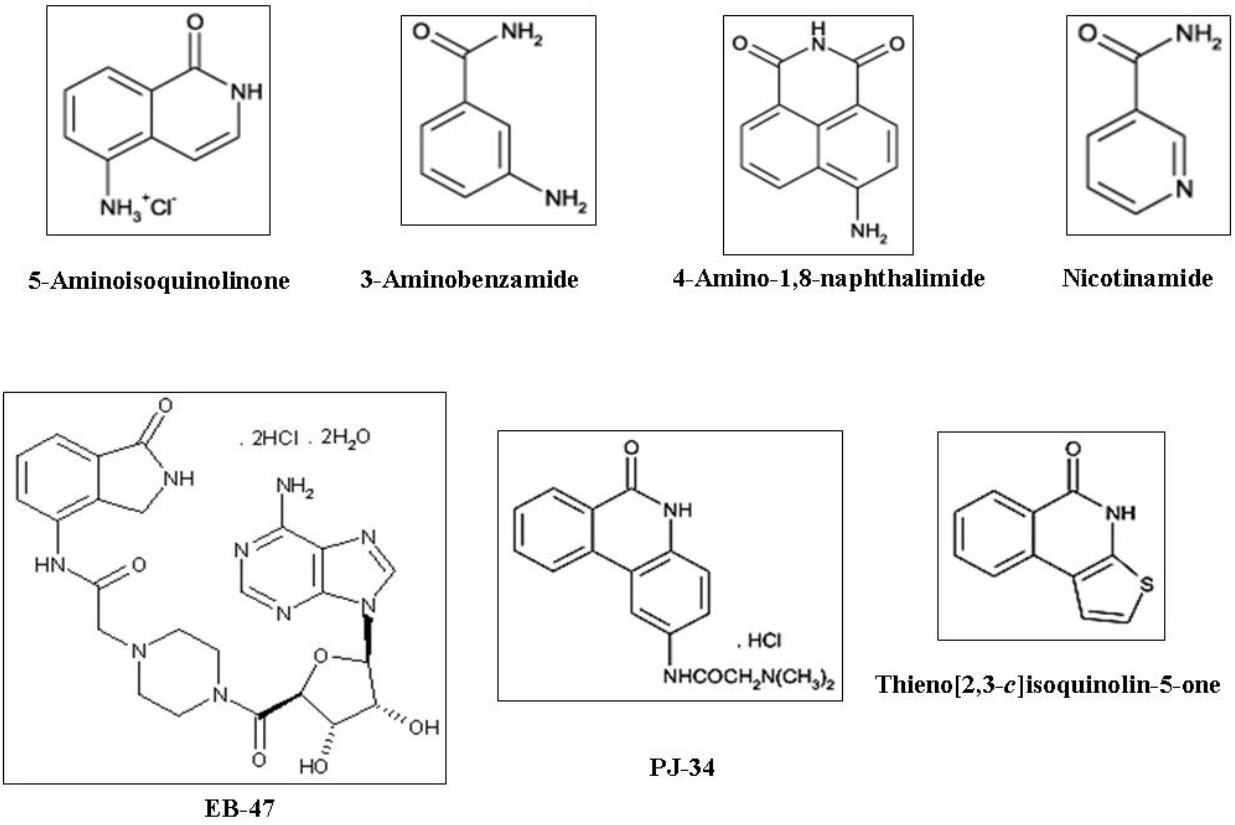

Figure 5. Several PARP inhibitors. Some of these inhibitors are entering clinical trials, some as chemotherapic agent, other for the treatment of acute cardiac ischemia. PARP inhibitors all share a carboxamide group attached to an aromatic ring, like the normal PARP substrate NAD^+, or a carbamoyl group built in a polyaromatic heterocyclic skeleton. Some of PARP inhibitors are 3-aminobenzamide, 1,5-dihydroxyisoquinoline, and 1,11b-dihydro- (2H)benzopyrano (4,3,2-de)isoquinolin-3-one (GPI 6150), which can be free radical scavengers. Newer, more potent inhibitors such as 3,4-dihydro-5- (4- (1-piperidinyl)butoxy)-1 (2H)-isoquinolinone (DPQ), indeno-isoquinolinone, *N* - (6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N* -dimethylacetamide (PJ34), and 5-chloro-2- (3- (4-phenyl-3,6-dihydro-1 (2*H*)-pyridinyl)propyl)-4 (3*H*)-quinazolinone inhibit PARP-1 activity at nanomolar or low micromolar concentrations.

syndrome as well as in the heart and blood vessels of diabetic animals (256-260).

7.1. PARP inhibitors

Several PARP inhibitors are now known, and some of these are entering clinical trials, some as chemotherapic agent, other for the treatment of acute cardiac ischemia (261) (Figure 5). The PARP inhibitors all share a carboxamide group attached to an aromatic ring, like the normal PARP substrate NAD^+, or a carbamoyl group built in a polyaromatic heterocyclic skeleton (262). Some of PARP inhibitors are 3-aminobenzamide, 1,5-dihydroxyisoquinoline, and 1,11b-dihydro-(2H)benzopyrano (4,3,2-de)isoquinolin-3-one (GPI 6150), which can be free radical scavengers (263). GPI 6150 induces hypothermia, by an uncertain mechanism (264). Evidence obtained with pharmacological PARP inhibitors of various structural classes, as well as animals lacking the PARP-1 enzyme, indicate that PARP plays an important role in cerebral ischemia/reperfusion, stroke and neurotrauma.

Newer, more potent inhibitors such as 3,4-dihydro-5- (4-(1-piperidinyl)butoxy)-1 (2H)-isoquinolinone (DPQ) (263), indeno-isoquinolinone, *N* - (6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N* -dimethylacetamide (PJ34) (265), and 5-chloro-2- (3- (4-phenyl-3,6-dihydro-1 (2*H*)-pyridinyl)propyl)-4 (3*H*)-quinazolinone (266) inhibit PARP-1 activity at nanomolar or low micromolar concentrations. Antioxidant effects are unlikely at such low concentrations, but effects at other sites, especially at NAD^+ -binding sites, cannot be excluded. It should also be noted that the currently available PARP inhibitors do not discriminate well between PARP-1 and the other PARP species.

Recently, it has been demonstrated that PARP-deficient cells are defective in NF-kB-dependent transcriptional activation (267, 268). Similarly, pharmacological inhibitors of PARP abolish mRNA expression of inducible nitric-oxide synthase, interleukin-6, and tumor necrosis factor-α in *in vitro* cultured cells (269).

Interestingly, tempol prevents the increase in PARP activity caused by hydrogen peroxide in cultured human cardiomyoblasts exerting beneficial effects and reducing the degree of inflammation and the associated tissue injury in animal models of diseases associated with local or systemic inflammation. These include rodent models of carrageenan-induced pleurisy, colitis, zymosan-induced multiple organ injury and uveoretinitis.

However, tempol did not inhibit the activity of inducible nitric oxide synthase in the lungs. Treatment of rats with tempol significantly reduced (i) the formation of peroxynitrite, (ii) the DNA damage, (iii) the impairment in mitochondrial respiration, and (iv) the fall in the cellular level of NAD^+ observed in macrophages harvested from the pleural cavity of rats treated with carrageenan. Tempol also attenuated the cell injury caused by hydrogen peroxide (1 mM) in cultured human endothelial cells (270).

Moreover, tempol attenuates the degree of chronic inflammation and tissue damage associated with collagen-induced arthritis in the rat.

Zamir and colleagues have recently reported that tempol exerts beneficial effects in a rodent model of experimental autoimmune uveoretinitis (EAU) (271). Poly-ADP-ribose polymerase is target for therapeutic intervention and its inhibitors such as nicotinamide and 3-aminobenzamide attenuate both acute and chronic inflammatory processes (272). SOD mimetics reduce poly-ADP-ribose polymerase immunofluorescence and attenuate the reduction of NAD^+ in models of acute and chronic inflammation (134). In light of the role of poly-ADP-ribose polymerase in inflammation, it is possible that poly-ADP-ribose polymerase inhibition by these SOD mimetics accounts for their anti-inflammatory response.

3-aminobenzamide (3-AB) (10 mg/kg 1h after carrageenan) attenuated the pleural exudation and the migration of polymorphonuclear cells caused by carrageenan. 3-AB also attenuated the lung injury as well as the increase in the tissue levels of myeloperoxidase caused by carrageenan in the lung. However, 3-AB did not inhibit the activity of inducible nitric oxide synthase in the lungs. Similarly, 5-aminoisoquinolinone (5-AIQ) reduced lung injury induced by intra-thoracic administration of zymosan-activated plasma. In particular, we have demonstrated that 5-AIQ attenuated the expression of P-selectin and intracellular adhesion molecules-1 (ICAM-1) as well as the recruitment of neutrophils into the injured lung (273). In addition, recent studies have clearly demonstrated that the infusion of INO-1001, a PARP inhibitor, significantly reduced the lalteration of the Pulmonary shunt fraction and the lung injury associated with smoke inhalation and Pseudomonas aeruginosa (5 x 109 cfu/kg) instillation in ovine (274, 275). Thus, PARP-1 inhibition attenuates the degree of acute inflammation and tissue damage associated with experimental lung injury. Recently it has been demonstrated that PARP-1 play an important role in the regulation of leukocyte recruitment within the lung with regard to the localization in the pulmonary microcirculation and in correlation to hemodynamics in the respective vascular segments and expression of intercellular adhesion molecule 1. The genetic (PARS knockout mice) or pharmacological (3-aminobenzamide) inhibition of PARP-1 reduced the endotoxin-induced leukocyte recruitment within pulmonary arterioles, capillaries, and venules in rabbits (276). In addition, inhibition of PARP suppressed the endotoxin-induced adhesion molecules expression. Therefore, PARP activation mediates the leukocyte sequestration in pulmonary microvessels through upregulation of adhesion molecules. As reactive oxygen species released from leukocyte are supposed to cause an upregulation of adhesion molecules, PARS inhibition contributes to termination of this vicious cycle and inhibits the inflammatory process.

Recent studies in a variety of rodent models of experimental colitis (induced by trinitrobenzene or diinitrobenzene sulfonic acid, dextrane sulfate solution or genetic interleukin (IL)-10 deficiency) support the role of PARP activation in the pathogenesis of inflammatory bowel disease (IBD) (277-279). Genetic ablation of the PARP gene or pharmacological inhibition of PARP with 3-aminobenzamide or 1,5-dihydroxyisoquinoline resulted in significant resistance to the damage induced by trinitrobenzene sulfonic acid administration, reduced nitrotyrosine formation and tissue levels of malondialdehyde, and reduced neutrophil recruitment into the injured tissue (280). Resolution of the damage was associated with reduction of neutrophil infiltration, nitrotyrosine formation and apoptosis. Treatment with PARP inhibitors also reduced DNA binding of NF-kappa B and AP-1 in the colon (280). Similarly, PJ34 exerts marked protective effects against the histological damage, lipid peroxidation, neutrophil infiltration, and mortality in a dextrane sulfate colitis model in the mouse (278). Moreover, recent study have assessed the role of PARP in the colitis seen in IL-10 gene-deficient mice. IL-10 gene-deficient mice demonstrated significant alterations in colonic cellular energy status in conjunction with increased permeability, proinflammatory cytokine release, and nitrosative stress. After 14 days of treatment with 3-aminobenzamide, IL-10 gene-deficient mice demonstrated normalized colonic permeability; a significant reduction in TNF-α and IFN-γ production, iNOS expression, and nitrotyrosine levels; and significantly reduction of inflammation.

Recently, 1,11b-dihydro- (2*H*) benzopyrano (4,3,2-*de*) isoquinolin-3-one (GPI 6150) caused a substantial reduction of (i) the degree of colon injury, (ii) the neutrophils infiltration, (iii) the increase in the tissue levels of lipid peroxidation, (iv) the increase in staining (immunohistochemistry) for poly (ADP ribose), as well as (v) the up regulation of ICAM-1 and P-selectin caused by dinitrobenzensulfonic acid in the colon, and (vi) the increase in TNF-α and IL-1β as observed in colonic tissues (279). All these data demonstrate that pharmacological inhibition of PARP ameliorates experimental colitis.

Several studies have been carried out in order to clarify the key role of PARP activation in the

pathophysiology of arthritis. In murine models of arthritis, inhibition of PARP with nicotinamide reduced the onset of the disease (281, 282). Inhibition of PARP not only prevented the development of arthritis, it also inhibited the progress of established collagen-induced arthritis. Furthermore, studies with 5-iodo-6-amino-1,2-benzopyrone, a novel PARP inhibitor that lacks oxyradical scavenging properties, also protected in a mouse model of collagen-induced arthritis: the PARP inhibitor reduces both the incidence of arthritis and the severity of the disease throughout the experimental period (102). Similarly, GPI 6150 was also found to be highly effective in a rodent model of adjuvant-induced arthritis (283). PJ34, another potent novel PARP inhibitor, was found to be highly effective in a murine model of collagen-induced arthritis (278). Finally, recently Virag and Szabo have indicated that PARP-1-deficient mice were found to be resistant against collagen-induced joint swelling and inflammation when compared with wild-type animals (284). These findings support the role or PARP in arthritis as well as the potential use of PARP inhibitors as therapeutic agents in human RA.

Similar to the effect of the pharmacological inhibitors, PARPKO animals were found to be resistant against zymosan-induced inflammation and multiple organ failure when compared with the response of wild-type mice (285).

In addition, several evidence suggest that activation of PARS may alter macrophage function in inflammatory processes (286). Macrophages harvested from the peritoneal cavity exhibited a significant production of peroxynitrite, as measured by the oxidation of the fluorescent dye dihydrorhodamine 123. Furthermore, zymosan-induced shock caused a suppression of macrophage mitochondrial respiration, DNA strand breakage, activation of PARS, and reduction of NAD+ cellular levels. *In vivo* treatment with 3-aminobenzamide (10 mg/kg intraperitoneally, 1 and 6 h after zymosan injection) or nicotinamide (50 mg/kg intraperitoneally, 1 and 6 h after zymosan injection) significantly inhibited the decrease in mitochondrial respiration and the activation of PARS, and partially restored the cellular level of NAD+. 3-aminobenzamide or nicotinamide significantly prevent the cardiovascular alteration (vascular hyporeactivity and endothelial dysfunction) associated with zymosan-induced multiple organ failure (287).

Various studies have clearly demonstrated that in isolated cells and tissues, peroxynitrite is capable of mimicking many of the pathophysiological alterations associated with shock (endothelial and epithelial dysfunction, vascular hyporeactivity, and cellular dysfunction), and these alterations are, in part, mediated by PARP activation (288). In studies in anesthetized rats, the inhibition of PARP with 3-aminobenzamide and nicotinamide reduced the suppression of the vascular contractility of the thoracic aorta *ex vivo* (289). In a more recent study in pigs injected with *Escherichia coli* endotoxin, pretreatment with 3-aminobenzamide eliminated the LPS-induced increase in pulmonary and total respiratory resistance, indicating that PARP activation plays an important role in the lung dysfunction associated with endotoxin- shock (290).

It is well demonstrated the ROS contribute to endothelial injury in circulatory shock (289). Various data have also demonstrated that 3-aminobenzamide significantly reduced the development of endothelial dysfunction in vascular rings obtained from LPS-treated rats suggesting that DNA strand breakage and PARP activation occur in endothelial cells during septic shock (289, 291). Therefore Zingarelli and colleagues have also demonstrated that the pretreatment of the animals with PARP inhibitors 3-aminobenzamide or nicotinamide significantly reduced the cellular energetic failure (289). On the contrary the use of 1,5-dihydroxyisoquinoline have demonstrated a small protective effect, whereas PJ34 treatment resulted in significant protection against liver and kidney dysfunction in endotoxic shock in the rat (292, 293). Another water-soluble, potent PARP inhibitor, 5-aminoisoquinolinone, significantly reduced the liver injury and dysfunction in hemorrhagic shock (294). Similarly it has been also demonstrated a protective effect against shock-induced intestinal epithelial permeability changes by different PARP inhibitors. In particular the treatment with by 3-aminobenzamide or by PJ34 exerts a protects effect against the intestinal hyperpermeability induced by endotoxic shock in rodents (295). Finally there are also various evidence that different PARP inhibitor such us 3-aminobenzamide (295), 5-iodo-6-amino-1,2,-benzopyrone (296), or PJ34 (293) improves survival rate in mice challenged with high-dose endotoxin. Therefore, recent studies have been carried out using wild-type and PARP deficient mice in response to high-dose endotoxin in order to evaluate the role of PARP in the development of liver damage during septic shock. 100% of the PARP-deficient animals survived at the treatment with (20 mg/kg) LPS-mediated shock, which killed 60% of wild-type animals (297). Similar results were obtained by another independent group (268). Moreover, LPS-induced necrotic liver damage was significantly reduced in the PARP-deficient mice (297). In contrast, when apoptotic liver damage was induced via injection of low concentrations of LPS into D-galactosamine-sensitized mice or via activation of hepatic cell-death receptors, PARP-deficient animals were not protected (297). Thus, PARP activation is involved in systemic LPS toxicity.

7.2. PARG inhibitor

PARG (EC 3.2.1.143) is a key enzyme for poly (ADP-ribose) catabolism by hydrolyzing both terminal ADP-ribose units from poly (ADP-ribose) polymers via exoglycosidic activity and removing larger oligo (ADP-ribose) fragments via endoglycosidic cleavage (298, 299). Since the *K*m value of PARG is much lower for larger (ADP-ribose) polymers than for smaller ones the enzyme probably removes and catabolizes bigger fragments first. PARG then switches to exoglycosidic mode and removes ADP-ribose units one by one. The specific activity of PARG compensates for the low abundance of the enzyme. Nearly 90% inhibition of PARG activity is required for cellular poly (ADP-ribose) accumulation. PARG is present in mammalian cells as multiple isoforms resulting from

alternative splicing of the primary mRNA transcript (300) or arising as alternative translation products (301).

PARG may serve regulatory functions and may also participate in the digestion of poly (ADP-ribose) synthesized by cytoplasmic PARP enzymes.

PARG has not been investigated as intensively as PARP-1, most likely because of difficulties in obtaining pure PARG enzyme. The difficulty lies in the low cellular abundance of the enzyme and its sensitivity to proteolytic degradation during purification (302, 303).

Poly (ADP-ribose) glycohydrolase (PARG) negatively regulates the cellular amount of PAR by degrading the PAR chains synthesised by PARP-1 and a number of additional PARP isoforms (304).

As summarized by Zhang and Li, only two classes of PARG inhibitors have been used extensively. They are ADP- (hydroxymethyl)pyrrolidinediol (ADP-HPD) and the tannin-carbohydrate complexes, such as oenothein B (305). The ADP-HPD, designed to mimic the structural ADP-ribose, is the most potent known PARG inhibitor (IC_{50}, = 0.12 μM, MW = 542). However, the poor membrane permeability of this class of PARG inhibitors did not favoured the development of this compounds as a drug. The tannin derivatives which are weak PARG inhibitors (IC_{50}, > 1.0 μM, MW = 1,000-10,000), have been most frequently used to inhibit PARG *in vitro*. The heterogeneous composition and significant vendor-to-vendor and batch-to-batch variation of tannin represents a major obstacle in PARG pharmacology (298, 306).

In cell-free assays, gallotannin, a complex mixture of tannins purified from oak gall, has been known to inhibit PARG and the compound also showed protection in cell culture models of oxidative stress-induced cell death (306-309). The lack of specific, potent and membrane permeable PARG inhibitors, however, hinders *in vivo* animal testing to definitively establish PARG's role in pathogenesis. Nevertheless, preliminary studies with a number of PARG inhibitors *in vitro* revealed their cellular protective effects.

Its cytoprotective actions were associated rather with the radical-scavenging potential of the compound. On the contrary, in astrocytes exposed to high concentrations of the nonoxidative DNA-damaging agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), PARP inhibitors were fully protective, while gallotannin enhanced the damage (310).

Two important disadvantages of these compounds, i.e., non-specific protein interaction and high molecular weight, limit the use of them as therapeutic drugs (311). Other compounds have also been used as PARG inhibitors, such us ethacridine, tilorone analogues, daunomycin or daunorubicin hydrochloride, ellipticine, and proflavine (312). Recently, Li *et al.* identified several families of small-molecule PARG inhibitors with improved potency such us *N*-bis- (3-phenyl-propyl)9-oxo-fluorene-2,7- diamide (GPI 16552 , IC_{50} = 1.7 μM, MW 503) or GPI 18214 (IC_{50} = 4.2μM) (313).

GPI-16552 was reported to significantly reduce the infarct volume in a middle cerebral artery occlusion model of cerebral ischemia (314).

The data obtained on utilzation of GPI 16552 are contrasted by a study in which the compound GPI 16552, had no effect in the different astrocyte death models compared with PARP inhibitors. In an *in vitro* PARG activity assay, the maximal inhibition that could be achieved with GPI 16552 was only 40% at a drug concentration of 80 microM. These conclude that neither GPI 16552 nor gallotannin are suitable for the evaluation of PARG in cellular death models, and that previous conclusions drawn from the use of these compounds should be interpreted with caution (310).

Furthermore, it was postulated that PARG might affect inflammation by inhibition of inducible nitric oxide synthase (iNOS) (315).

8. PERSPECTIVE

A growing body of data has emerged supporting a fundamental role of reactive oxygen species and in particular superoxide and/or peroxynitrite in septic shock. Understanding the signal transduction mechanisms used by these species to modify the course of disease will undoubtedly elucidate important molecular targets for future pharmacological intervention. The development of low molecular weight SOD synzymes such as M40403, might provide the tool necessary to achieve this. These SODm are manganese (II)-containing, non-peptidic molecules that possess the function and catalytic rate of native SOD enzymes, but with the advantage of being a much smaller molecule (MW 483 *vs* MW 30,000 for the mimetic and native enzyme, respectively). One important property is that they catalytically remove superoxide at a high rate without interacting with other reactive species including nitric oxide, peroxynitrite, hydrogen peroxide, oxygen or hydroxyl radicals. The s*electivity* of the SODm for superoxide is not shared by other classes of SOD mimetics or scavengers including several metalloporphyrins such as tetrakis- (N-ethyl-2-pyridyl) porphyrin and tetrakis- (benzoic acid)porphyrin, that interact with other reactive species such as nitric oxide and peroxynitrite.

The SOD mimics are *catalytic* drugs; the compounds do not involve a stoichiometric interaction with a biological target, such as a receptor, but instead enhance the rate of conversion of superoxide to molecular oxygen and hydrogen peroxide without the complex itself being consumed. *In vitro* and *in vivo* studies have demonstrated that the SOD mimetics have potent anti-inflammatory properties, attenuate myocardial ischemia-reperfusion injury, and prolong the half-life of nitric oxide, an anti-thrombotic and vascular relaxant.

A multitude of studies have demonstrated the role of PARP activation in a wide range of pathophysiological conditions. Furthermore, a series of animal experiments have proved that PARP-inhibition therapy represents an effective approach to treating a variety of diseases. The key to this effectiveness is that PARP inhibition targets a relatively late event of oxidative cell injury. The wide variety of disease models in which PARP inhibition proved beneficial also indicates that PARP inhibitors block a common pathway (s) of tissue injury, such as NF-kB activation or oxidative stress-induced cytotoxicity. It must be emphasized that data obtained from knockout studies cannot always be extrapolated to situations in which PARP is present but is inhibited by pharmacological agents. The development of isoform-selective PARP inhibitors and generation of knockout mice deficient in the PARP enzymes will clarify the biological roles of new PARP homologs. The marked beneficial effect of PARP inhibitors in many animal models of various diseases suggests that PARP inhibitors can be exploited to treat human inflammatory diseases. However, before potent PARP inhibitors can be used in humans, crucial safety issues must be addressed. Because PARP has been implicated in DNA repair and maintenance of genomic integrity, one possible risk associated with long-term PARP inhibition might be increased mutation rate and cancer formation.

That superoxide play a role in human disease has also been substantiated by encouraging results obtained in various clinical trials performed with Orgotein® (174, 175).

Despite the apparent clinical benefit, SOD enzymes as drug candidates were removed from the markets primarily because of immunogenic response. In certain cases where an enzyme of potential therapeutic benefit does not have the appropriate properties for a drug, a synthetic, small molecule enzyme mimetic can conceivably be designed with chemical and physical characteristics suitable for a therapeutic.

The unique *selectivity* of mimetics such as M40403 resides in the nature of the manganese (II) center in the complex. The resting oxidation state of the complex is the reduced Mn (II) ion; as a consequence, the complex has no reactivity with reducing agents until it is oxidized to Mn (III) by protonated superoxide, whereupon, the complex is rapidly reduced back to the Mn (II) state by the superoxide anion at diffusion-controlled rates. Since the complex is so difficult to oxidize (> +0.74 volts (vs. SHE) many one-electron oxidants cannot oxidize this and its related complexes (including nitric oxide and oxygen). Further, since the superoxide dismutase mimetics operate via a facile one-electron oxidation pathway, other two-electron non-radical, but nevertheless, potent oxidants are not kinetically competent to oxidize the Mn (II) complex. Although these agents are clearly protective in animal models, one general limitation of these agents is that they react not only with superoxide but also with a wide variety of reactive oxygen species. M40403 has successfully completed a Phase I safety clinical trial in healthy human subjects.

Like above mentioned, toxic effects of ROS and NO/peroxynitrite can also involve damage to DNA and increases in DNA single-strand breakage. Although it is difficult to establish the definitive mechanism by which the PARP inhibitors reduce the DNA binding of nuclear factors in *in vivo* experiments, data support the possibility that PARP may be an important modulator of transcription during inflammation (316).

As a central element of the network of inflammatory shock mediators, superoxide and/or peroxynitrite contribute significantly to organ dysfunction through multiple mechanisms. Even so, the most important feature of the shock state that ultimately determines survival is the reversibility of inadequate organ perfusion secondary to loss of vasomotor tone, which in turn leads to reduced venous return, cardiac output, and severe arterial hypotension. Despite such aggressive therapy successful outcomes are limited because of the development of vascular hyporeactivity to exogenously administered dopamine and norepinephrine. This hyporeactivity hampers the ability of the clinician to sustain blood pressure as exhibited in nonsurvivors of septic shock, in which blood pressure continues to drop despite administering progressively larger doses of dopamine and norepinephrine. Such inability to successfully restore and maintain an appropriate blood pressure leads to severe hypoperfusion of critical organs and eventually death. Therefore, the use of norepinephrine in septic shock results in a therapeutic paradox: thus it is one of the most commonly used vasopressors but its vasoconstrictor activity is broken down and deactivated by superoxide as soon as it is infused. Furthermore, endotoxin induced hypotension is completely abolished by the administration of M40403 a synthetic and selective (for superoxide) low molecular weight enzyme of superoxide dismutase. It has been known that superoxide interacts with catecholamines (these are in fact considered as antioxidants) converting them to adrenochromes. Some evidence exists to support a role of adrenochromes as specific mediators of cytotoxicity and cell damage, although their mechanism (s) of actions at this stage are not known. More related to the cardiovascular abnormalities of septic shock is the fact that adrenochromes have been shown to be cardiotoxic and to cause myocardial necrosis. Such adrenochrome-mediated cardiotoxicity would have adverse consequences for subjects with pre-existing compromise of ventricular function and systemic oxygen delivery owing to coronary artery disease, hypertension, and other conditions. Inhibition of endotoxin-induced hypotension by M40403 is associated with a reduction in the plasma levels of adrenochromes.

Based on these results, the use of a superoxide dismutase mimetic represents a new paradigm for the treatment of septic shock; namely, enhancement of host vasopressor responses by attenuation of superoxide-induced autooxidation of endogenous and exogenous catecholamines.

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Abbreviations: Reactive oxygen species, ROS; hydrogen peroxide, H_2O_2; superoxide anion, $O_2^{\cdot-}$**;** hydroxyl radical, $HO^{\cdot}$; superoxide dismutase, SOD; nitric oxide, NO; nitric oxide synthase, NOS; lipopolysaccharide**,** LPS; nuclear factor-kB, NF-kB

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