Superoxide dismutase as toxicity modulator

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Superoxide dismutase as toxicity modulator

G.J.M. den Hartog

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nutrim

The Graduate School

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Superoxide dismutase as toxicity modulator

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I

Superoxide dismutase as toxicity modulator:

General Introduction

G.J.M. den Hartog, G.R.M.M. Haenen, W.J.F. van der Vijgh, A. Bast

Superoxide or dioxide(-1), chemical formula: $O_2^{\cdot -}$ is the one-electron reduction product of oxygen. The chemist prepares $O_2^{\cdot -}$ by exposing metallic potassium (which is one of the strongest reductants known) to dry air. This results in the formation of a reactive, canary-yellow coloured salt: potassium superoxide or KO_2 which finds its daily application in self-contained rebreathers used by divers: Reaction of this compound with water results in the generation of oxygen. Interest in this radical increased when it was shown that it was formed *in vivo* where it plays an important, yet double role. Many enzymes produce $O_2^{\cdot -}$, e.g. NADPH oxidase in for example the phagocytes. Here $O_2^{\cdot -}$ is involved in the protection against invading microorganisms but it may also lead to lung tissue damage during inflammation.

1. Sources of $O_2^{\cdot -}$

Ischemia-reperfusion

$O_2^{\cdot -}$ is formed continuously in the body. However, there are certain pathological states where the generation of $O_2^{\cdot -}$ is dramatically increased. Following reestablishment of blood flow to previously ischemic organs and tissues, the damage inflicted is known as postischemic injury or ischemia-reperfusion injury. It was first observed in patients where blood flow was reestablished to ischemic extremities, but has also been found to be a problem following heart surgery, organ transplantation, thrombotic stroke, embolic vascular occlusions, angina pectoris and lung embolisms (1, 2). Overproduction of $O_2^{\cdot -}$ when oxygen is reapplied to the ischemic tissues appears to be largely responsible for the observed postischemic injury. How does $O_2^{\cdot -}$ generation occur after reperfusion?

Within a cell, mitochondria largely contribute to the production of $O_2^{\cdot -}$ via the respiratory chain. The respiratory chain is located in the inner membrane of the mitochondria where reduced $FADH_2$ and $NADH$ produce the proton gradient that subsequently incites ATP synthesis. Normally, the end product of oxygen in this process is water (Fig. 1) when oxygen accepts 4 electrons at complex IV (cytochrome c oxidase, the enzyme that handles approximately 98% of the oxygen we metabolize (3)). However, approximately 2 to 5% of the oxygen consumed during respiration (daily oxygen consumption is approximately 500 liter or 20 moles) is incompletely (i.e. 1 electron instead of 4) reduced to $O_2^{\cdot -}$. The major sites of $O_2^{\cdot -}$ production are within the complexes I and III (4) (Fig. 2). It is expected that exposure to high levels of oxygen increases the production of $O_2^{\cdot -}$. Rats that breath

100% oxygen die within 72 hr because pure oxygen causes damage to pulmonary tissues (5). While too much oxygen has toxic effects, for aerobic organisms a (temporary) shortage of oxygen also has toxic effects. During ischemia, when oxygen is absent to accept the electrons, an 'electron jam' emerges.

Figure 1. Molecular orbital diagram of oxygen (O_2). The two unpaired electrons in the $2p$ molecular orbitals give oxygen its biradical character and its exceptional oxidizing power. The ability to pick up a maximum of 4 additional electrons which results in the formation of H_2O render O_2 suitable to act as the final electron acceptor in the mitochondria.

Chemically, this can be described as the complete reduction of the electron transport chain; i.e. all complexes in the electron transport chain are in the reduced form. After reperfusion, when oxygen reaches the mitochondria, it reacts directly with the reduced complexes. This results in a massive production of $O_2^{\cdot -}$. Catecholamine levels are elevated in response to ischemia. Catecholamines can auto-oxidize, therefore reoxygenation might result in $O_2^{\cdot -}$ generation (1).

Figure 2. Representation of the respiratory chain in the inner membrane of the mitochondria. Normally electrons flow out of the chain at complex IV where oxygen is turned into water. Leakage of electrons (which occurs here at UQ (ubisemiquinone)) results in the formation of $O_2^{\bullet-}$. Protection against $O_2^{\bullet-}$ is offered by SOD2 in the mitochondrial matrix and by SOD1 in the cytosol.

Another process that takes place during ischemia is the transformation of xanthine dehydrogenase (XDH) into xanthine oxidase (XO) (Fig. 3). This transformation is a two-step process. The initial step involves the oxidation of important protein thiol groups. Cleavage of a 20 kDa fragment from each of the two subunits by a Ca^{2+} dependent protease subsequently converts XDH into XO (6). XO and XDH catalyze the oxidation of xanthine to uric acid. The major difference between the two is that XDH uses NAD^+ as an electron acceptor during oxidation of xanthine while XO uses oxygen as oxygen acceptor (Fig. 3). The change from XDH to XO in the ischemic tissues will lead to $O_2^{\bullet-}$ formation upon reoxygenation. A role for neutrophils in causing ischemia reperfusion injury has been suggested by many investigators (as reviewed by Welbourn et al. (7)). The NADPH oxidase system (Fig. 4) which is located in the neutrophil membrane is an important source of $O_2^{\bullet-}$. When the neutrophil is stimulated its oxygen consumption is dramatically increased (respi-

ratory burst) reducing it to $O_2^{\cdot -}$. Intracellular NADPH serves as the electron donor in this process (Fig. 4) (8).

Figure 3. The NAD dependent xanthine dehydrogenase (XDH) can be converted, by oxidation of sulhydryl groups or through proteolysis, to the O_2 dependent xanthine oxidase (XO). The conversion of XDH to XO during ischemia leads to the formation of the superoxide radical when oxygen is readmitted at reperfusion. Oxypurinol is a widely used inhibitor of XO.

Inflammation

During inflammation $O_2^{\cdot -}$ is used to kill pathogenic bacteria, fungi, and parasites. Phagocytic leukocytes (neutrophils, eosinophils, monocytes, and macrophages) contain NADPH oxidase (9). NADPH oxidase is dormant in the resting phagocyte but comes to life when the cell is activated by any of a variety of stimuli, including bacteria, inflammatory polypeptides or phorbol esters (protein kinase C (PKC) activation). To increase the bactericidal function of $O_2^{\cdot -}$, activated neutrophils also release the enzyme myeloperoxidase, which catalyzes the reaction between H_2O_2 and chloride anions to yield the bactericidal hypochlorous acid (HOCl) (10).

Figure 4. Simplified representation of NADPH oxidase showing the routes of passage for the electrons (e^-) and protons (H^+) generated from the oxidation of NADPH. Indicated are the reduction potentials (E_0') for the consecutive redox reactions that shuttle the electrons from the NADPH to O_2 .

Furthermore, in response to bacteria, phagocytes upregulate the inducible form of nitric oxide synthase (iNOS). Nitrosyl radicals (NO^*) that are generated by iNOS react with O_2^- which results in the generation of the cytotoxic peroxynitrite ($ONOO^-$)¹ (11). NADPH oxidase and its product O_2^- are therefore prominent participants in host defense. This becomes even more clear when NADPH oxidase is unable to shuttle electrons from NADPH to O_2 . Chronic granulomatous disease (CGD) is an inherited group of disorders in which phagocytes fail to undergo a respiratory burst when stimulated. As a result of the failure to activate the respira-

¹ Peroxynitrite ($ONOO^-$) should not be confused with nitrate (NO_3^-) which possesses the same brutto formula. In nitrate the three oxygen atoms are bound to a central nitrogen atom. In peroxynitrite, two oxygen atoms are bound to the nitrogen atom, the third is bound to another oxygen atom.

tory burst in their phagocytes, most CGD patients suffer from severe recurrent infections (12).

Biotransformation

Biotransformation of xenobiotics often results in the formation of $O_2^{\cdot-}$. Bioactivation by peroxidases is the proposed mechanism of toxicity for a number of drugs including the antipsychotics clozapine and chlorpromazine (Fig. 5).

Figure 5. Bioactivation of the antipsychotic drugs clozapine and chlorpromazine by peroxidases to reactive intermediates (X = N or S).

The aromatic amine structure of clozapine and chlorpromazine renders them susceptible to peroxidation by peroxidases (13). This is generally a one-electron transfer resulting in the formation of a positive radical nitrenium ion (Fig. 5). These strong electrophilic compounds will react very rapidly with thiols, including the ubiquitous glutathione or NADPH yielding thiyl radicals or the NADP $^{\cdot}$ radical (Fig. 6). The thiyl radical can react with a glutathione thiolate anion to the reduced form of glutathione disulfide (GSSG). This anion subsequently shuttles its electron to oxygen yielding $O_2^{\cdot-}$ (Fig. 6).

Figure 6. Reactions of carbon centered radicals ($R^{\bullet}$) with reduced glutathione (GSH) or NADPH under aerobic conditions results in the formation of $O_2^{\bullet-}$.

$NADP^{\bullet}$ can donate its extra electron directly to oxygen resulting in the formation of $O_2^{\bullet-}$. This mechanism strongly resembles that of the 'free radical sink hypothesis', where $O_2^{\bullet-}$ is also formed as oxygen serves as the acceptor of unpaired electron of carbon-centered radicals and glutathione serves as H-atom donor (14). The $O_2^{\bullet-}$ radicals that are formed this way are held responsible for the agranulocytosis observed in 1 - 2% of the patients that receive clozapine for the treatment of schizophrenia (15, 16). Another route by which exposure to xenobiotics can lead to the formation of reactive oxygen species (ROS) is (an attempt) to metabolize them via cytochrome P-450 (CYP450) enzymes. CYP450s catalyze a range of oxidative transformations, including carbon hydroxylation, heteroatom oxidation, π bond oxidation, and hydrocarbon desaturation. This makes compounds more polar and prepares them for phase II transformations. A key step in the catalytic cycle of the CYP450s is the formation of the ferrous dioxy complex ($Fe^{2+}-O_2$). From this complex, dissociation of $O_2^{\bullet-}$ can occur, resulting in the uncoupling of NAD(P)H consumption from organic substrate oxidation. A low degree of uncoupled turnover is normally observed with the resting enzyme, but the extent of uncoupled oxygen and NAD(P)H consumption is greatly increased by the binding of certain substrates. Especially barbiturates and chlorinated hydrocarbons are known to induce CYP450-catalyzed $O_2^{\bullet-}$ formation (17). CYP450 uncoupling is thus a substrate-dependent catalytic process that produces $O_2^{\bullet-}$ instead of oxidized metabolites. Redox cycling is a third way by which compounds can increase $O_2^{\bullet-}$ production. Compounds that are well-known for their redox cycling behavior include the herbicide paraquat, menadione (vitamin K_3) and many useful drugs. One of the most

widely studied redox cycling drugs is the anthracycline antibiotic doxorubicin, clinically known as Adriamycin®. The quinone structure that these compounds have in common permits them to act as electron acceptors, the transfer being mediated by flavoprotein enzymes including mitochondrial NADH dehydrogenase (18), microsomal NADPH-cytochrome P-450 reductase (19) and cytochrome b_5 reductase (20). Acceptance of the electron turns the quinone into the semiquinone radical. The semiquinone then shuttles the electron to oxygen, generating $O_2^{\cdot-}$ while at the same time the quinone form is regenerated. The quinone containing structure combined with the reductase thus serve as catalysts to make the reaction between NAD(P)H and oxygen possible which is otherwise kinetically restricted, i.e. an energy barrier prevents the spontaneous reaction.

2. Actions of $O_2^{\cdot-}$

$O_2^{\cdot-}$ with its odd number of electrons is a radical. A radical is defined as any atomic or molecular species capable of independent existence that contains one or more unpaired electrons in one of its molecular orbitals (21).² Since most atoms and molecules strive for a paired number of electrons, radicals are highly reactive.

$O_2^{\cdot-}$ can act as both an oxidant and a reductant. A $O_2^{\cdot-}$ anion will try to pick up another electron (thereby oxidizing the compound that provides this electron) or will try to get rid of its extra electron (thereby reducing the compound that picks it up). Furthermore, upon reaction of a radical with a non-radical molecule which possesses an even number of electrons, another radical is formed. This results in a chain reaction that can only be stopped upon reaction with either another radical or an electron donating species. $O_2^{\cdot-}$ is thus capable of initiating chain reactions in bioorganic compounds or reducing or oxidizing transition metal ion containing proteins. Abstraction of a hydrogen atom from polyunsaturated fatty acids by $O_2^{\cdot-}$ (Fig. 7), yielding lipid radicals, is a fast reaction with rate constants exceeding $10^7 \text{ M}^{-1}\text{s}^{-1}$ (22). Lipid radicals react with oxygen to lipid peroxyl radicals which abstract hydrogen atoms from other polyunsaturated fatty acid. This process, lipid peroxidation (LPO), is a chain reaction which is only interrupted when two radicals meet or when a stable radical is formed, for example after reaction with vitamin E. LPO results in an altered permeability of the membrane, resulting eventually in the death

² A deviation on this rule is formed by the paramagnetic transition metal ions which are not considered to be free radicals, although by technical definition they are (3).

of the cell (23-26). Indeed, during reperfusion, the most acute problem resulting from the increased production of $O_2^{\cdot -}$ appears to be the greatly increased rate of LPO. $O_2^{\cdot -}$ acts as a two-edged sword in the process of LPO. Apart from being able to initiate LPO $O_2^{\cdot -}$ also seems capable of terminating the lipid peroxidation chain reaction, probably by donating an electron to a lipid radical or a lipid peroxy radical (27). Other compounds from which $O_2^{\cdot -}$ can abstract hydrogen atoms are the catecholamines (Fig. 7). $O_2^{\cdot -}$ mediated inactivation of catecholamines has been shown to contribute to the loss of vascular reactivity to norepinephrine and the subsequent hypotension that develops in endotoxic shock (28). Very sensitive to oxidation by $O_2^{\cdot -}$ are the [4Fe-4S] clusters of dehydratase enzymes, such as the aconitases and fumarase A and B. The oxidized clusters are unstable which results in the release of Fe^{2+} (Fig. 7). The combination of Fe^{2+} and hydrogen peroxide leads to the formation of the very reactive hydroxyl radical (29).

Another important target for $O_2^{\cdot -}$ is protein kinase C (PKC) (Fig. 7), which $O_2^{\cdot -}$ activates via oxidation of thiol groups. Zinc is subsequently released from the zinc finger motif of the cysteine-rich region (30). Alternatively, $O_2^{\cdot -}$ increases the activities of receptor-regulated enzymes including phospholipase A_2 . The formation of lipid second messenger (diacylglycerol) that results from the increased activity of the phospholipases has an activating activity on PKC as well as on other protein kinases (31). Thus there are two ways by which $O_2^{\cdot -}$ can activate PKC. Interesting in this respect is that activation of PKC results in increased $O_2^{\cdot -}$ production via activation of NADPH oxidase (32). This example of a positive feed-forward mechanism ($O_2^{\cdot -}$ activating PKC which in turn induces $O_2^{\cdot -}$ generation) could have serious consequences for the tissue in which it takes place. PKC is known to influence a wide variety of cellular processes including proliferation, differentiation, immune response and synaptic transmission. It is therefore likely that PKC plays an important role in sensing oxidative stress and helping the cell to survive this threat, i.e. adaptation to oxidative stress. Other cellular constituents that are closely linked to protein kinases and $O_2^{\cdot -}$ are the transcription factors NF- κ B and AP-1 (Fig. 7).

Figure 7. Superoxide as ‘the root of cellular evil’. Superoxide is the precursor of an astonishing arsenal of highly reactive oxidizing species and reacts with important cell constituents such as DNA/RNA, lipids, proteins. Furthermore, superoxide is capable of activating protein kinases and important nuclear transcription factors including NF- κ B.

These transcriptional regulators are activated in response to increased $O_2^{\bullet-}$ concentrations in various tissues. The mechanism of this activation probably involves oxidation of critical cysteine residues in the transcription factors and/or increased phosphorylation mediated by protein kinases. Activation of NF- κ B and AP-1 results in the increased transcription of pro-inflammatory mediators including tumor necrosis factor- α , the interleukins 1 and 6 and inducible nitric oxide synthase (33). Currently, evidence is piling up that $O_2^{\bullet-}$ serves as an essential participant of cellular communication, in addition to its oxidative properties. Ligands such as angiotensin II and serotonin which act via G protein-coupled receptors have been shown to initiate $O_2^{\bullet-}$ production in peripheral tissues but also in the CNS (34). This occurs via coupling to enzymes that resemble NAD(P)H oxidases and that produce $O_2^{\bullet-}$ at the expense of reducing equivalents. Receptor mediated production of $O_2^{\bullet-}$ has been shown to induce hypertrophy, p38 activation, extra-cellular signal related kinase (ERK) activation and proliferation in smooth muscle cells (35).

3. Fate of $O_2^{\cdot -}$

Hydrogen peroxide (H_2O_2)

Because $O_2^{\cdot -}$ is both an oxidant and a reductor, one $O_2^{\cdot -}$ can react with another resulting in the formation of oxygen and hydrogen peroxide (H_2O_2) (Fig. 7). This reaction is called a dismutation because the substrate ($O_2^{\cdot -}$) reacts with another $O_2^{\cdot -}$ to give an oxidized product (O_2) and a reduced product (H_2O_2).

An alternative source of H_2O_2 are the monoamine oxidases (MAOs) which catalyze the oxidative deamination of primary aromatic amines and are located on the outer mitochondrial membrane (36). H_2O_2 is found in very high concentrations inside peroxysomes where it is important for peroxidase catalyzed oxidations of numerous compounds (37). H_2O_2 is a weaker oxidant than $O_2^{\cdot -}$. However, in the presence of transition metals such as iron or copper, H_2O_2 can give rise to the reactive hydroxyl radical ($HO^{\cdot}$). This radical is formed from hydrogen peroxide in a reaction catalyzed by reduced transition metal ions (Fe^{++} or Cu^+), often complexed with different proteins or other molecules. These ions transfer a third electron to H_2O_2 , causing lysis of the O-O bond, and remain in the oxidized form after the reaction. One fragment is reduced to the state of water, while the other fragment is the hydroxyl free radical, one of the most potent oxidants known, with a standard reduction potential of 2.8 V (second only to F_2 with has a standard reduction potential of 3 V). The formation of $HO^{\cdot}$ from H_2O_2 has been named after Fenton who used transition metal ions to increase the oxidative strength of H_2O_2 in organic reactions (38):

Their Fenton catalytic property is the major reason that free copper and iron ions are toxic even in relatively low concentrations and very efficient transport systems for these ions exist (39, 40). $O_2^{\cdot -}$ plays a three-fold role in the generation of hydroxyl radicals. First of all dismutation of $O_2^{\cdot -}$ leads to formation of hydrogen peroxide. Secondly, $O_2^{\cdot -}$ is capable of releasing iron from its stores. Thirdly, oxidized transition metal ions can be reduced to their Fenton active form by $O_2^{\cdot -}$. $HO^{\cdot}$ can

initiate lipid peroxidation, cause DNA strand breaks, and, due to its very high reduction potential, indiscriminately oxidize virtually any organic molecule (3). Its high reactivity also implies that its half-life is very short and that the volume in which $\text{HO}^\cdot$ is formed and subsequently acts as an oxidant, is highly limited.

In contrast to $\text{O}_2^\cdot$ (41), the neutral H_2O_2 is capable of penetrating biological membranes which renders H_2O_2 more useful to serve as an intracellular signaling molecule. H_2O_2 is removed by at least three antioxidant systems viz. catalases, (glutathione) peroxidases and peroxiredoxins. Catalases catalyze the dismutation of hydrogen peroxide, because H_2O_2 can be a weak reductant as well as a fairly strong oxidant:

Peroxidases catalyze the reduction of H_2O_2 to water. In the case of glutathione peroxidase (GPx) this reaction is coupled to the oxidation of GSH to GSSG. GPx can also reduce other peroxides (e.g., lipid peroxides in cell membranes) to alcohols. The peroxidases in the peroxysomes couple the reduction of H_2O_2 to the oxidation of a wide variety of organic compounds which they do so efficiently that no hydrogen peroxide escapes from the peroxisomal confinement (37). Peroxiredoxins are recently discovered enzymes capable of directly reducing H_2O_2 and other peroxides. The peroxiredoxin becomes oxidized in this process and is regenerated by the thioredoxin system. The thioredoxin system consists of two enzymes, thioredoxin and thioredoxin reductase. The latter uses NADPH as an electron source for the reduction of the active site disulfide in thioredoxin (42).

Hypochlorous acid (HOCl)

Apart from $\text{O}_2^\cdot$ generation, activation of phagocyte cells also results in the release of the heme-containing enzyme myeloperoxidase (MPO) (10). Unlike haemoglobin and most other heme-containing proteins (except chlorophyll), MPO is green, not red, and is the substance that imparts the greenish color to pus. This unusual color arises because the heme group is linked to MPO by three covalent bonds, which force the heme into a dome configuration, altering its optical spectrum (9). MPO catalyzes the reaction of H_2O_2 with chloride anions to give hypochlorous acid (HOCl) (43) (Fig. 7). HOCl is a major bactericide (10), which is also used in household cleaning and for purification of drinking water. HOCl reacts rapidly with

many biological molecules including DNA/RNA, proteins, lipids, cholesterol and polysaccharides (44). Especially proteins are targeted by HOCl since this oxidant favours reaction with thiol groups and thioethers, and to a somewhat lesser extent with amine and amide functions which form the peptide bonds (45, 46). This can result in side chain damage, fragmentation, aggregation, and an increased susceptibility to proteolytic degradation. The protein will thus lose at least part of its function (46-48). Overproduction or misplaced production of HOCl is very damaging for neighbouring cells and is believed to play a prominent role in certain human diseases. In the lungs, a prominent effect of HOCl is the inactivation of α_1 -antiprotease (49) by oxidation of methionine-358 (50). Due to this inactivation, the ability of α_1 -antiprotease to inhibit elastase is lost. The resulting higher activity of elastase leads to increased breakdown of elastin, eventually resulting in a complete loss of elasticity of lung tissue which manifests itself as emphysema (49, 50). The reaction of HOCl with amines results in the formation of chloramines (RNHCl) (44). These compounds can react with $O_2^{\cdot -}$ to form reactive nitrogen centered radicals (Fig. 7).

Reaction with nitric oxide: Formation of peroxynitrite

Nitrosyl radicals, in biomedical and biochemical literature known as nitric oxide (51) or $NO^{\cdot}$ react with $O_2^{\cdot -}$ at near diffusion-limited rates when both are produced simultaneously (11). $NO^{\cdot}$ is therefore one of the few biomolecules that is able to outcompete superoxide dismutases for $O_2^{\cdot -}$. The reaction product, peroxynitrite ($ONOO^{\cdot}$, PON) (Fig. 7) is a potent oxidant that, when protonated (pK_a 7.49) has $HO^{\cdot}$ like activity (52), i.e. it readily abstracts hydrogen atoms, resulting in LPO and oxidation of proteins and DNA.

The reaction of $NO^{\cdot}$ with $O_2^{\cdot -}$ is generally regarded as an inactivation of $NO^{\cdot}$. Because $NO^{\cdot}$ is such an important regulator of vascular tone, the role of $O_2^{\cdot -}$ in blood pressure regulation is well investigated. Increased aortic adventitial $O_2^{\cdot -}$ production contributes to hypertension by blocking the vasodilatory effects of NO (53). The origin of the $O_2^{\cdot -}$ in spontaneously hypertensive rats (SHR) is not known. Xanthine oxidase activity was not higher, although higher uric acid levels were found in SHR compared to normal rats. Inhibition of xanthine oxidase decreased blood pressure in SHR but not in normal rats (54). Furthermore, angiotensin II-induced hypertension is mediated in part by the interaction of $O_2^{\cdot -}$, produced by an NAD(P)H oxi-

dase and $\text{NO}^\bullet$ in vascular smooth muscle cells (55). The endothelial dysfunction observed during hyperglycemia is also believed to be mediated by $\text{O}_2^{\bullet-}$. Scavenging of $\text{O}_2^{\bullet-}$ by superoxide dismutases or restoring $\text{NO}^\bullet$ levels using $\text{NO}^\bullet$ donors can prevent the development of endothelial dysfunction (22).

Superoxide dismutases

A blue copper-containing protein was obtained from bovine erythrocytes by Mann and Keilin in 1939. It was called hemocuprein but its function remained unknown until the 1960s, when Huber discovered that hemocuprein possessed antiinflammatory properties. Hemocuprein soon found an application as an antiinflammatory drug under the name Orgotein which was used for the treatment of racing horses and dogs (56). In 1969, it was discovered that hemocuprein or erythrocuprein catalyzes the dismutation of $\text{O}_2^{\bullet-}$ to H_2O_2 and O_2 (57, 58). Erythrocuprein was renamed superoxide dismutase or SOD and was the first enzyme that was discovered with a free radical as substrate (57). The uncatalyzed or spontaneous dismutation of $\text{O}_2^{\bullet-}$ is already quite fast so why do we need SOD? The answer lies in the fact that the spontaneous dismutation has a rate constant in the order of $10^{4.5} \text{ M}^{-1}\cdot\text{s}^{-1}$. This is relatively slow when compared to the rate at which $\text{O}_2^{\bullet-}$ can abstract a hydrogen atom from biological targets such as catecholamines or lipids where the rate constant exceeds $10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$ (22). Spontaneous dismutation is relatively slow because the uncatalyzed dismutation of $\text{O}_2^{\bullet-}$ requires collision of two $\text{O}_2^{\bullet-}$ radicals, which repulse each other because they are both negatively charged. Because the chance that two $\text{O}_2^{\bullet-}$ radicals collide decreases in proportion to the square of the $\text{O}_2^{\bullet-}$ concentration, the rate of dismutation is greatly reduced at low $\text{O}_2^{\bullet-}$ concentrations:

$$\text{Rate (spontaneous dismutation)} = k_{\text{sd}} \cdot [\text{O}_2^{\bullet-}]^2$$

In theory this would lead to a steady state concentration of $\text{O}_2^{\bullet-}$ of approximately $10 \mu\text{M}$ in a biological environment which would have deleterious consequences. To remove $\text{O}_2^{\bullet-}$ rapidly, a class of superoxide dismutase enzymes has evolved which dramatically speed up the spontaneous dismutation. All superoxide dismutases possess transition metal ions in their catalytic center. Transition metal elements are characterized by having partially filled *d* orbitals. They generally have more than one stable valence or oxidation state. For example, Cu^+ and Cu^{2+} or even Cu^{3+} in the case of copper. As a consequence, transition metal ions have the ability to me-

diate electron transfer by valence-shuttle mechanisms. This is clearly visible in the reaction scheme of the enzyme-catalyzed dismutation of O_2^- :

In which M^{n+} is Cu^{2+} , Mn^{2+} , Fe^{3+} or Ni^{2+} depending on the type of SOD.

When added, these three reactions yield the overall reaction (1) (page 21). The rate of the dismutation reaction is determined by the first step. This rate-determining step requires only 1 superoxide anion and one molecule of SOD of which the concentration is constant. Furthermore, although Cu,Zn-SOD has a net charge of -4 at pH 7, the three dimensional structure of this SOD gives the complex protein an electrostatic potential shape which mimics that of isolated cupric ions (59). In other words O_2^- is attracted toward SOD as if it were attracted toward an isolated cupric ion, while negatively charged amino acid residues present in SOD do not repulse O_2^- . The three dimensional structure of SOD also ensures very efficient tunneling of the negatively charged O_2^- towards the cupric ion. Taken together this results in a reaction rate:

$$\text{Rate (catalyzed dismutation)} = k_{cd} \cdot [O_2^-]$$

Thus, at low concentrations of O_2^- , the rate of the SOD-catalyzed reaction falls in direct proportion to the decrease in O_2^- concentration and not in proportion to the square of the O_2^- concentration, as occurs in the spontaneous dismutation reaction. In this way, the presence of SOD results in a much lower steady-state concentration of O_2^- than would occur if the enzyme was absent.

SOD1 (copper,zinc-superoxide dismutase)

Cu,Zn-SOD (SOD1) is a homodimeric enzyme with a molecular weight of ~ 32 kDa. SOD1 contains in its active site an imidazolate bridged mixed metal centre with one Cu^{2+} ion and one Zn^{2+} ion (Fig. 8) (60).

Figure 8. Imidazolate bridged mixed metal center that forms the active site in SOD1. The Cu^{2+} ion is coordinated in a square plane to four histidine imidazoles (His-44, -46, -61 and -118). The Zn^{2+} ion is coordinated tetrahedrally to three histidine imidazoles (His-61, -69 and -78) and to Asp-81.

The structure of SOD1, established by radiograph crystallography, indicates that access to the Cu^{2+} is via a narrow channel (61). Loss of the Cu^{2+} ion from the enzyme results in complete loss of dismutase activity. The subunits are stabilized by an intrachain disulfide bond and associated by noncovalent forces. Although considered primarily a 'cytosolic' enzyme, SOD1 has also been identified in peroxisomes, lysosomes and the nucleus. Moreover, Fridovich and colleagues reported that a fraction of SOD1 is localized within mitochondria (58). While intracellular free copper is highly toxic due to its Fenton catalytic capacity, a copper chaperone for SOD1 (CCS) is essential for in vivo Cu^{2+} incorporation into SOD1 to efficiently activate this metalloenzyme (40). It is not easy to determine the importance of SOD1. SOD1 knockout mice get along quite well and develop normally. Problems only arise when they are stressed: showing increased neurological and histological damage after focal cerebral ischemia and reperfusion (62) and increased sensitivity to the redox cycling agent paraquat and reduced female fertility (40). Nevertheless, SOD1 concentrations are high (10 μM in *E. coli*) indicating that SOD1 is necessary for correct functioning of the cell.

SOD2 (manganese or mitochondrial superoxide dismutase)

MnSOD (SOD2) resides in the mitochondrial matrix at a concentration of up to 30 μM . It consists of four identical subunits and weighs 88 kDa (63). SOD2 is be-

lieved to represent the major means of protection against mitochondrial $O_2^{\cdot-}$, keeping mitochondrial $O_2^{\cdot-}$ concentrations close to 10^{-10} M (64). One of the most striking functional differences between SOD's 1 and 2 is that the animals lacking SOD2 (mitochondrial form) have a very short life span. The effect of knock-out of the SOD2 genes in transgenic mice is dramatic. They are unable to survive for more than 2 weeks and develop a series of biochemical defects (including a dilated cardiomyopathy) ascribed to oxidative stress (65). The importance of SOD2 illustrates the relevance of mitochondrial production of $O_2^{\cdot-}$. Another major difference with SOD1 is that SOD2 is inducible. Its concentration increases under conditions of stress, such as circumstances that elicit the production of tumor necrosis factor- α , or when animals are forced to breath 100% oxygen (9). Interestingly, insertion of SOD1 into mitochondria can protect cells lacking SOD2 from $O_2^{\cdot-}$ -induced apoptosis, suggesting that these two SOD species can take over each other's functions and that localization supercedes differences between SOD species (42).

SOD3 (EC-SOD)

Outside the eukaryotic cell, a third SOD resides: extracellular SOD or endothelial cell (EC-SOD). EC-SOD is a 135 kDa protein that exists primarily as a tetramer of four identical subunits. The tetramer consists of two dimers, each containing a disulfide bond that links heparin-binding domains in the C-terminuses of two subunits together.

EC-SOD acts in the extracellular space localized on the surface of the endothelium and vascular smooth cells, intracellularly it exists in much smaller amounts than SOD1 or SOD2 (66). EC-SOD is targeted to the surface of the endothelium and vascular smooth muscle cells by means of the heparin-binding site. Heparin binding affinity can be modulated through proteolysis of the heparin-binding domain. The activity of many proteolytic enzymes is increased during inflammation and reperfusion after ischemia. This increase in proteolytic activity may further exacerbate the oxidative stress associated with inflammation and ischemia-reperfusion injury by removing EC-SOD from the affected area (67). Further research might elucidate how a proteolytic enzyme sensitive can provide protection against oxidative stress. The synthesis of SOD3 is highly responsive to various inflammatory cytokines (68). In bacteria both copper,zinc and manganese containing SODs are found. Additionally, depending on the type of bacterium, iron containing SOD (69) and nickel

containing SOD (70) have been identified. SOD in bacteria will provide resistance to the $O_2^{\cdot -}$ produced by the host defense reaction that the bacteria trigger. It is therefore not surprising that the pathogenicity of bacteria, e.g. *Salmonella* shows a correlation with the amount of periplasmic Cu,Zn-SOD present, which is capable of intercepting the $O_2^{\cdot -}$ produced by host's innate immune response (71).

4. SOD toxicity

Additional catalytic activities

Superoxide dismutases dramatically reduce the concentration of $O_2^{\cdot -}$. The major function of SOD1 appears to be the regulation of the $O_2^{\cdot -}$ concentration. However, SOD1 has also been demonstrated to possess a nonspecific peroxidase activity. It was found that SOD1 was slowly inactivated by its own dismutation product, H_2O_2 . In first instance this was ascribed to the formation of $HO^{\cdot}$ by Fenton chemistry, where the Cu^+ ion acts as the catalyst. This was thought to be confirmed by spin trapping experiments where the combination of H_2O_2 and SOD1 in the presence of 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) resulted in the formation of DMPO-OH adducts, which are stable radicals that can be studied by electron spin resonance spectrometry (Fig. 9)(72). For the inactivation of SOD1 the $HO^{\cdot}$ does not have to travel a large distance. Oxidation by $HO^{\cdot}$ of His-61 which forms the imidazolate bridge in the mixed metal center of SOD1 results in an inactivation of SOD1 and simultaneous release of Cu^{2+} from the enzyme (Fig. 8). The inactivation of SOD1 and the formation of DMPO-OH adducts could be prevented by many antioxidants including xanthine, urate and formate. However, some well known $HO^{\cdot}$ scavengers such as ethanol or benzoate, known to react very rapidly with $HO^{\cdot}$, did not prevent inactivation of SOD1, indicating that $HO^{\cdot}$ does not leave the active site of SOD1. This means that the strong oxidant formed by SOD1 and H_2O_2 was $HO^{\cdot}$ bound to Cu^{2+} . A large molecule such as DMPO cannot reach the active site of SOD1, implying that DMPO is oxidized to DMPO-OH outside SOD1. Large molecules cannot reach this oxidant but small anions like the ubiquitous HCO_3^- can. HCO_3^- plays an important role as the intermediate oxidant in both the inactivation of SOD1 as well as exporting oxidation from the active site and out of SOD1 (73).

Figure 9. Electron spin resonance spectrum of DMPO-OH adduct generated by the reaction of SOD1 and H_2O_2 in the presence of the radical trapping agent DMPO. Initially this was seen as evidence that $\text{HO}^\bullet$ radicals were generated by the combination of H_2O_2 and SOD1. However, it is now widely accepted that $\text{HO}^\bullet$ is not released from the copper ion in SOD1 but that the $\text{HCO}_3^\bullet$ radicals that are responsible for the oxidation of DMPO to DMPO-OH.

$\text{HCO}_3^\bullet$ is oxidized by the Cu^{2+} bound $\text{HO}^\bullet$ radical to $\text{HCO}_3^\bullet$. Liochev and Fridovich demonstrated that $\text{HCO}_3^\bullet$ is an effective oxidant which is capable of oxidation of the active site His-61 but also of diffusing out of SOD1 and subsequently oxidize DMPO to DMPO-OH (74). In addition to the release of $\text{HCO}_3^\bullet$, inactivation of SOD1 by $\text{HCO}_3^\bullet$ leads to release of Cu^{2+} ions which results in the formation of $\text{HO}^\bullet$. Because the combination of SOD1 and H_2O_2 results in oxidative damage including lipid peroxidation (75) mediated by $\text{HO}^\bullet$ and $\text{HCO}_3^\bullet$ radicals, it is essential for cells to possess a proper distribution of catalases and other peroxidases. This ensures that the H_2O_2 that is produced by the SODs can be removed in a safe manner. How crucial this can be is illustrated by the efficacy of SOD1-catalase conjugate in reducing reperfusion injury (76, 77). In reperfused ischemic hearts, working at concentrations of free SOD1 which were either toxic or failed to give any protection against reperfusion injury, an equivalent dose of the SOD1-catalase conjugate resulted in an almost complete return to normal mechanical function of the reperfused hearts (76).

Another side function of SOD1 is the catalysis of tyrosine residue nitration by peroxynitrite (78, 79); this catalytic activity disappeared upon removal of the copper from SOD1. This nitration reaction may be pathologically relevant by nitrating critical tyrosine on proteins important for cell regulation. Increased expression of

SOD1 and SOD2 has been found in nerve and muscle fibers where vacuolation has occurred. This increased expression of SOD1 and SOD2 is associated with increased nitrotyrosine levels indicating that dysfunction of SOD1 and SOD2 might be involved and that this loss of function could be induced by peroxynitrite (80). Moreover, it has been demonstrated that certain SOD enzymes are inactivated by reaction with peroxynitrite. Because inactivation of SOD results in much higher superoxide concentrations and hence increased ONOO⁻ production, inactivation of SOD by peroxynitrite constitutes a positive feedback mechanism for peroxynitrite formation.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurological disease that rapidly progresses from mild motor symptoms to severe motor paralysis and death with a prevalence of 5 to 10 per 100,000. Mutations in SOD1 are responsible for ~10% of the familial cases of ALS (fALS). Transgenic mice that express a mutant SOD1, Gly93 → Ala (G93A), have been shown to develop ALS symptoms. Eum and Kang (81) reported that another fALS SOD1 mutant, D90A, exhibited an enhanced HO[•] generating activity, while its dismutation activity was identical to that of the wild-type enzyme. The mutation observed in patients with fALS is therefore associated with a 'gain of function', not a 'loss of function'. The most convincing evidence for such a gain of toxic function is the observation that the expression of fALS mutant SOD1 in transgenic mice causes motor neuron disease, while expression of wild type human SOD1 does not. The gain of function hypothesis is also supported by the observation that expression of a fALS mutant SOD1 is pro-apoptotic in cultured neuronal cells, whereas wild type human SOD1 is anti-apoptotic (82). These higher free radical-generating activities of mutants facilitated the release of copper ions from their own molecules (wild-type < D90A < G93A). The released copper ions can enhance the Fenton-like reaction to produce HO[•] and play a major role in the oxidative damage of macromolecules. Thus, the ALS-like symptoms may be associated with the enhancements in both the free radical-generating activity and the release of copper ions from the mutant enzyme. Mice with one of these mutations in SOD1 are a well accepted fALS model now. These mice demonstrate a robust phenotype of neurodegeneration, the severity of which

is correlated with the level of transgene expression. These animals are used to study the effectiveness of drugs for the treatment of ALS (83).

Down syndrome / trisomy 21

Down syndrome (trisomy 21) is the most common chromosomal abnormality associated with early mental retardation and neurological abnormalities followed by precocious age dependent Alzheimer-type neurodegeneration later in life (84). Overexpression of genes residing in chromosome 21 was considered to be the cause for the Down syndrome phenotype (85). Located on chromosome 21 is the gene coding for SOD1. Indeed, SOD1 protein levels, SOD1 activity and mRNA levels are approx. 1.5 – 2.5 times higher in patients with trisomy 21 than in their controls (84, 86). The peroxidase activity that SOD1 possesses might explain part of the neurodegeneration observed in Down's syndrome.

5. Therapeutic application of SOD

The bulk of evidence implicating that $O_2^{\cdot -}$ can be an initiating or a contributing factor in several pathological states is based on experiments that examine the ability of SOD to alter the injury response. Intriguingly, SOD was already used as a therapeutic (Orgotein) before its biochemical activity was known. Orgotein showed promising results as a therapeutic in acute and chronic conditions, including rheumatoid arthritis and osteoarthritis, as well as side effects associated with chemotherapy and radiation therapy (87). There are drawbacks associated with the use of native enzymes as therapeutic agents. These include immunogenicity of non-human enzymes and a high susceptibility to proteolytic digestion. Furthermore, they usually have short plasma half-lives, which seriously limits their therapeutical applicability. Native SOD has a high glomerular filtration rate (40% that of inulin) and is effectively reabsorbed in the tubules, while no significant uptake is observed from the capillary side (88). Glomerular filtration rate could be reduced by covalent linkage of SOD1 to macromolecules such as polyethylene glycol (PEG-SOD), galactose (Gal-SOD), mannose (Man-SOD), carboxymethyl-dextran, hexamethylene diamine (AH-SOD), lecithine or phosphatidyl choline (PC-SOD) (88) or fatty acids (Ac-SOD) (89). The increased plasma-half lifes that result from covalent linkage increase the pharmacological potency of SOD dramatically (90). In the following overview of the therapeutic application of the (modified) SODs the biological ac-

tivity of these SOD conjugates but also of gene therapeutical application of SODs are presented (Table 1). Only a small fraction of the papers regarding the therapeutic application of SODs were reviewed here. One can nevertheless understand that there has been great interest in this field for more than 15 years. It is likely that this interest is due to the complexity of $O_2^{\cdot -}$ formation and the importance of this radical in a wide variety of diseases. Unfortunately, no SOD has reached the stage of advanced clinical trials yet. It could be that the decrease in immunogenicity and the long-lasting *in vivo* properties are still not sufficient for successful applications.

To circumvent some of the limitations that are associated with native enzyme therapy, several low molecular weight SOD mimetics have been developed. One of the new compounds that possess superoxide dismutase activity is M40401. This non-peptidyl low-molecular weight (511.4 Da) manganese superoxide dismutase mimetic has been evaluated in neurodegenerative disorders where superoxide anions are thought to play an important role. M40401 has been demonstrated to protect cerebral tissue from ischemic insult when administered before middle cerebral artery occlusion in the rat (91). Pretreatment of rats with M40401 prevented the neuropathological effect of paraquat microinfused into the substantia nigra in the rat (92). M40401 is also protective after intraperitoneal administration, suggesting that it is capable of crossing the blood-brain barrier, which makes it the prototype drug for a new approach in the treatment of neurodegenerative disorders where oxidative processes occur, including Parkinson's disease (92). Post-infection treatment with M40401 protected against hypotension, vascular hyporeactivity to catecholamines, and mortality associated with septic shock in rat (28). Another promising SOD mimetic is MnTBAP. In this compound, Mn^{2+} ions are complexated in a porphyrin ring. MnTBAP prevented doxorubicin-induced damage to adult rat cardiomyocytes *in vitro* (93). MnTBAP and its Zn^{2+} containing analog, ZnTBAP, prevented damage in endothelial cells caused by paraquat (94). Spin traps react with radicals to form stable adducts. These compounds have been tested as superoxide dismutase mimetics. Treatment of hyperglycemic dogs with the spin trap Tempol prevented high glucose-induced coronary wall shear stress and endothelial dysfunction (95).

Table 1. Therapeutic application of superoxide dismutases

SOD	Modification	Disease	Effect	Ref.
1	PC-SOD	Colitis	Reduction of bloody stools	(101)
1	PC-SOD	Asthma	140 U.kg ⁻¹ inhibited Forsman serum-induced bronchial obstruction in guinea pigs	(102)
1	PC-SOD	Neurodegeneration	100 U.kg ⁻¹ attenuated progression of motor disfunction, prevented denervation muscle atrophy, and delayed degeneration of spinal motor neurones in wobbler mice.	(103)
1	PC-SOD	Myocardial infarction	Cardioprotective during myocardial ischemia-reperfusion. PC-SOD reduced infarct size.	(104)
1	PC-SOD	Drug toxicity	PC-SOD attenuated the increase in serum creatinine observed after i.v. cisplatin	(105)
1	PC-SOD	Hypertension	induced dose-dependent vasodilation of aortic ring preparations. ... indicating that superoxide anions play an prominent role in modulating vascular tone by enhancing the breakdown of NO.	(106)
1	PC-SOD	Inflammation	at both low (4,800 U.kg ⁻¹) and high (48,000 U.kg ⁻¹) doses phorbol-12-myristate-13-acetate (PMA) induced increase in vascular permeability in the isolated dog lung was prevented. Unmodified recombinant human SOD1 was unable to prevent the increased vascular permeability.	(107)
1	PC-SOD	Neurotrauma	Injected 30 min after spinal cord injury, motor function was improved and spinal cord edema was suppressed.	(108)
1	PC-SOD	AIDS	PC-SOD, inhibited HIV-1 replication at 2,500 U.ml ⁻¹ in vitro. PC-SOD did not decrease reverse transcriptase and proteinase activity which demonstrates that its mechanism of action is entirely different from those of the currently available. PC-SOD does show synergistic interaction with AZT and inhibits the oxidative stress-induced depletion of thiols observed in HIV infected patients.	(109, 110)
1	PC-SOD	Drug toxicity	Low doses (1 mg.kg ⁻¹ daily) inhibited the development of pulmonary fibrosis and interstitial pneumonia induced by bleomycin in mice. This indicates the potential usefulness of PC-SOD as a new treatment agent for idiopathic pulmonary fibrosis, or at least for bleomycin-induced pulmonary fibrosis in humans.	(111)
1	transgene	Myocardial infarction	10-fold expression of SOD1 yields 2.2 fold recovery of contractile function after heart ischemia-reperfusion in transgenic mice while it decreases infarct size with a factor 2.	(112)
1	transgene	Diabetes	Overexpression in streptozosin diabetic rats prevented diabetic renal injury and stimulation of MC matrix protein synthesis. Hyperglycemia is associated with generation of superoxide radicals (95).	

1	liposomal	Rheumatoid arthritis	Reduced infiltration of inflammatory cells in a rat model of adjuvant arthritis.	(113, 114)
1	none	Glaucoma	Treatment improved recovery after retinal ischemia caused by elevated intraocular pressure, as measured by electoretinography. Recovery was more efficient when SOD1 was administered just after ischemia than when animals were pretreated with SOD1.	(115)
1	none	Drug toxicity	SOD1, prevented ECG alterations, decreased atrial contractility, impairment of body-weight gain in rats receiving doxorubicin. Moreover, morphologic lesions were significantly reduced in rats receiving doxorubicin + SOD1 compared to rats receiving doxorubicin only.	(116)
1	none	Inflammation	PAF-induced disturbances of endothelial barrier function were partially attenuated by SOD1.	(117)
1	none	Alcohol toxicity	Chronic alcohol consumption-induced impairment of dilatation of the basilar artery in response to acetylcholine after was improved. SOD1 improves, and may thus prevent, cerebrovascular disorders, including stroke, observed in chronic alcoholics	(118)
1	PEG-SOD	Ototoxicity	10,000 U.kg ⁻¹ ameliorated the acute cochlear dysfunction induced by the environmental ototoxicant trimethyl tin.	(119a)
1	PEG-SOD	Brain infarct	Intravenous administration before brain ischemia reduced infarct size in rats (focal cerebral ischemia model).	(119)
1	transgene	Hearing loss induced by noise or I/R	Effective in reducing cochlear cellular damage and hearing loss associated with ischemia and exposure to noise.	(62)
1	heparin binding site (HB-SOD)	Hypertension	SOD1, specially targeted to endothelial cells by means of a heparin-binding site decreased blood pressure of SHR but not of normal rats. This indicates that superoxide radicals in and around vascular endothelial cells play critical roles in the pathogenesis of hypertension of SHR.	(54)
1	AH-SOD	Drug toxicity	Accumulates in proximal tubuli and prevents cisplatin-induced oxidative kidney injury.	(120)
2	transgene	Drug toxicity	Prevented doxorubicin-induced cardiotoxicity.	(121)
2	adenovirus	Transplantation	Islet replacement therapy is a promising cure for diabetics. Islet grafts overexpressing SOD2 functioned approximately 50% longer than control grafts.	(122)
3	adenovirus	Impotence	Gene transfer to the penis of aged rats improved erectile function.	(123)
3	transgene	Herbicide toxicity	Secreted from genetically modified cells (rat lung epithelial-like and rat skin fibroblasts) protected against extracellular but not intracellular paraquat-induced cytotoxicity.	(124)

It has often been reported that copper complexes can exhibit SOD activity. Bijloo et al. have tested a series of copper complexes and confirmed that the redox potential of these complexes determine their superoxide dismutating potency for the greater part (96). Low molecular weight copper complexes have been shown to possess antiarthritic effects in man (97). The most prominent of these compounds is copper(II)₂(3,5-diisopropylsalicylate)₄ or (Cu(II)₂(3,5DIPS)₄. Sorenson and co-workers have described the antiulcer, anticancer, anticonvulsant, antimutagenic, antidiabetic and analgesic activities of this compound. CuDIPS has also been found to reduce radiation (98) and ischemia-reperfusion damage (99). The radioprotective properties of CuDIPS suggest that copper complexes might be developed for protection of normal tissues in association with cancer radiotherapy and protection against occupational exposures to hazardous radiation (98). The hepatotoxicity of CCl₄, which induces LPO, was inhibited by CuDIPS as well. Interesting in this respect was the inability of CuDIPS to decrease the hepatotoxicity of paracetamol which depletes glutathione levels (100). Superoxide-related diseases may result from either increased production of O₂^{•-} due to hyperoxia, activation of granulocytes and macrophages, conversion of xanthine dehydrogenase to xanthine oxidase, autooxidation of catecholamines and glucose, exposure to ionizing radiation or biotransformation of xenobiotics. Evidence is emerging that O₂^{•-} also plays a role in the replication of the HIV virus. Modified SODs and non-protein SOD mimetics lower O₂^{•-} concentration and thus reduce the damage that superoxide inflicts.

One of the aspects of the therapeutic application of SODs or SOD mimetics that is largely ignored is the production of H₂O₂ that takes place in the presence of SOD or a reducing O₂-scavenger. A step ahead may come from the preparation of more complex conjugates, containing both SOD and catalase activity. An interesting additional activity that may be conveyed to these complex conjugated is a chelating action. The chelating activity could prevent the participation of free transition metal ions in the formation of HO[•] radicals from H₂O₂. The development of suitable targeting moieties to direct SODs and, if needed, to facilitate their extravasation and entrapment into cells may be another aspect of the therapeutic application of SODs well worth investigating.

6. Scope and outline of this thesis

$O_2^{\cdot -}$ plays many roles in physiologic and pathologic processes, including inflammation, ischemia-reperfusion, hypertension and drug toxicity. Reactive oxygen species derived from $O_2^{\cdot -}$ cause additional damage. Superoxide dismutases are enzymes that nature developed for aerobic organisms to form a first line of defense against $O_2^{\cdot -}$. These enzymes might have therapeutic value in the diseases where $O_2^{\cdot -}$ plays an important role. SOD levels might be increased by overexpressing the enzyme or by administration of modified SODs or SOD mimetics. A huge body of evidence demonstrates that SODs and SOD mimetics are effective therapeutics in an ever increasing array of diseases ranging from rheumatic arthritis to AIDS. However, just like $O_2^{\cdot -}$ has two faces or maybe even because $O_2^{\cdot -}$ has two faces, superoxide dismutase has two faces as well: Lowering $O_2^{\cdot -}$ concentrations is not necessarily beneficial as can be observed in CGD patients. One of the problems associated with the use of SOD is the fact that the combination of SOD and its dismutation product H_2O_2 has peroxidase activity. In other words, SOD might shift the toxicity from the superoxide side to the hydrogen peroxide side. Here, secondary ROS such as the hydroxyl radical and hypochlorous acid might be able to induce more damage than superoxide would have caused. Indeed, many investigators have observed bell-shape dose-response curves for protective effects of SOD. The central question in this thesis is therefore: Does superoxide dismutase modulate the toxicity of $O_2^{\cdot -}$, where modulation can be read as changing the extent, severity, amplitude, frequency or location. Secondary questions that arise from the first are: What makes an antioxidant a good therapeutic agent? What are the considerations when an antioxidant is chosen to influence a certain pathophysiological process?

In order to answer these questions the protective effect of a lecithinized form of SOD1, PC-SOD (which resides longer in the circulation than native SOD1), was investigated in the mouse model of doxorubicin-induced cardiotoxicity. Furthermore, the effects of the combination of SOD1 and hydrogen peroxide were investigated. Also the toxic effects of the hydrogen peroxide derived oxidant hypochlorous acid were investigated. Finally, the potency of sulphur containing compounds to modulate the toxicity of hypochlorous acid was evaluated.

7. References

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II

Cardiotoxic drugs and measurement of electrocardiographical alterations in the mouse

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Cardiotoxic drugs

1. Anthracyclines

Since the late 1960s, the anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DAU) (Fig. 1) have been among the most effective and widely used anti-neoplastic agents. Apart from the adverse effects that are associated with the use of many cytotoxic agents (gastrointestinal disturbances, nausea and vomiting, alopecia and haematopoietic suppression) the clinical usefulness of DOX and DAU is limited by their cardiotoxic effects.

Figure 1. Anthracyclines (R = -OH doxorubicin, R = -H daunorubicin)

The acute and subacute manifestations of this cardiotoxicity are cardiac dysrhythmias and myocarditis. More prominent however, are the chronic and the delayed cardiotoxicity (1). The chronic cardiotoxicity is the most common form of anthracycline-induced cardiotoxicity. It manifests itself near the end of the therapy or shortly after the therapy has ended in the form of a dilated cardiomyopathy. The delayed cardiotoxicity is of special importance for the survivors of childhood cancers. It develops 10-15 years after the therapy with anthracyclines has ended and is characterized by a small thin left ventricle with decreased compliance (dp/dV) (1, 2).

2. Antidepressants

Another class of drugs which affects the heart are the antidepressants, especially those containing a tricyclic structure (e.g. imipramine, clomipramine, amitriptyline) but also the non-tricyclic antidepressant trazodone (Fig. 2) (3).

Figure 2. Structures of antidepressants with cardiovascular side-effects.

These drugs influence especially electrical excitation and conduction of cardiac tissue, resulting in prolonged ventricular repolarization. Abnormal ventricular repolarization mimicking myocardial infarction has frequently been observed after heterocyclic antidepressant overdose, a phenomenon that requires a long recovery time to return to normal (4).

To study the onset and development of cardiotoxicity, and the treatment or prevention of this adverse effect several methods are available. Cardiac histopathology with scoring of myocardial lesions according to Billingham (5) is the most widely used method to study the chronic toxicity of anthracyclines and the efficacy of potential cardioprotectors (6-12). Another method which is often employed to assess

anthracycline-induced cardiotoxicity is the measurement of lipid peroxidation products (TBA reactive substances) and antioxidant levels (13, 14). These methods have in common that the animal has to be sacrificed in order to obtain the material needed to perform histopathology and/or biochemical determinations. To refine the animal models and to reduce the number of animals needed to study cardiotoxicity, a non-invasive marker of cardiac performance has been developed.

Telemetry model

1. ECG

The electrocardiogram (ECG) is an important medical tool, not only for diagnosis of adverse cardiac events (15), but also to predict the risk that such an event might occur (16). For the antidepressant amitriptyline, changes in the ECG form important indicators for toxicity. Burgess et al. demonstrated that trazodone and imipramine had significant effects on the ECG in healthy human volunteers at therapeutic doses (3). Both agents increased the QT interval and decreased T wave amplitude (3). Amitriptyline affected ventricular depolarization as well, which was reflected by a short but maximal QRS widening. The effect on ventricular repolarization (QT interval lengthening) was less pronounced but persistent.

That the cardiotoxicity caused by DOX could be monitored using ECG was already noted by Shimamoto et al. who observed that QRS complexes in the rat ECG were widened after treatment with DOX (17). Villani et al. also observed in the rat ECG a widening of the QRS complex which depended on the dose of DOX but which was also reversible (18). Jensen et al. observed variable R-, S-, and T voltages (19). The progressive lengthening of the QT and ST intervals were irreversible and therefore a useful parameter for monitoring the DOX-induced cardiotoxicity (18, 19). A good correlation was found between the morphologic score indicating the degree of observed tissue damage and ST prolongation (18, 20). Closer to the clinical setting, Sarubbi et al. demonstrated that after only a short period of treatment with doxorubicin there was a significant increase in ventricular recovery time. Thus, increased regional variation in ventricular repolarization could be, in the absence of a significant modification of the echocardiographic parameters, an early marker of an electropathy, due to the early cardiotoxic action of doxorubicin on myocardial cells, eventually leading to heart failure (21). Clinical and ex-

perimental data have demonstrated that prolongation of the QT interval is also a risk factor for ventricular arrhythmias and sudden cardiac death (16).

Although the mouse has been used in many experiments involving the acute doxorubicin cardiotoxicity, it has been used less extensively in studies of chronic toxicity because it is difficult to administer multiple i.v. injections of the drug over an extended period of time (8). The telemetry model, as developed previously by us (20), nevertheless employed the mouse to study the chronic doxorubicin cardiotoxicity because rats develop a nephrotic syndrome after doxorubicin administration which may influence the severity of the cardiotoxicity (22). Using the telemetry model, the effectiveness of two distinct flavonoids, monoHER (23) and Frederine (24) to prevent doxorubicin-induced cardiotoxicity was demonstrated.

2. Subcutaneous implantation and data acquisition

In the original telemetry model the biopotential transmitters (Data Sciences International, St. Paul, MN, USA) were placed intraperitoneally. To make implantation easier for animal and investigator the transmitter was implanted on the back of the mouse subcutaneously, a method described in the manual for larger animals (25) and for mice by Mitchell et al. (26). For that purpose a 2 cm midline incision was made on the back along the spine, and a subcutaneous pocket was formed by blunt dissection. The transmitter was sutured to the muscle just to the left of the midline with the sensing leads pointing toward the back of the animal. The cathodal lead (red) was tunneled subcutaneously (using a trocar) to the lower left chest and was anchored in place with a permanent suture. The anodal lead (colorless) was tunneled in a similar way subcutaneously to the right shoulder. This operation poses less risk for the mouse (reduced chance of infections) and requires a much shorter recovery period than after i.p. implantation. The procedure is also much shorter, and therefore less anaesthetic is needed. This is important because it has been shown that ketamine anesthesia markedly increases the duration and variability in RR and QT intervals (26). Transmitter signals were collected by receivers (Data Sciences International, St. Paul, MN, USA) that were placed under the cages. Signals were sent via multiplexors (Data Exchange Matrices) to a microcomputer. Here the signals were redirected to the analog output (A.R.T. analog) to which a PowerLab (ADInstruments, Castle Hill, Australia) AD converter was connected.

Signals were then recorded, stored and analyzed with Chart for Windows 4.1 (AD-Instruments, Castle Hill, Australia) (Fig. 3).

3. Oversampling and automatic detection of intervals

As already mentioned, the two ECG sensing leads of the telemeter are located subcutaneously on the ventral surface of the thorax (25). This positioning of the electrodes suffers from somatomotor activity which has been demonstrated to be non-different from animals without implanted telemeter (20). The resulting superimposed electrical noise causes a markedly lowered signal-to-noise ratio in the received ECG signal. In addition, the amplitude of the measured signals is highly variable due to changes of the spatial arrangement between the sensing leads and the heart, which is in agreement with the solid angle theory (27).

Figure 3. Telemetry system with A.R.T. Analog Output. Signals from the receivers (REC) are collected by the Data Exchange Matrices and redirected by the microcomputer to the analog output. The analog output can be connected to a suitable data acquisition system which is the major advantage of the ART analog system.

Not only were the ECG traces obscured by noise, determination of the length of the ST interval in the ECG was done by hand. The manual determination of the ST interval makes the experiment prone to inter-experimentalist variation and increases the concern that the results may be biased. Therefore, an alternative was developed which aimed to solve two problems:

1. Reduction of the noise levels
2. Automatic detection of the start and end-point of the ST interval

The first problem was overcome by oversampling or signal averaging. Averaging of a number (n) of ECG complexes results in an increase in the signal to noise ratio of $\sqrt{n}$. A raw recording trace (10 sec, which at an average heart rate of about 600 amounts to 100 ECG complexes) stored in Chart was divided into separate ECGs by detection of the R peaks. R peaks could be detected automatically by their high voltage combined with the reversal of sign of the derivative in the ECG. The individual ECG complexes were averaged using the R peak of each ECG as a fiducial point yielding an average ECG complex (Fig. 4). This average ECG complex is not the definitive complex. From the average ECG complex and all separate complexes 4 parameters were determined, the R voltage, QRS width, QT length and ST length. ECG complexes deviating more than 2 standard deviations with the parameters of the average ECG (usually about 15 to 20) were excluded from the ECG set from which the definitive average ECG was calculated (Fig. 4).

The second problem could also be solved mathematically. The exact determination of the S position is easy because it is the first reversal of sign of the derivative after the R peak. Determination of the length of the ST interval is therefore essentially the same as determination of the end of the T wave (T_{end}) (Fig. 4). One has two options for the automatic determination of T_{end} : 1. The crossing with the 0 mV line or 2. the point where the derivative (dV/dt) becomes 0. In many cases this is difficult because the end of the T wave does not always converge with the 0 mV line or x-axis or because the end of the T wave has a very shallow end where the derivative decreases very slow to 0. This was solved by defining T_{end} as the time at which the height of the T wave has decreased by 90 or 95%, yielding a $T_{\text{end},90}$ or $T_{\text{end},95}$ from which the ST_{90} interval or ST_{95} interval was easily obtained. Both values proved to be useful in quantification of ST interval lengthening induced by doxorubicin in the mouse.

4. Limitations

Currently available transmitter systems permits recording of a single lead (mostly lead II) ECG only. However, for our purpose the single lead ECG is sufficient. Because QT and ST intervals may vary among leads on a electrocardiogram (15), standardization of lead placement is of utmost importance (28). Constraining the mouse to facilitate intravenous administration of drugs to the mouse imposes stress on the mouse body and therefore ECG sensing leads. In extreme cases, this may cause an alteration of the position of the ECG sensing lead. Alteration of the location of the ECG sensing lead changes the ECG and might therefore be a cause for false results employing this model.

Figure 4. The individual ECG complexes are averaged yielding an average complex. Each individual complex is compared with the average complex. Complexes that differ more than 2 s.d. from the average complex are excluded from the set that is used to calculate the final average ECG. In the final average ECG, the ST interval is indicated. The ST interval is defined as the time from the S peak to the end of the T wave.

The final limitation of this technique is that the transmitter damages the skin at the site of implantation. Usually, this occurs after 8 to 10 weeks which limits the duration of the study to about 3 months.

Conclusions

Cardiotoxicity is an adverse effect of several important classes of drugs. Limiting and/or prevention of this potentially lethal side effect is therefore clinically important. An animal model allowing to monitoring cardiotoxicity over a long period of time is a useful tool to investigate the potency of (potential) cardioprotective compounds or the absence of cardiotoxic effects of analogues. Research on protective substances also offers information on the mechanism(s) of the toxic action. The telemetry model by van Acker was a major improvement in the study of the onset and prevention of doxorubicin-induced cardiotoxicity in the mouse. The current improvements only increase the applicability of the telemetry model. The subcutaneous implantation, the signal averaging technique combined with the automated determination of the ST interval is a reliable, easy and fast method of monitoring cardiac function for studies lasting up to 3 months.

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III

Lecithinized superoxide dismutase (PC-SOD) as a protector against doxorubicin-induced cardiotoxicity in mice

G.J.M. den Hartog, G.R.M.M. Haenen, E. Boven, W.J.F. van der Vijgh, A. Bast

Introduction

The involvement of oxygen radicals such as superoxide anion ($O_2^{\bullet-}$) in doxorubicin-induced cardiotoxicity is widely accepted (1-3). This cardiotoxicity, which manifests itself as congestive heart failure even years after completion of treatment, limits the maximum cumulative dose of doxorubicin that can be administered. Redox cycling of doxorubicin (Fig. 1) generates oxygen radicals.

Figure 1. Doxorubicin redox cycling. Doxorubicin is enzymatically reduced to the doxorubicin semi-quinone radical. This semi-quinone radical is subsequently oxidised by molecular oxygen yielding doxorubicin and superoxide anion.

Doxorubicin is enzymatically reduced to the doxorubicin semi-quinone radical by NADPH and cytochrome P450 reductase, NADH and NADPH dehydrogenase or cytochrome P450 (3-5). This semi-quinone radical directly donates its extra electron to molecular oxygen, yielding $O_2^{\bullet-}$.

Copper,zinc-superoxide dismutase (SOD1) is normally present in sufficient amounts to keep intracellular concentrations of $O_2^{\bullet-}$ low. However, excessive $O_2^{\bullet-}$ production may overwhelm the defence system leading to doxorubicin induced cardiotoxicity (6). Supplementing with exogenous SOD1 would be a rational rem-

edy against increased production of $O_2^{\bullet-}$, but the clinical application of SOD1 has thus far been limited by its low cell membrane affinity and its rapid clearance (7). In order to increase the cell membrane affinity and the plasma half-life of SOD1, Igarashi et al. have developed lecithinized SOD1 (PC-SOD), in which 4 molecules of a phosphatidylcholine (PC) derivative are covalently bound to recombinant human SOD1 (8, 9). It has been reported that PC-SOD at dose levels of $50,000 \text{ U.kg}^{-1}$ was effective in several animal models for inflammatory diseases, such as the dextran sulphate sodium-induced rat colitis model (10) and in the bleomycin-induced interstitial pneumonia mouse model (11, 12). Because of the alleged role of $O_2^{\bullet-}$ in doxorubicin-induced toxicity it is expected that PC-SOD protects the heart during doxorubicin treatment. The aim of the present investigation was to evaluate the potential of PC-SOD as a cardioprotective compound during doxorubicin treatment in the mouse. Interference of PC-SOD with the anti-tumour activity of doxorubicin was investigated both *in vitro* and *in vivo*.

Materials and methods

Chemicals

PC-SOD was provided by Seikagaku Corporation (Tokyo, Japan). Doxorubicin (Adriablastina[®]) was obtained from Pharmacia Nederland BV (Woerden, The Netherlands). PC-SOD was dissolved in 5% mannitol solution. Doxorubicin was dissolved to a concentration of 2 mg.ml^{-1} in saline. Doxorubicin for the *in vitro* experiments was further diluted in complete cell culture medium.

Effect of PC-SOD on doxorubicin-induced cardiotoxicity

The protocol was approved by the ethics committee for animal experiments of the Maastricht University. In male BALB/c mice ECG sensing telemeters (DataScience International, St. Paul, USA) were implanted subcutaneously. After a recovery period of two weeks the mice were submitted to one of the following weekly dose-schedules for 6 weeks:

Group 1 (n=3)	0.9% NaCl solution i.v.
Group 2 (n=6)	Doxorubicin 4 mg.kg^{-1} i.v.

- Group 3 (n=6) PC-SOD 5,000 U.kg⁻¹ i.v., followed by doxorubicin 4 mg.kg⁻¹ after 15 min
- Group 4 (n=6) PC-SOD 20,000 U.kg⁻¹ i.v., followed by doxorubicin 4 mg.kg⁻¹ after 15 min
- Group 5 (n=6) PC-SOD 80,000 U.kg⁻¹ i.v., followed by doxorubicin 4 mg.kg⁻¹ after 15 min
- Group 6 (n=6) 5% mannitol solution i.v., followed by doxorubicin 4 mg.kg⁻¹ after 15 min

The doses of PC-SOD used in this experiment were based on the observation of Yamazaki et al. (11) who observed that 50,000 U.kg⁻¹ was necessary to suppress the progression of pulmonary fibrosis in the bleomycin-induced interstitial pneumonia mouse model. All injections were administered on the first day of each week via the tail vein. After 6 weeks of treatment, animals were observed for another two weeks. The ECG was registered in the freely moving animal on the second day after treatment and on the same day of the week during the observation period. For interpretation of the ECG 9 complexes were analysed in detail. The ST interval was defined as the period from the S-peak to the end of the T-peak in milliseconds (Fig. 2).

Figure 2. Effect of doxorubicin treatment (4 mg.kg⁻¹ weekly for 6 weeks) on the mouse ECG. Doxorubicin causes flattening of the T top and lengthening in the ECG

Increases in ST interval were expressed as mean $\pm$ S.E. After the observation period, mice were sacrificed, the hearts were excised and taken into buffered formalin for 24 hours and stored in 70% ethanol until processing. Hearts were spliced and embedded in paraffin. Sections (4 μ m) were cut and stained with haematoxylin and eosin. Myocardial damage was evaluated by light microscopy according to Billingham (13). Specific attention was paid to the occurrence of lesions in cardiomyocytes.

Effect of PC-SOD on the anti-tumour effects of doxorubicin

For the *in vitro* part of the study three cell lines were used: the human ovarian cancer cell lines A2780 (14) and OVCAR-3 (15) and the human breast cancer cell line MCF-7 (16). The cells were routinely grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, Irvine, Scotland) supplemented with 10% heat inactivated foetal calf serum (GIBCO, New York, USA).

Exponentially growing cells were harvested and plated as single cell suspensions in 96-wells flat bottom microtiter plates (Greiner, Solingen, Germany). Cells were seeded in 100 μ l medium at a density of 3000 cells per well (A2780) or 5000 cells per well (OVCAR-3 and MCF-7). After 24 hr doxorubicin was added resulting in a range of concentrations of 10 fM to 10 μ M. PC-SOD was added resulting in concentrations of 30 μ g.ml⁻¹, 120 μ g.ml⁻¹ and 480 μ g.ml⁻¹, corresponding to an SOD activity (17) of 100, 400 and 1600 U.ml⁻¹. All concentrations were studied in quadruplicate wells. After 96 hr the IC₅₀ (defined as the doxorubicin concentration that decreases cell growth by 50% when compared to control cell growth) was calculated. The effect of PC-SOD on cell growth was expressed as a percentage of control cell growth. The experiments were carried out four times.

The protocol for the *in vivo* part of the study was approved by the ethics committee for animal experiments of the Vrije Universiteit Amsterdam. Nude female mice (Hsd:Athymic-nu) were obtained from Harlan Olac (Horst, The Netherlands) at the age of 6 weeks. The animals were maintained in isolation under controlled atmospheric conditions (temperature 23-25 °C, humidity 50-60%). Animal handling was carried out under sterile conditions. First, it was assessed whether PC-SOD had an influence on the weight loss in non-tumour-bearing nude mice induced by doxorubicin in groups of 5 mice each.

Thereafter, OVCAR-3 xenograft fragments of 3 mm diameter were implanted s.c. into both flanks of 8-week-old nude mice. As xenografts, OVCAR-3 shows a pattern of a poorly differentiated serous adenocarcinoma and has a doubling time of approximately 5 days. Treatment was started when the tumour size was approximately 100 mm³; the first treatment day was designated as day 0. The mice were subjected to the following weekly dose schedules for two weeks:

- Group 1 (n=6): Control
- Group 2 (n=6): Doxorubicin 8 mg.kg⁻¹ i.v.
- Group 3 (n=6): PC-SOD 24.8 mg.kg⁻¹ i.v.
- Group 4 (n=6): PC-SOD 6.2 mg.kg⁻¹ i.v. followed by doxorubicin 8 mg.kg⁻¹ i.v. after 15 min
- Group 5 (n=6): PC-SOD 24.8 mg.kg⁻¹ i.v. followed by doxorubicin 8 mg.kg⁻¹ i.v. after 15 min

The dose of doxorubicin 8 mg.kg⁻¹ weekly x 2 is maximally tolerated in the OVCAR-3 nude mouse xenograft model (18). Mice were weighed twice-a-week and weight loss was used as a measure of toxicity. Toxic deaths were defined as animals that died within 14 days after the last injection.

Tumour volume was measured in three dimensions twice a week. The anti-tumour activity was expressed as the percentage of growth inhibition, which is 100% - T/C%. T/C% was calculated by the ratio of the mean volume of treated tumours / mean volume of control tumours × 100%.

Statistical evaluation

Differences were evaluated with SPSS 8.0 for Windows (SPSS Inc., Chigaco, USA) using analysis of variance (ANOVA) with Fisher's least-significant differences (LSD) test for multiple comparisons when ANOVA indicated significant differences.

Results

Effect of PC-SOD on doxorubicin-induced cardiotoxicity

In Fig. 3 the effect of doxorubicin alone or in combination with PC-SOD (5,000; 20,000 or 80,000 U.kg⁻¹) on the length of the ST interval in the mouse ECG is pre-

sented. Treatment with doxorubicin ($4 \text{ mg.kg}^{-1} \text{ i.v.}$) for 6 weeks increased the length of the ST interval by $7.9 \pm 1.3 \text{ msec}$ at week 8. Treatment with saline did not cause any changes in the ST length. Treatment with mannitol, the solvent for PC-SOD prior to administration of doxorubicin, did not prevent the doxorubicin-induced lengthening of the ST interval. Pretreatment with $5,000 \text{ U.kg}^{-1}$ PC-SOD completely prevented the lengthening of the ST interval ($P = .000$). Increasing the dose of PC-SOD to $20,000$ or $80,000 \text{ U.kg}^{-1}$ also prevented the ST interval increase induced by doxorubicin ($P = .001$ and $.000$, respectively). The ST interval in the PC-SOD $20,000 \text{ U.kg}^{-1}$ treatment group was, however, also longer than that in the saline-treated group ($P = .037$) (Fig. 3).

Figure 3. Increase in ST interval per treatment group. Mean $\pm$ standard error ($n=6$) * $P < 0.05$ relative to animals treated with saline.

The hearts were removed from the mice 2 weeks after completion of treatment. Microscopic evaluation of haematoxylin- and eosin-stained sections revealed that

treatment with doxorubicin induced small lesions in the cardiomyocytes (Fig. 4B). In mice that received saline only, these lesions were not observed (Fig. 4A). The lesions were also not observed in mice that received PC-SOD prior to doxorubicin (Fig 4C).

Figure 4. Light micrographs showing haematoxylin and eosin stained sections of male BALB/c mouse hearts. A. Control. B. Treatment with 4 mg.kg^{-1} per week of doxorubicin for 6 weeks, resulting in myocardial lesions. C. Treatment with $80,000 \text{ U.kg}^{-1}$ PC-SOD and 4 mg.kg^{-1} doxorubicin per week for 6 weeks. Arrows indicate the lesions in cardiac tissue.

Effect of PC-SOD on doxorubicin-induced cell growth inhibition

PC-SOD alone seemed to stimulate the proliferation in MCF-7 cells, but did not affect the growth of A2780 and OVCAR-3 cells (Fig. 5A).

Figure 5A. The effect of PC-SOD on cell proliferation in three different human malignant cell lines. Cells were exposed to PC-SOD for a period of 96 h. Control cell growth is set at 100%. Mean $\pm$ standard deviation * $P < 0.05$

MCF-7 cells were approximately 4 times more sensitive to doxorubicin than A2780 and OVCAR-3 cells, IC_{50} 's were 4.2 ± 0.5 nM, 16.9 ± 4.6 nM, 12.6 ± 9.3 nM, respectively. In A2780 cells, the inhibitory effect of doxorubicin on cell proliferation was enhanced by PC-SOD (Fig. 5B). The IC_{50} of doxorubicin on A2780 cell proliferation was decreased from 16.9 ± 4.6 nM (no PC-SOD present) to 4.7 ± 1.9 nM ($480 \mu\text{g.ml}^{-1}$ PC-SOD) ($P = .048$). In MCF-7 cells, the IC_{50} of doxorubicin on cell proliferation was significantly increased in the presence of 30 and $120 \mu\text{g.ml}^{-1}$ PC-SOD (from 4.3 ± 0.3 nM to 7.2 ± 0.6 nM ($P = .003$) and 7.9 ± 0.7 nM ($P = .002$) respectively). However, the IC_{50} of doxorubicin was unchanged with PC-SOD pre-

sent at the highest concentration tested ($480 \mu\text{g}.\text{ml}^{-1}$) (Fig. 5B). In OVCAR-3 cells, PC-SOD at all tested concentrations had no effect on the doxorubicin-induced inhibition of cell proliferation (Fig. 5B).

Figure 5B. The effect of PC-SOD on the IC_{50} of doxorubicin on cell proliferation in A2780, MCF-7 and OVCAR-3 tumor cell lines. Cells were exposed to doxorubicin or the combination of doxorubicin and PC-SOD for a period of 96 hr. Mean $\pm$ standard deviation * $P < 0.05$

Effect of PC-SOD on doxorubicin-induced inhibition of tumour growth

Non-tumour-bearing nude mice treated with doxorubicin alone suffered a maximum weight loss of $5.7 \pm 3.7\%$ (Fig. 6). In nude mice that were treated with PC-SOD alone or the combination of doxorubicin and PC-SOD maximum weight loss was $0.9 \pm 3.0\%$ and $2.2 \pm 4.1\%$, respectively (Fig. 6). In all groups one mouse developed ascites which resulted in the death of one animal each in 2 groups.

Subsequently, treatment was given to nude mice bearing well established OVCAR-3 xenografts. Treatment with PC-SOD alone did not affect tumour growth (Fig. 7). Doxorubicin significantly inhibited tumour growth (71% at day 28 and 81% at day

35). Pre-treatment with PC-SOD (both 6.2 and 24.8 mg.kg⁻¹) did not influence the doxorubicin-induced growth inhibition (Fig. 7). In the group of PC-SOD 6.2 mg.kg⁻¹ + doxorubicin one mouse died from ascites.

Figure 6. Effect of doxorubicin, PC-SOD or the combination on body weight of mice. Mean $\pm$ standard error (n=5). Arrows indicate treatment (day 0 and day 7).

Discussion

Cardiotoxicity caused by treatment with doxorubicin can be life-threatening and may occur even years after completion of therapy (19). It is widely accepted that oxygen free radicals generated during doxorubicin redox cycling are responsible for the damage that doxorubicin causes to the heart. Oxygen radical generation specifically affects the heart because of the low antioxidant defence systems in cardiac tissue. Pre-treatment with antioxidants is therefore a rational approach to prevent doxorubicin-induced cardiotoxicity and has been shown to be successful in several animal models (20, 21).

We have investigated the cardioprotective efficacy of PC-SOD using the telemetry model in mice as described earlier by Van Acker (20,22). In this model, the toxic effect of doxorubicin on the heart is monitored by measuring the length of the ST interval in the mouse ECG using implanted telemeters. A good correlation has been demonstrated between the extent of tissue damage and ST interval prolongation as a result of the total cumulative doxorubicin dosage administered (22, 23).

Figure 7. Effect of doxorubicin, PC-SOD and combination of both on relative tumour volume in mice bearing well-established OVCAR-3 xenografts. Mean $\pm$ standard error ($n=6$). Arrows indicate treatment (day 0 and day 7).

A compound that attenuates doxorubicin-induced damage to cardiac tissue is therefore expected to inhibit doxorubicin-induced ST interval prolongation. By ECG measurements and histology we have demonstrated that PC-SOD reduces cardiac damage in mice treated with doxorubicin. To our knowledge PC-SOD is the first semi-synthetic enzymatic antioxidant that has been shown to be effective against doxorubicin-induced cardiotoxicity.

Furthermore, we have demonstrated that PC-SOD is already effective at doses of $5,000 \text{ U.kg}^{-1}$. Pre-treatment with $20,000$ and $80,000 \text{ U.kg}^{-1}$ did not increase cardioprotection. Apparently, PC-SOD was so potent in our model that a dose-dependency for the cardioprotection offered by PC-SOD could not be shown.

In comparison to other antioxidant cardioprotectors, such as the flavonoid monohydroxyflavone (20) the lipid lowering agent probucol (24) and the iron chelator ICRF-187 (25) PC-SOD has the advantage of having a very specific mode of action: only superoxide anions are scavenged. PC-SOD was well tolerated and did not cause any signs of general toxicity or loss of body weight. PC-SOD appears to be suitable as a cardioprotector during treatment with doxorubicin.

A main prerequisite for a compound to be used as a cardioprotector during treatment for cancer, is that it does not interfere with the anti-tumour activity of the chemotherapy. In order to evaluate the possible clinical application of PC-SOD, the influence of PC-SOD on the anti-tumour activity of doxorubicin was investigated *in vitro* as well as *in vivo*. *In vitro*, it was found that PC-SOD at concentrations that protected the heart against doxorubicin, did not interfere with the inhibition of cell proliferation induced by doxorubicin in three different cell types. Also *in vivo*, PC-SOD did not attenuate the doxorubicin-induced inhibition of xenograft growth. It can therefore be concluded that scavenging superoxide anions specifically decreases the cardiotoxic properties of doxorubicin, which provides further evidence that superoxide anions mediate the cardiotoxic effects of doxorubicin. The absence of an effect of PC-SOD on the anti-tumour activity of doxorubicin suggests that superoxide anions are not involved in the mechanism of the anti-tumour effect of this anticancer drug which mainly depends on inhibition of topoisomerase II (26).

Before the start of the present study, we speculated that high doses of PC-SOD might lead to toxic effects. The combination of hydrogen peroxide and high SOD1 concentration could lead to peroxidative damage to bioorganic compounds and might thus increase damage at the site of oxygen radical formation (27, 28). Even at the highest dose of PC-SOD ($80,000 \text{ U.kg}^{-1}$) however, no reduction of cardioprotection or amplification of doxorubicin-induced cardiotoxicity was observed. Moreover, no signs of toxicity due to the high dose of PC-SOD were found, indicating that PC-SOD has a large therapeutic window.

In summary, it was shown that PC-SOD protects against doxorubicin-induced cardiotoxicity in the mouse when administered 15 min before doxorubicin. PC-SOD

is well tolerated, shows no signs of toxicity even at very high doses. Very importantly, PC-SOD does not interfere with the anti-tumour effect of doxorubicin. Therefore, PC-SOD is a suitable candidate for the prevention of doxorubicin-induced cardiotoxicity.

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IV

Superoxide dismutase:

The balance between prevention and induction of oxidative damage

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Introduction

Oxidative stress, an imbalance between oxidant and antioxidant activity, occurs during a variety of pathologies (1). Cu,Zn-superoxide dismutase (SOD1), the first enzyme identified to have a free radical as its substrate (2), provides a first line of defense against oxidative stress by catalyzing the dismutation of $O_2^{\cdot-}$ into H_2O_2 plus O_2 . The therapeutic application of SOD1 has been examined for various diseases in which oxidative stress plays a role. However, SOD1 is rapidly cleared from the blood by glomerular filtration (3). To improve the retention of SOD1 in the body as well as its cellular uptake, the enzyme has been either conjugated to a variety of moieties such as polyethylene glycol (4), hexamethylenediamine (5) and lecithine (6) or encapsulated in liposomes (7, 8). SOD and its derivatives have been evaluated in a multitude of diseases including bleomycin-induced pulmonary fibrosis (9), cis-platin-induced renal dysfunction (5), dextran sulphate sodium-induced colitis (10), traumatic brain injury (11), and ischemia reperfusion in a variety of animal models. Transgenic mice overexpressing SOD1 show reduced ischemia reperfusion injury (12) and decreased diabetic glomerular injury (13). However, some reluctance is appropriate when SOD is applied therapeutically. SOD1 appears to have an additional peroxidase activity which in combination with large quantities of its dismutation product, hydrogen peroxide is capable of oxidizing a large number of bioorganic molecules. The ubiquitous bicarbonate anions have also been shown to be a target for SOD1 peroxidase activity. The resulting carbonate radical ($CO_3^{\cdot-}$) induces damage in a similar fashion to that caused by hydroxyl radicals (14). This implies that in case of $O_2^{\cdot-}$ formation, SOD1 at low concentrations would offer protection while high SOD1 concentrations could promote oxidative stress. The aim of the present study was to investigate the balance between antioxidant activity, $O_2^{\cdot-}$ scavenging and pro-oxidant activity, catalysis of peroxidations and hydroxylations by SOD1 *in vitro* and *in vivo*.

Materials and methods

Animals

Male BALB/c mice (20-25 g) were obtained from Charles River Nederland B.V. (Someren, The Netherlands). Animals were acclimatized for two weeks prior to starting the studies. They had free access to food and acidified water and were

housed under light, temperature and humidity controlled conditions. All protocols were approved by the local ethics committee for experimental animals of Maastricht University (DEC-UM) and were in accordance with the EU guidelines for research involving experimental animals.

Chemicals

Superoxide dismutase, xanthine, xanthine oxidase, nitro blue tetrazolium (NBT), coumarin-3-carboxylic acid and heparin (lithium salt) were obtained from Sigma Chemical Co. (St. Louis, USA). Doxorubicin (2 mg.ml^{-1} in sterile saline) was obtained from Pharmacia & Upjohn. Lecithinized SOD1 (PC-SOD) was supplied by Seikagaku Co. (Tokyo, Japan). All other reagents were of analytical grade.

In vitro detection of oxidative damage

Superoxide radicals were generated by the reaction of xanthine (1 mM) and xanthine (10 mU.ml^{-1}) oxidase in air-saturated potassium phosphate buffer ($\text{pH } 7.8$ at 25°C). $\text{O}_2^{\cdot-}$ radicals were detected by nitroblue tetrazolium (NBT, $50 \text{ }\mu\text{M}$). NBT undergoes reduction by $\text{O}_2^{\cdot-}$ to its formazan. The rate of this reduction was monitored spectrophotometrically at 560 nm . SOD1, by scavenging $\text{O}_2^{\cdot-}$, diminishes the rate of the reduction of NBT. The extent of hydroxylation catalysed by SOD1 was monitored using coumarin-3-carboxylic acid (3-CCA, $50 \text{ }\mu\text{M}$) as described by Makrigiorgos et al. (15). The resulting 7-hydroxycoumarin-3-carboxylic acid was quantitated fluorometrically with an excitation wavelength of 400 nm and an emission wavelength of 450 nm . Control experiments (data not shown) demonstrated that 3-CCA did not influence the formation of $\text{O}_2^{\cdot-}$.

Determination of protection by lecithinized SOD1 in vivo

ECG sensing telemeters (DataSciences International) were implanted subcutaneously in male BALB/c mice. Following surgery the mice were allowed to recover for 2 weeks. All mice were treated with 24 mg.kg^{-1} doxorubicin equally divided over 6 weekly doses. 3 groups of mice were treated with PC-SOD 15 min before doxorubicin was administered at dose levels 5,000; 20,000 or $80,000 \text{ U.kg}^{-1}$ i.v. weekly. The control group of mice received 5% mannitol, the vehicle for PC-SOD. As a measure of general toxicity, the body weight of the mice was determined weekly.

Differences were evaluated with SPSS 8.0 for Windows using analysis of variance (ANOVA) with Fisher's least-significant differences (LSD) test for multiple comparisons when ANOVA indicated significant differences from the control group (0 U.kg⁻¹ PC-SOD and doxorubicin). The level of significance chosen was 95% ($P < 0.05$).

Determination of SOD1 activity in plasma time course

Blood was collected from male BALB/c mice treated with PC-SOD (5,000; 20,000 and 80,000 U.kg⁻¹) at 11 time-points (5, 10, 15, 30, 60 min and 2, 4, 6, 8, 16 and 24 hr) after intravenous administration. For each time point and each dose 3 mice were used. Blood was rapidly mixed with lithium heparin (5 U.ml⁻¹) and centrifuged (5,000g for 10 min). Plasma was stored at -80 °C until analysis. SOD1 activity was calculated from the ability of the plasma to decrease the rate of NBT reduction induced by xanthine and xanthine oxidase. A calibration curve for SOD activity was constructed with human SOD1 in phosphate buffer.

Figure 1. Superoxide scavenging and hydroxyl radical formation by SOD1 (closed squares) and PC-SOD (open circles) in a system consisting of xanthine and xanthine oxidase and detector molecules for superoxide anion radicals (NBT) and hydroxyl radicals (3-CCA). The rate of NBT reduction is plotted on the left y-axis. On the right y-axis the extent of 3-CCA hydroxylation is plotted. Values are means of three separate experiments. Standard errors are not presented for clarity; the determination of NBT reduction and 3-CCA hydroxylation were reproducible within 5%.

Results

In vitro

Superoxide ($O_2^{\cdot -}$) produced by xanthine and xanthine oxidase resulted in the reduction of nitroblue tetrazolium (NBT) to the corresponding formazan. The production of this formazan was monitored spectrophotometrically by the increase in absorption at 560 nm. SOD1 (0.02 to 2 U.ml^{-1}) scavenged $O_2^{\cdot -}$ concentration dependently (Fig. 1). Under the experimental conditions, an SOD1 concentration above 2 U.ml^{-1} completely prevented $O_2^{\cdot -}$ mediated NBT reduction. At SOD1 concentrations up to 60 U.ml^{-1} fluorescence due to hydroxylation of 3-CCA did not rise above the background value. Above 60 U.ml^{-1} SOD1 hydroxylation of 3-CCA occurs. The fluorescence due to the formation of hydroxylated 3-CCA was dependent on the SOD1 concentration (Fig. 1). Similar effects were found for lecithinized SOD1 (PC-SOD) (Fig. 1).

Figure 2. The effect of PC-SOD on doxorubicin induced toxicity in Balb/c mice. Mice received 4 mg.kg^{-1} doxorubicin weekly for six weeks in combination with PC-SOD $5,000$; $20,000$ or $80,000\text{ U.kg}^{-1}$ or vehicle. Doxorubicin-induced lengthening of the ST interval in the mouse ECG (filled squares) and reduction of body weight (filled circles) are presented as doxorubicin toxicity as percentage $\pm$ standard error of three to five separate experiments. * $P < 0.05$ compared to the control group (0 U.kg^{-1} PC-SOD + doxorubicin).

*In vivo**Protection against doxorubicin cardiotoxicity*

Doxorubicin treatment induced an increase in ST interval length (+11 msec, 100%)(Fig. 2). retreatment with PC-SOD at a dose of $5,000 \text{ U.kg}^{-1}$ largely inhibited the increase in ST interval length caused by 6 weeks of doxorubicin treatment (Fig. 2). After pretreatment with $20,000$ or $80,000 \text{ U.kg}^{-1}$ PC-SOD a similar protection against doxorubicin-induced ECG changes was found. Treatment with doxorubicin caused a reduction of the body weight of mice (Fig. 2). Pretreatment with PC-SOD ($5,000 \text{ U.kg}^{-1}$) prevented part of the reduction in body weight caused by doxorubicin (Fig. 2). Higher doses of PC-SOD provided the same level of protection against the reduction in body weight caused by doxorubicin treatment.

PC-SOD plasma levels

Prior to treatment of mice with PC-SOD the background SOD activity of plasma was below the lower level of quantification. Treatment of mice with PC-SOD resulted in a dose-dependent increase in plasma SOD activity (Fig. 3). In mice treated with $80,000 \text{ U.kg}^{-1}$ plasma SOD activity reached a maximum ($A_{\max}$) of $23.2 \pm 0.4 \text{ U.ml}^{-1}$. After administration of $20,000$ and $5,000 \text{ U.kg}^{-1}$ $A_{\max}$ was 20.7 ± 1.1 and $10.8 \pm 0.7 \text{ U.ml}^{-1}$, respectively. Increased plasma SOD activity was detected until 24 hours after administration of $80,000 \text{ U.kg}^{-1}$. After administration of $20,000$ and $5,000 \text{ U.kg}^{-1}$, increased plasma SOD activity was detected for 8 and 4 hours respectively.

Discussion

The effects of SOD1 in a $\text{O}_2^{\bullet -}$ producing environment were investigated in a simple test tube assay. It was observed that SOD1 and its lecithinized derivative (PC-SOD) in a wide range of concentrations are both protectors by scavenging $\text{O}_2^{\bullet -}$. Hydroxylation of 3-CCA occurred only at concentrations higher than 60 U.ml^{-1} of either SOD1 or PC-SOD. Thus, at high concentrations the protective $\text{O}_2^{\bullet -}$ scavenging effect of SOD1 and PC-SOD is overshadowed by the ability of SOD1 to catalyze hydroxylation of (bio)organic compounds. Combined, this results in a U-shaped curve when plotting the protective effect against the SOD1 or PC-SOD

Figure 3. Time course plasma SOD1 activity after single intravenous administration of PC-SOD in the mouse. Closed triangles: 80,000 U.kg⁻¹; Open circles: 20,000 U.kg⁻¹; Closed circles: 5,000 U.kg⁻¹. Values are means $\pm$ standard error of three separate experiments. The upper graph displays the time course from 0 to 30 min. The lower graph displays the time course from 0 to 24 hr. The table presents the values for maximum activity (A_{max}) which is reached and the time at which maximum activity is reached (t_{max}).

concentration. The concentration range from 2 to 60 U.ml⁻¹ which forms the 'bottom of the U' could be regarded as the protective window of SOD in a O₂^{•-} mediated pathology or toxicity.

Copper-induced hydroxylation and/or peroxidation of biological compounds could be the mechanism by which high concentrations of SOD1 induces its toxic effects. In previous studies similar U-shaped dose-response relationships when protecting biological systems against oxidative stress have been reported (16-18). However, it should be noted that pro-oxidant effects of SOD1 have only been demonstrated in cell culture systems or isolated organs. No toxicity has been reported in any of the *in vivo* models in which the efficacy of SOD1 or its derivatives has been evaluated (5, 9-11, 19). Therefore the effect of SOD1 *in vivo* was investigated.

The effect of SOD1 on O₂^{•-} toxicity *in vivo* was studied in a mice model. The redox cycling compound doxorubicin was used to generate O₂^{•-} (20, 21). Doxorubicin toxicity is most prominent in the heart due to accumulation of doxorubicin in the heart and the comparatively low antioxidant levels in the heart. A major advantage of this model is that doxorubicin-induced cardiotoxicity can easily be monitored by changes in the ECG, most notably a lengthening of the ST interval (22). PC-SOD was used to investigate the balance between protective and toxic effects of SOD1 because of its longer retention within the circulation compared to unmodified SOD1 (23). Lecithinization also greatly improves the delivery of SOD1 into cardiomyocytes (24).

PC-SOD provided protection against superoxide mediated doxorubicin-induced cardiotoxicity. At a dose level of 5,000 U.kg⁻¹, protection already reached its maximum value. Administration of higher doses of PC-SOD did not result in additional protection against doxorubicin-induced cardiotoxicity compared to the lowest dose. PC-SOD also attenuated the general toxic effect of doxorubicin which is reflected by the induction of weight loss. In contrast with the results obtained *in vitro*, no sign of toxicity due to SOD1 overdose is found *in vivo*. The ST interval does not increase at higher doses of PC-SOD. To our knowledge, 80,000 U.kg⁻¹ is the highest dose of SOD1 ever administered intravenously to an animal. This means that PC-SOD possesses a therapeutic window of at least 5,000 to 80,000 U.kg⁻¹ in the mouse. While the very high dose of 80,000 U.kg⁻¹ of PC-SOD does not lead to toxic effects, mutations of SOD1 can render it toxic. In some familial cases of amyotrophic lateral sclerosis (25) abnormalities in the structure of SOD1 have been reported. The copper ion appears to be more loosely bound than in the normal

SOD1 and is therefore more likely to catalyze hydroxylation of bioorganic compounds which results in impaired motoneuron function. In this case, promotion of oxidative stress by a mutated form of SOD1 is possible.

In order to compare the results obtained in the *in vitro* experiment with those obtained in the *in vivo* experiment, the SOD1 activity in blood plasma after PC-SOD administration was assessed. Remarkably, the maximum values of SOD1 activity do not increase linearly with the dose of PC-SOD administered. It is reasonable to assume that this is caused by the disposition of PC-SOD from the blood into the tissues. In rats, tissue SOD1 concentrations of 7-10 U per gram wet weight after intravenous administration have been demonstrated (26). It was found that after administration of 80,000 U.kg⁻¹ PC-SOD the peak plasma SOD1 activity which is reached *in vivo* (23.2 U.ml⁻¹) after intravenous administration is lower than the activity which is necessary to catalyze OH[•] formation from superoxide *in vitro* (> 60 U.ml⁻¹). Although the experimental conditions are different, it is therefore not surprising that administration of high amounts of SOD1 does not lead to toxic effects even when O₂^{•-} production is enhanced.

Down's syndrome (trisomy 21) is associated with an approximate 50% overproduction of SOD1, since the gene coding for this enzyme is located on human chromosome 21. It has been suggested that this overproduction causes the symptoms of this syndrome (27). Our experiments indicate that a 50 to 100 fold increase in SOD concentration is needed to shift the balance between prevention and induction of damage in the direction of induction of damage. Although we examined acute effects, it is unlikely that a 50% overdosage of SOD would be responsible for the symptoms observed in trisomy 21. This has been suggested previously by Fridovich (28).

The toxic effects of SOD1 are not caused by SOD1 on its own, but by the combination of SOD1 with its dismutation product, H₂O₂. A rapid removal of H₂O₂ by catalase, which dismutates hydrogen peroxide into water and oxygen, should prevent the toxic effects of high doses of SOD1. The protective effect of catalase-SOD conjugates (29) but also catalase alone has also been investigated in O₂^{•-} caused injury. Conjugation of catalase to SOD1 resulted in complete protection against O₂^{•-} radicals generated in a rat heart model of ischemia reperfusion whereas the unconjugated SOD1 was either toxic or did not provide protection (29). Interestingly, when catalase expression in transgenic mice was increased a U-shaped curve was observed *ex vivo* for the protection against doxorubicin-generated super-

oxide-induced lipid peroxidation (30). Catalase inhibited doxorubicin-induced formation of lipid peroxides until 70-fold expression. Protection was lost at 200-fold expression, while at 500-fold expression damage was even increased. The very high expression levels at which toxic effects become noticeable indicate that catalase also has a large therapeutic window. Increasing the expression of a protein to such a high level is likely to have a negative effect on the expression level of other proteins, some of which might protect tissues against the noxious effects of doxorubicin. Therefore the effects of the 500-fold expression of catalase found by Kang et al. (30) might also have been caused by a decreased expression of protective cell constituents instead of excessive catalase activity.

Many *in vitro* investigations have pointed out that too high concentrations of SOD1 may paradoxically induce oxidative stress. In this study, however, we have shown that the lecithinized form of SOD1 appears to have a large therapeutic window. Strengthening the first line of defense against oxidative stress by administering lecithinized SOD1 can therefore be classified as both sound and safe.

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**Lack of inhibition of endothelial nitric oxide synthase by
doxorubicin**

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Toxicology in Vitro (accepted)

Introduction

Doxorubicin is a potent antineoplastic agent. Its therapeutic application is limited by the occurrence of cardiotoxicity (1-3). Previous studies (4, 5) have shown that the vascular tissue is also affected by doxorubicin. Recently, (6) reported that doxorubicin binds to, and is reduced by the reductase domain of endothelial NOS (eNOS). This would lead to increased superoxide ($O_2^{\bullet-}$) production while nitric oxide ($NO^{\bullet}$) formation is decreased. Because $NO^{\bullet}$ is one of the major vasorelaxant substances synthesized in the vascular endothelium (7, 8), the inhibition of eNOS by doxorubicin would impair vascular relaxation. Indeed, many cardiovascular disorders are associated with reduced $NO^{\bullet}$ -mediated effects and an increased production of vascular $O_2^{\bullet-}$ (9). Until now, eNOS inhibition by doxorubicin has been demonstrated in cultured cells only. To investigate the relevance of this inhibitory effect, we determined the effect of doxorubicin on endothelial nitric oxide synthase in the isolated rat aorta.

Materials and Methods

Effects of doxorubicin on the endothelium dependent relaxation

Experiments in which experimental animals were used were approved by the local ethics committee and protocols comply with the EC guidelines for the use of experimental animals. Male Wistar rats (3-6 months old) were killed by cervical dislocation. The thoracic aortas were excised. They were immediately placed in Krebs-Ringer solution (mM: NaCl 118, KCl 5.6, $CaCl_2$, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 10, $NaHCO_3$ 25) and excessive fat and connective tissue were removed. Rings (1.5 mm in length) were cut and mounted between stainless steel hooks in 20 ml organ baths. Changes in contraction were monitored using isotonic transducers. After 1 hr equilibration, tissues were incubated with doxorubicin (0-50 μM) or the known eNOS inhibitor L-NAME (10 μM). After 10 minutes, tissues were contracted with phenylephrine (10 μM). Carbachol concentration-response curves were constructed when the phenylephrine-induced contraction reached a plateau. To reach maximum relaxation sodium nitroprusside (0.1 mM) was added at the end of each experiment. The relaxant response to carbachol was expressed as a percentage of the relaxation induced by sodium nitroprusside. The resulting concentration-response data was

fitted using SigmaPlot v. 4.01 for Windows (SPSS Inc.) yielding values for the pD_2 ($-\log EC_{50}$) and maximum effect (E_{max}) for the concentration response curves.

Effect of doxorubicin on NO production in rat aortic rings

Nitrite, the metabolite from NO, was measured according to the method of (10). Aortic rings (5 mm) were washed in Krebs solution and divided into different experimental groups, which were treated with saline, carbachol (10 μ M) or the combination of carbachol (10 μ M) and doxorubicin (50 μ M) and incubated at 37 °C in a shaker for 30 min in a total volume of 1 ml. After reduction of NO_3^- to NO_2^- using nitrate reductase (0.1 U) over 30 min, ring supernatants were obtained by centrifugation. Equal volumes of Griess reagent (1% sulfanilamide and 0.1 % naphthylethylene diamine in 5% phosphoric acid) were added and the mixture was incubated at 37 °C for 30 min; absorbance was measured at 548 nm to determine total nitrite content. Data are expressed as μ mole/mg dry weight of tissue.

Figure 1. The effect of doxorubicin on the concentration-response curves for carbachol-induced relaxation of rat aorta. Open triangles: Control; Closed circles: doxorubicin 10 μ M; Open circles: doxorubicin 50 μ M; Closed triangles: L-NAME 10 μ M. Values are the mean $\pm$ standard error from four of five separate experiments. The inserted table presents the values for the pD_2 and the E_{max} obtained by fitting of the data.

Results

After precontraction with 30 μM phenylephrine, carbachol induced a relaxation with a pD_2 ($-\log \text{EC}_{50}$) value of 6.6. This relaxation was 84% of the maximum relaxation induced by 0.1 mM sodium nitroprusside (Fig. 1). The relaxation by carbachol was endothelium dependent, i.e. when the endothelium was not present, relaxation in response to carbachol was not observed (data not shown). Addition of doxorubicin (10 or 50 μM) or the known eNOS inhibitor L-NAME (10 μM) prior to phenylephrine did not cause an increase in basal tension or an increased response to phenylephrine (data not shown). Addition of doxorubicin (10 or 50 μM) did not affect carbachol-induced relaxation of phenylephrine contracted rat aorta. Pre-incubation with doxorubicin did also not affect the response to carbachol. L-NAME (10 μM) decreased both pD_2 (4.34 vs 6.58) and E_{max} (45.1 % vs 83.1 %) of the carbachol-induced relaxation.

Figure 2. Nitrite production by rat aortic rings. Aortic rings were stimulated with saline (CTRL), carbachol (10 μM) alone (CAR) or combined with 50 μM doxorubicin (CAR/DOX). Values are the mean $\pm$ standard error from four of five separate experiments.

Nitrite, an indicator of NO production was increased in the supernatant of carbachol-stimulated aortic rings in comparison to the supernatant of the unstimulated rings (Fig. 2). Nitrite concentrations in supernatant of rings that were stimulated with carbachol in the presence of doxorubicin were not decreased compared to supernatant of aortic rings that were stimulated with carbachol only (Fig. 2).

Discussion

A major problem with anthracyclines is their cardiotoxicity. In addition, these drugs have vascular actions as well. Nicholson and Custead reported that several antitumor drugs can damage the vascular endothelium (11). Endothelial cells contain high levels of endothelial nitric oxide synthase (eNOS) which is responsible for the production of NO[•]. This gaseous free radical mediates vasorelaxation by acetylcholine and histamine. The results of the present discussion clearly show that doxorubicin does not reduce NO[•] production and the relaxation which depends on NO[•] production. In contrast, in purified bovine eNOS doxorubicin was a potent inhibitor of NO[•] production (6). This discrepancy cannot be related to the concentration used. In the cell culture the IC₅₀ equaled 5 μM, while in the isolated aorta doxorubicin at a concentration of 50 μM had no effect. In patients, peak concentrations up to 5 μM are reported. Also, inability of doxorubicin to reach eNOS directly in the aorta cannot explain the lack of effect of doxorubicin because the endothelium is directly exposed to the doxorubicin containing medium. Furthermore, the therapeutic activity of doxorubicin requires high intracellular concentrations of this drug. Apparently, a straightforward explanation for the discrepancy between the results obtained in the cultured cells and the isolated aorta cannot be given. Nevertheless, the inability of doxorubicin to attenuate the carbachol-induced NO[•] production and relaxation of rat aorta implies that the impact of the doxorubicin-induced inhibition of NO[•] formation by purified eNOS is limited. The present results indicate that an impaired NO[•] formation by eNOS is not involved in the toxic effects of doxorubicin on vascular tissue.

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VI

SOD1 is inactivated by hypochlorous acid

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Abstract

HOCl is a powerful oxidant generated from hydrogen peroxide (H_2O_2) by activated neutrophils to kill invading microorganisms. Because it is well known that HOCl attacks proteins we hypothesized that HOCl could influence its own formation by effects on the proteins which play a role in HOCl generation, including SOD1. In the present study the effect of HOCl on SOD1 has been investigated. It was found that HOCl inactivates SOD1, the IC_{50} was $0.12 \mu\text{M}$.

Introduction

Activated neutrophils generate superoxide ($\text{O}_2^{\cdot-}$) and release the haem enzyme myeloperoxidase (MPO) (1, 2). Superoxide dismutates to hydrogen peroxide (H_2O_2) and oxygen, a reaction catalyzed by superoxide dismutase (SOD1). Hydrogen peroxide subsequently drives the oxidation of Cl^- anions to Cl^+ cations, which are so reactive that HOCl is formed almost immediately (Fig. 1).

Figure 1. Myeloperoxidase (MPO) oxidizes Cl^- anions to Cl^+ cations. The electrons are accepted by H_2O_2 which is reduced to H_2O . The Cl^+ cations react almost instantaneously with H_2O to yield HOCl.

HOCl reacts with lipids, proteins and DNA causing lipid peroxidation (3), protein inactivation (4) and DNA damage (5). Cysteine and methionine residues in proteins and reduced glutathione (GSH) appear to be the main targets for HOCl (6) thereby altering structure and function of proteins and lowering the antioxidant status in the target cell. It is therefore not surprising that HOCl plays a significant role in the

bactericidal function of the neutrophil. Although most of the HOCl generated in the inflammatory locus is directed at the target microorganism, the surrounding tissues can also be affected (7, 8). One of the proteins that might be damaged by HOCl is SOD1, which is highly sensitive to inactivation by oxidants (9). In this way, HOCl would inhibit the formation of one of its precursors, H_2O_2 . The aim of the present study was to determine the effect of HOCl on SOD1.

Materials and Methods

Chemicals

Human Cu,Zn-SOD (from human erythrocytes), xanthine, xanthine oxidase (grade IV: from milk suspension), nitro blue tetrazolium, were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Sodium hypochlorite (NaOCl, the conjugated base of hypochlorous acid ($pK_a = 7.46$) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Determination of SOD1 activity

SOD1 activity was determined according to McCord and Fridovich (10) except that nitrobluetetrazolium (NBT) instead of cytochrome c was used as detector molecule for $O_2^{\cdot -}$. $O_2^{\cdot -}$ were generated using xanthine (1 mM) and xanthine oxidase (11 $U \cdot ml^{-1}$) in 50 mM KP_i buffer containing 0.1 mM EDTA, pH 7.8 at 25 °C. $O_2^{\cdot -}$ reduces NBT to the corresponding formazan. The rate of this transformation can be monitored spectrophotometrically as the $\Delta A_{560} \cdot min^{-1}$. SOD1, by scavenging $O_2^{\cdot -}$, decreases the rate of NBT reduction. The effect of HOCl on SOD1 activity was determined by incubating SOD1 (1.2 $U \cdot ml^{-1}$) with HOCl (0.01 to 0.25 μM) for 15 min. The $O_2^{\cdot -}$ scavenging effect of HOCl treated SOD1 was subsequently determined as described above.

Results

SOD1 (1.2 $U \cdot ml^{-1}$) almost completely inhibited the xanthine/xanthine oxidase mediated reduction of NBT. This inhibition of NBT reduction was defined as 100% SOD1 activity (Fig. 2). Incubation of SOD1 with HOCl reduced the ability of SOD1 to inhibit the xanthine/xanthine oxidase mediated reduction of NBT. The

inhibition of SOD1 by HOCl was concentration-dependent with an IC_{50} of 0.12 μM (Fig. 2).

Figure 2. Concentration dependent inhibition of SOD1 by HOCl. SOD1 activity is expressed as the percentage inhibition of xanthine/xanthine oxidase mediated reduction of nitroblue tetrazolium. Data points are means $\pm$ standard error ($n=3$).

Discussion

HOCl generated by neutrophils reacts readily with thiols and thioethers and amines (6). It can therefore be expected that in case of neutrophil activation proteins will form an important target. HOCl affected proteins have immunogenic properties. The present results demonstrate that HOCl inactivates SOD1. This observation was recently confirmed by Aucherre and Capeillere-Blandin (11). The fact that HOCl inactivates SOD1 may account for the observation that SOD activity is rapidly lost during an acute asthmatic response.

Loss of SOD activity will result in a lowering of the rate of the dismutation reaction of $O_2^{\cdot -}$. Consequently, the $O_2^{\cdot -}$ concentrations will become higher at the ex-

pense of the H_2O_2 concentration. Because H_2O_2 serves as the electron acceptor for the MPO catalyzed oxidation of Cl^- this will result in a decreased production of HOCl by MPO. This is not expected to cause problems. Patients who are myeloperoxidase deficient and are unable to produce HOCl deficient rarely show symptoms (12). This contrast dramatically with chronic granulomatous disease (CGD), where the NADPH oxidase is absent (13). In CGD, common pathogens including *S. aureus* cause life threatening problems. Reduced formation of H_2O_2 , resulting in reduced formation of HOCl is therefore not expected to undermine host defense.

A likely consequence of the increased superoxide levels is a shift in toxic effects from the HOCl side to the $\text{O}_2^{\cdot -}$ side. Furthermore, the oxidative inactivation of SOD1 might be accompanied by a loss of copper ions from the active site. Release of copper out of the imidazolate mixed metal center in SOD1 has also been observed in the H_2O_2 induced inactivation of SOD1. Because copper ions can catalyze the formation of $\text{HO}^{\cdot}$ radicals some of the HOCl toxicity is shifted to the $\text{HO}^{\cdot}$ side. The inactivation of SOD1 by HOCl might shift. The relevance of the present finding needs further investigation.

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CHAPTER VI

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VII

Hypochlorous acid is a potent inhibitor of acetylcholinesterase

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Abstract

The role of hydrogen peroxide and peroxynitrite in the induction of airway hyperreactivity has been well described. Another reactive species which is formed during airway inflammation is hypochlorous acid (HOCl). In the present investigation the effect of HOCl on cholinergic innervation of the airway was investigated. It was observed that HOCl was capable of increasing the basal tension of electrically stimulated tracheal smooth muscle. It was found that HOCl inhibits purified acetylcholinesterase with an IC_{50} value of $0.66 \mu M$. Decreased acetylcholinesterase activity could allow accumulation of acetylcholine and increased airway muscle tension. The effects of HOCl on the isolated organ and the enzyme preparation could be precluded with thiol group containing compounds such as reduced glutathione and N-acetylcysteine. The present findings indicate that HOCl can act as inhibitor of acetylcholinesterase. The implications of this finding for the induction of airway hyperreactivity are discussed.

Introduction

Lung diseases such as (adult) respiratory distress syndrome, chronic bronchitis and asthma are characterized by pulmonary inflammation and a hyperreactivity of the airways toward several stimuli including methacholine. In the past it has been demonstrated that reactive products generated by inflammatory cells such as hydrogen peroxide (1) and peroxynitrite (2) or ozone in inhaled air (3) can be involved in the development of this hyperreactivity. During the inflammatory reaction macrophages and mast cells become activated and release chemotactic products such as interleukin-8 and leukotriene B_4 . Neutrophils and eosinophils are then attracted to the inflamed pulmonary tissue. Activated macrophages produce superoxide anions by a membrane bound NADPH oxidase. These superoxide anions are converted to hydrogen peroxide by enzymatical or spontaneous dismutation. Neutrophils release myeloperoxidase which catalyzes the formation of hypochlorous acid (HOCl) from chloride ions and hydrogen peroxide. The strong oxidizing species HOCl plays a highly significant role in the bactericidal function of the neutrophil. However, inappropriate and/or excessive activation of neutrophils leads to oxidative stress and collateral damage to surrounding tissues (4, 5). Cysteine and methionine residues in proteins and reduced glutathione (GSH) appear to be the main targets for HOCl

(6) thereby altering structure and function of proteins and lowering antioxidant status in the cell. Research on the role of HOCl in lung diseases has focused on the ability of HOCl to inhibit α_1 -antiprotease by oxidizing the methionin residue on position 358 (7). Due to this oxidation α_1 -antiprotease is not able to inhibit elastase. An increased elastase activity leads to break down of elastine which results in alveolar wall destruction (emphysema) and mucus hypersecretion (8). Thiol containing compounds including GSH and N-acetylcysteine are able to protect α_1 -antiprotease by scavenging HOCl (9). While it has been demonstrated that other reactive oxygen species are involved in the pathogenesis of airway hyperreactivity, this has not been shown for HOCl. The aim of the present study is to examine the effect of HOCl on the cholinergic control of airway smooth muscle. Cholinergic nerve fiber density remains relatively constant throughout the airway tree (10). Therefore, the effect of HOCl on the response of electrically stimulated guinea pig tracheal smooth muscle was investigated.

Materials and methods

Functional experiments

Experiments in which experimental animals were used were approved by local ethics committee and protocols comply with the EC guidelines for the use of experimental animals. Male Dunkin-Hartley guinea pigs, weighing 300-350 g and obtained from Harlan CPB (Zeist, the Netherlands) were killed by a blow on the head. The trachea was removed, dissected free from surrounding tissue and cut into longitudinal direction opposite the smooth muscle layer. Tracheal strips (each containing one cartilage ring) were mounted in a 20 ml water jacketed organ bath containing Krebs-buffer solution of pH 7.4 at 37 °C of the following composition (mM): NaCl, 117.5; KCl, 5.6; MgSO₄, 1.2; CaCl₂, 2.5; NaH₂PO₄, 1.3; NaHCO₃, 25; glucose, 5.5; gassed continuously with 5% CO₂-95% O₂. Indomethacin (10 μ M) was added to the buffer to maintain constant tension. Measurements were started after an equilibration period of 60 min with 4 intermediate changes of buffer solution. To determine the effect of HOCl on the basal tone, the tracheal preparation was incubated with HOCl (0.1; 1 or 10 mM) with or without N-acetylcysteine (in equimolar amounts). The increase of basal tone was recorded isometrically and expressed as a percentage of the maximal response to methacholine (30 μ M).

To determine the effect of HOCl on the electric field stimulated trachea, platinum electrodes were placed above and under the tracheal preparation. Electrical field stimulation (50 V, 1.0 ms with train pulses of 5 Hz during 3 s) was applied. Changes in smooth muscle contraction were recorded isotonicly using 0.1 g counterweight. The tracheal preparation was incubated with HOCl (1, 10 and 100 μM). Physostigmine (0.1 and 1 μM) was added to control tracheal preparation. At the end of each experiment atropine (10 μM) was added.

Determination of acetylcholinesterase activity

Purified acetylcholinesterase (0.014 $\text{U}\cdot\text{ml}^{-1}$) was incubated with HOCl (0 to 20 μM) in phosphate buffer pH 8.0 for 15 min. Acetylcholinesterase activity was subsequently determined according to Ellman *et al.* (11), using acetylthiocholine (0.5 mM) as substrate and dithiobisnitrobenzoic acid (0.33 mM). Thiocholine, formed by the action of acetylcholinesterase on acetylthiocholine reacts nonenzymatically with dithiobisnitrobenzoic acid to form thionitrobenzoic acid. Thionitrobenzoic acid formation was monitored spectrophotometrically at 412 nm for 2 min. A significant interaction between remaining HOCl and thionitrobenzoic acid or (acetyl)thiocholine can be excluded because the sulphur containing compounds are present in 20-25 fold excess. Moreover, acetylthiocholine is added after HOCl has reacted with acetylcholinesterase. The IC_{50} value for inhibition of acetylcholinesterase by HOCl was determined by curve fitting (4 parameter logistic curve) using SigmaPlot 2001 for Windows. To investigate the type of inhibition, the substrate (acetylthiocholine) concentration was varied (60 to 600 μM) while the concentration inhibitor (HOCl) was kept constant (0.66 μM). A Lineweaver-Burke plot was constructed. Data fitting by linear regression yielded values for the K_m (reciprocal value of intercept with x-axis) and V_{max} (reciprocal value of intercept with y-axis). The ability of GSH, N-acetylcysteine and lipoic acid to protect acetylcholinesterase against inactivation by HOCl was investigated by mixing these compounds (0-1 μM) with acetylcholinesterase prior to addition of HOCl (0.9 μM).

Drugs and reagents

Sodium hypochlorite, indomethacin, physostigmine, atropine, methacholine, , acetylthiocholine iodide, DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetylcholinesterase (type III

from Electric Eel, Sigma Cat. No. C-2629) had a specific activity of 1,000 units per mg of protein. One unit will hydrolyze 1.0 mmole of acetylcholine to choline and acetate per min at pH 8.0 at 37 °C. All other reagents were of analytical grade.

Statistical evaluation

Data are expressed as mean $\pm$ S.E.M, when appropriate. Differences were evaluated with SPSS 8.0 for Windows using analysis of variance (ANOVA) with Fisher's least significant differences (LSD) test for multiple comparisons when ANOVA indicated significant difference between groups. The level of significance chosen was 95% ($P < 0.05$).

Figure 1. The HOCl dependent increase in basal tone of guinea pig tracheal smooth muscle and the influence of N-acetylcysteine (1 mM). The data are expressed as a percentage of the maximal response to methacholine (30 μ M) ($\pm$ S.E.M.) (N=3). * $P < 0.05$ compared to baseline (no contraction).

Results

Functional experiments

Addition of HOCl (0.1, 1 and 10 mM) to the tracheal preparation resulted in a dose dependent increase in basal tone of guinea pig tracheal smooth muscle (Fig. 1). The increase in basal tone caused by 1 mM HOCl could be blocked by pre-incubating with N-acetylcysteine (1 mM) (Fig. 1). Electrical field stimulation of guinea pig tracheal strips *in vitro* induced a monophasic contraction. The twitch could be blocked by atropine (10 μ M).

Figure 2. Twitch response of guinea pig tracheal smooth muscle induced by electric field stimulation (50 V, 1.0 ms with train pulses of 5 Hz during 3 s). In the left trace the effect of HOCl is shown. In the right trace the effect of physostigmine is shown. Typical recordings are shown. In both cases atropine blocks the increase in basal tone and the twitch response.

Addition of 10 μ M HOCl to the electrically stimulated tracheal smooth muscle strips caused a small decline of the twitch height. Addition of 100 μ M HOCl caused an increase in basal tone which could be abolished using atropine (10 μ M) (Fig. 2). The increase in basal tone, the tension to which the contraction returns after the twitch has vanished, of the electrically stimulated tracheal preparation

caused by HOCl could be mimicked using the acetylcholinesterase inhibitor physostigmine (0.1 and 1 μM) (Fig. 2).

Figure 3. Inhibition of acetylcholinesterase purified from electric eel by HOCl. Values are means of two separate experiments. Determination of the acetylcholinesterase activity was reproducible within 5%. (AU = arbitrary units)

Acetylcholinesterase assay

HOCl decreased the rate of acetylthiocholine hydrolysis by purified acetylcholinesterase (Fig. 3). Curve fitting showed that the IC_{50} was 0.66 μM . Enzyme kinetics analysis (Fig. 4) revealed that HOCl induced a lowering of the V_{max} (8.2 versus 14.7 $\mu\text{M.s}^{-1}$) while the K_m remained unchanged (120 versus 128 μM). This indicates that the inhibition of acetylcholinesterase by HOCl is non-competitive. Co-incubation of acetylcholinesterase with reduced glutathione, N-acetylcysteine or lipoic acid dose-dependently prevented the inhibition of acetylcholinesterase by HOCl (Fig. 5).

Figure 4. Lineweaver-Burk plot showing non-competitive inhibition of purified acetylcholinesterase by 0.66 mM HOCl (open circles) versus control (closed circles). Values are means of two separate experiments. Determination of the acetylcholinesterase activity was reproducible within 5%.

Discussion

HOCl plays a central role in the antimicrobial action of neutrophils. It is also linked to the tissue damage which is seen in many inflammatory diseases, including those in the airways such as asthma, respiratory distress syndrome and chronic obstructive pulmonary disease. In inflammatory lung diseases, an exaggerated response towards cholinergic stimuli, which are responsible for contraction of airway smooth muscle, is frequently observed (12). The increased tone of smooth muscle contraction is directly linked (13) to the drastic decrease in FEV_1 and deterioration of other respiratory parameters (14). In the present study the role of HOCl in the induction of airway hyperresponsiveness toward cholinergic stimuli was investigated. In the organ bath studies, it was demonstrated that HOCl was capable of increasing the basal contraction level. This process could be reversed by addition of atropine indicating that this increase in basal contraction level was indeed acetylcholine mediated. Apart from a small effect on the twitch height caused by HOCl, addition of

physostigmine caused an effect on the electrically stimulated trachea comparable to that caused by HOCl. Therefore, acetylcholinesterase was suspected to be the target of HOCl.

Figure 5. The concentration-dependent effect of N-acetylcysteine, glutathione and lipoic acid on the inhibition of purified acetylcholinesterase by HOCl (0.9 mM). (AU = arbitrary units). Control: No HOCl was added. Values are means of two separate experiments. Determination of the acetylcholinesterase activity was reproducible within 5%.

Using acetylcholinesterase purified from electric eel, it was confirmed that HOCl is indeed a very efficient inhibitor of acetylcholinesterase. Enzyme kinetics analysis (Fig. 4) clearly demonstrated that the HOCl induced inhibition of acetylcholinester-

ase is non-competitive. Analogue to the inhibition of α_1 -antiprotease, the inhibition of acetylcholinesterase is probably caused by oxidation of an amino acid critical for enzyme function. It has been suggested that oxidatively damaged α_1 -antiprotease peptide can be reduced by methionine sulfoxide reductase (PMSR, EC 1.8.4.6), resulting in reactivation of protein function (15). If cysteine or methionine residues in acetylcholinesterase are oxidized by HOCl, a similar reduction might also result in restoration of protein function. In our *in vitro* experiments using purified acetylcholinesterase no enzymes or reducing equivalents are present which might repair the oxidative damage to acetylcholinesterase. Therefore the inhibition of acetylcholinesterase *in vitro* is irreversible. The IC_{50} value of HOCl for acetylcholinesterase inhibition is approximately 30-fold lower than for α_1 -antiprotease inhibition (0.66 vs. 20 μ M (9)) which is remarkable because α_1 -antiprotease is generally regarded as the primary target of HOCl (7). The IC_{50} of HOCl is only 20-fold higher than the reported IC_{50} value of 30 nM for physostigmine (16). The capacity of HOCl to inhibit acetylcholinesterase in the tracheal preparation appears to be lower than its capacity to inhibit acetylcholinesterase purified from electric eel. This can be explained by the presence of other biomolecules which can also react with exogenously applied HOCl. It is difficult to determine the exact concentration of HOCl that can be reached *in vivo* since it is formed locally and HOCl is very reactive. It has been calculated that during inflammation superoxide can be formed at the rate of 5 to 10 mM per second, implicating a hydrogen peroxide formation rate of 2.5 to 5 mM per second. Locally, the released myeloperoxidase can reach a concentration of 1 to 2 mM [Hampton, 1998 #407]. Generation of amounts of HOCl large enough to induce substantial damage to acetylcholinesterase can therefore be expected. Calculations indicate that approximately 12,000 molecules of HOCl are necessary to completely inhibit 1 molecule of acetylcholinesterase. For 1 molecule of α_1 -antiprotease, which is 5 times lower in weight than acetylcholinesterase, 130 molecules are necessary for complete inhibition of activity.

A diminished activity of acetylcholinesterase results in an increased and prolonged action of acetylcholine at the neuromuscular junction. In this situation, basal cholinergic activity in the airways results in a stronger baseline obstruction of the airways. Increased parasympathetic activity will cause an exaggerated contractile response of airway smooth muscle. This hyperresponsiveness is often encountered in inflammatory diseases of the airways. The deleterious effects of HOCl could be prevented by pre-incubating with thiol group containing compounds. This is in

perfect agreement with (6) who reported that HOCl reacts rapidly with thiol groups.

In summary, our results show that HOCl causes an increased response to cholinergic stimuli which is most likely the result of decreased breakdown of acetylcholine. This points towards HOCl as an inhibitor of acetylcholinesterase. Although the exact relevance of this finding has to be substantiated by additional research, it could imply that HOCl, besides hydrogen peroxide (1) and peroxynitrite (2), also contributes to the observed airway hyperresponsiveness to cholinergic stimuli which is observed in inflammatory lung diseases. Thiol group containing compounds could play a protecting role during inflammatory processes and might prevent at least part of the observed airway hyperreactivity.

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VIII

Efficacy of HOCl scavenging by sulfur containing compounds.

Antioxidant activity of glutathione disulfide?

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Abstract

Hypochlorous acid (HOCl) is a bactericidal compound formed by activated neutrophils during inflammation. Overproduction of HOCl causes damage to tissues at the site of neutrophil accumulation. The deleterious effects of excessive HOCl formation can be attenuated using antioxidants. Thiols and thioethers are known to be very effective HOCl scavengers. In the present study, the potency of several sulfur containing compounds to protect acetylcholinesterase, glutathione S-transferase P1-1 (GST P1-1) and α_1 -antiproteinease against inactivation by HOCl was determined. Surprisingly, glutathione disulfide was an effective protector of acetylcholinesterase against hypochlorous acid. Glutathione disulfide did not provide protection for GST P1-1 and α_1 -antiproteinease against oxidative inactivation by HOCl. The implications of this finding are discussed.

Introduction

Hypochlorous acid (HOCl) is a powerful oxidant generated by the neutrophil enzyme myeloperoxidase from hydrogen peroxide and chloride ions (1). HOCl is thought to play a central role in the antimicrobial action of neutrophils when it is targeted to microorganisms (2). HOCl oxidizes a wide variety of biological compounds. The reaction of HOCl with thiols or thioethers are particularly fast (1). The oxidation of key enzymes and reduced glutathione (3) is probably important in the antimicrobial action of HOCl. Uncontrolled and excessive production of HOCl causes 'collateral damage' to surrounding tissues at the site of inflammation. Exposure to high levels of HOCl is cytotoxic and cell death can occur via an overwhelming onslaught to many vital cell components including membranes, proteins, and DNA (3). An important target for HOCl appears to be α_1 -antiproteinease (α_1 -AP), which is the most important inhibitor of elastase. The increased elastased activity after α_1 -AP inactivation by HOCl results in lung emphysema (4). It has been demonstrated that HOCl potently inhibits acetylcholinesterase (AChE) (5) and glutathione S-transferase P1-1 (GST P1-1) (6) *in vitro*. Inactivation of acetylcholinesterase results in exaggerated responses to cholinergic stimuli. This might contribute to the airway hyperreactivity that is observed during inflammation in the airways. GST P1-1 is, besides an important phase II detoxification enzyme, a regulator of Jun N-terminal kinases (JNKs) activities. It has been proposed that GST P1-

1 serves as a sensor of intracellular changes in redox potential that are elicited by various forms of oxidative stress (7). HOCl appears to be more than 1000 times as potent in inhibiting GST P1-1 than H_2O_2 *in vitro* (6). A potential way to circumvent the damaging effects of HOCl is the application of HOCl scavengers. It has been demonstrated *in vitro* that several sulfur-containing compounds efficiently protect α_1 -AP against inactivation by HOCl (8). These sulfur-containing compounds could therefore play a beneficial role in preventing and stabilizing lung emphysema. Antioxidants that protect AChE and GST P1-1 against HOCl may find an application during inflammation of the upper airways and control of cell growth respectively. In the present study the efficacy of several sulfur-containing compounds including thiols, thioethers and disulfides to protect α_1 -AP, AChE and GST P1-1 *in vitro* against HOCl was compared.

Materials and methods

Elastase/ α_1 -antiprotease assay

The efficacy of sulfur containing compounds to protect α_1 -antiprotease (α_1 -AP) against hypochlorous acid was determined by mixing α_1 -AP (20 μ g) with the scavenger/test compound. Subsequently HOCl (50 μ M) was added and the mixture was incubated at 25 °C for 5 min. The elastase inhibitory activity of this mixture was determined using the assay described by Wasil *et al.* (9) with minor modifications according to Haenen and Bast (1991). In short, 200 μ l buffer containing 5 μ g elastase was added. After 5 min, 700 μ l buffer and 50 μ l of a 10 mM solution of N-t-BOC-L-alanine-p-nitrophenol ester in methanol was added. Immediately after the addition of N-t-BOC-L-alanine-p-nitrophenol ester, the increase in absorbance at 410 nm was determined, which represents the activity of elastase. Control experiments pointed out that the test compounds neither affected the elastase activity directly nor the ability of α_1 -AP to inhibit elastase directly.

Acetylcholinesterase assay

The efficacy of sulfur containing compounds to protect acetylcholinesterase (AChE) against hypochlorous acid was determined by mixing AChE (0.014 U.ml⁻¹) with scavenger/test compound in 50 mM phosphate buffer pH 8.0. Subsequently

HOCl (1 μ M) was added and the mixture was incubated at 25 °C for 5 min. AChE activity was subsequently determined according to Ellman et al. (10). In short, acetylthiocholine (0.5 mM) was added as substrate with dithiobisnitrobenzoic acid (0.3 mM) as reagent to detect thiocholine formation. The increase in absorbance at 412 nm was monitored, which represents AChE activity. A significant interaction between remaining HOCl and TNB or acetylthiocholine can be excluded because the thiol group containing compounds are present in 20-25-fold excess. Moreover, acetylthiocholine is added after 15 min incubation of HOCl with AChE.

Glutathione S-transferase P1-1 assay

The efficacy of sulfur containing compounds to protect glutathione S-transferase P1-1 (GST P1-1) against hypochlorous acid was determined by mixing GST P1-1 (0.017 U.ml⁻¹) with scavenger/test compound in 100 mM potassium phosphate pH 6.5. Subsequently HOCl (1.5 μ M) was added and the mixture was incubated at 37 °C for 1 min. GST P1-1 activity was determined as described by Mannervik and Guthenberg (11) with minor modifications. In short, the reaction of 1 mM CDNB with 1 mM GSH in 100 mM potassium phosphate buffer pH 6.5, at 37°C was monitored spectrophotometrically by recording the increase in absorbance at 340 nm. A correction for the spontaneous reaction was made for the formation of the conjugate of GSH and CDNB in the absence of enzyme. Control experiments pointed out that α_1 -AP possesses no AChE activity and that AChE does not possess α_1 -AP activity.

Chemicals

Elastase (from human leukocytes), α_1 -antiproteinase (from human plasma), acetylcholinesterase (type III, from electric eel), glutathione S-transferase P1-1 (from human placenta), GSH, GSSG, S-methylglutathione (GSMe), N-t-BOC-L-alanine-p-nitrophenol ester, acetylthiocholine iodide, 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent, DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Sodium hypochlorite (NaOCl, the conjugated base of hypochlorous acid (pKa = 7.46) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Lipoic acid was a gift from Asta Medica AG (Frankfurt am Main, Germany). All other chemicals were of analytical grade.

Results

The efficacy of several sulfur containing compounds (reduced glutathione (GSH), glutathione disulfide (GSSG), S-methylglutathione (GSMe) and lipoic acid (LA) to protect acetylcholinesterase (AChE), glutathione S-transferase P1-1 (GST P1-1) and α_1 -antiproteinase (α_1 -AP) was determined. The concentrations of HOCl that have to be used to reduce the enzyme activities to 0 % are 0.6, 2.5 and 50 $\mu\text{M}/\mu\text{g}$ protein for GST P1-1, α_1 -AP and AChE, respectively. In order to be able to compare the efficacies of the test compounds to protect against HOCl, the ratio of the EC_{50} (concentration of scavenger needed to reduce HOCl damage by 50%) and the HOCl concentration used to reach 100 % inhibition is presented in Table 1.

Table 1. Protection of enzymes against inactivation by HOCl.

Compound	Enzyme		
	acetylcholinesterase	glutathione S-transferase P1-1	α_1 -antiproteinase
GSH	0.2	0.5	0.4
GSSG	0.3	>200	>200
GSMe	0.6	n.d.	0.7
Lipoic acid	0.4	2	0.6

The protective efficacy of the sulfur containing compounds is expressed as the ratio between EC_{50} and the HOCl concentration that is used to completely block the enzyme. The EC_{50} value is the concentration that reduces HOCl damage by 50%. The lower the ratio, the more potent the compound. GSMe acts as a competitive inhibitor of GST P1-1 in the same concentration range as it scavenges HOCl and is therefore not determined in this case (n.d.).

It is shown that GSH, GSMe and LA all have comparable efficacies when protecting AChE, GST P1-1 and α_1 -AP against oxidative inactivation by HOCl. GSSG does not protect GST P1-1 and α_1 -AP against oxidative inactivation by HOCl. The concentration of GSSG that is needed to reduce the HOCl-induced inactivation of these proteins by 50% is more than 200 times the concentration of HOCl that was used to completely block protect GST P1-1 and α_1 -AP. However, for AChE the amount of GSSG needed to reduce the HOCl-induced inhibition by 50% is only

0.3 times the concentration of HOCl that was used to completely inhibit AChE (Table 1).

Figure 1. Protection against HOCl mediated oxidation of lipoic acid by reduced glutathione (GSH), S-methyl glutathione (GSMe) and glutathione disulfide (GSSG). Protection was determined in a competition assay where the concentration of all three components (detector molecule, scavenger and oxidant) was 1 mM. Values are means $\pm$ standard error (n=3).

The order of potencies of sulfur containing compounds was also evaluated in a competition assay in which the protection of LA against HOCl was investigated. Preincubation with GSH (1 mM) protected 70% of LA (1 mM) against HOCl (1 mM) (Fig. 1). Preincubation with GSMe resulted in 50% LA remaining while GSSG did not scavenge HOCl at all, i.e. only 10% LA remained (Fig. 1).

The relative reactivities of AChE and α_1 -AP towards HOCl were investigated by determining the effect of HOCl on a mixture of the two proteins. An amount of 20 μ M HOCl was sufficient to inhibit both AChE and α_1 -AP when both enzymes were tested separately (data not shown). When AChE and α_1 -AP were mixed and 20 μ M HOCl was added, HOCl almost completely inactivated α_1 -AP, while AChE activity was not decreased (Fig. 2). Preincubation with GSSG (20 μ M) did not result in protection of α_1 -AP against inactivation by HOCl. Addition of 200 μ M

HOCl resulted in the inactivation of both α_1 -AP and AChE. Preincubation with 200 μ M GSSG prevented, although partially (50 %), only the inactivation of AChE, and not the inactivation of α_1 -AP (Fig. 2) by 200 μ M HOCl.

Figure 2. Effect of hypochlorous acid on a mixture of AChE and α_1 -AP. Addition of HOCl resulted in selective inactivation of α_1 -AP. An equimolar amount of GSSG could not prevent selective inactivation of α_1 -AP by HOCl. Addition of 200 μ M HOCl resulted in inactivation of both enzymes. An equimolar amount of GSSG partially prevented the inactivation of AChE by 200 μ M HOCl. Error bars indicate the reproducibility of the enzyme activity determinations which was within 5%.

Discussion

Reduced glutathione (GSH) and its methylated form (GSMe) were good scavengers of HOCl when tested in the α_1 -AP/elastase assay. The oxidized form of glutathione (GSSG) is not a potent scavenger of HOCl. The most striking result is that lipoic acid, which is also a disulfide compound like GSSG, is a very good scavenger in the α_1 -AP/elastase assay. The explanation for this difference may be found in

the nature of the disulfide bridge in both molecules. Lipoate contains an intramolecular S-S bridge. Because this bridge is fixed in a 5-membered ring, some ring strain exists. It has been reported that the angle between both sulfur atoms is energetically not optimal (12). In GSSG, no ring strain exists. The difference between the S-S bridges probably explains the difference between GSSG and lipoic acid in their ability to scavenge HOCl. Another reason for the relative inefficacy of GSSG compared to LA may be the fact that in GSSG, the disulfide moiety is more shielded by steric or electrostatic factors, as it is surrounded by 2 γ -glutamate and glycine residues.

When the ability of these compounds to protect GST P1-1 against oxidative inactivation by HOCl was investigated the same order of potencies was observed. It was however noted that GSH and LA appeared to be less effective in scavenging HOCl than in the α_1 -AP/elastase assay. GSH, GSMe and LA were shown to be good scavengers of HOCl when protecting AChE against oxidative inactivation by HOCl. Intriguingly, GSSG appeared to be a good scavenger in this assay as well, i.e. GSSG prevented the oxidative deterioration of AChE by HOCl. GSSG is generally regarded as a compound devoid of any antioxidant activity, whereas in the AChE assay its potency equalled that of GSH.

We were rather astonished by this protective effect of GSSG. To explain its protective effect, we initially thought that GSSG might shield the reactive centre of AChE so HOCl cannot reach its target or that disulfide exchange and the formation of mixed disulfide with a protein thiol function might occur. In retrospect, the explanation is more simple. In order to explain the different potencies of GSSG in protecting the different enzymes, the principle of the assays used in these studies has to be taken into consideration. The protective efficacy depends on the ratio of the reaction rates of detector molecule (i.e. the enzymes) and antioxidant with HOCl. A good protector reacts with HOCl at a higher rate than the detector molecule does. The efficacy of GSSG to protect AChE against HOCl might originate from the relatively slow reaction of HOCl with AChE, which appears to be even slower than the reaction of HOCl with GSSG.

This hypothesis was tested by investigating the relative reactivity towards HOCl by AChE and α_1 -AP when both proteins are present simultaneously. In this experiment it was clearly demonstrated that in a mixture of α_1 -AP and AChE, HOCl preferably reacts with α_1 -AP. This results in a decreased α_1 -AP activity and an unchanged AChE activity. GSSG did not protect α_1 -AP and only protected AChE.

Differences in rates of the reactions of α_1 -AP, GST P1-1 and AChE with HOCl are related to the (i) functional group in the respective enzymes which is attacked by HOCl (ii) the importance of this functional group for enzyme activity and (iii) the accessibility of these groups for HOCl. In GST P1-1 cysteine residues at the 47th and 101st position were found to be critical for activity (13, 14). In α_1 -AP, structural integrity of a methionine residue at position 358 is crucial for effective interaction with elastase. Oxidation of this residue to the corresponding sulfoxide by HOCl results in a strongly diminished elastase inhibiting activity (4). All AChEs contain three conserved intrasubunit disulfide bonds with the catalytic subunits (15). Oxidation by HOCl of these disulfide bridges, which disturbs the 3 dimensional arrangement of the enzyme, is probably the mechanism underlying AChE inactivation by HOCl.

HOCl reacts with GST P1-1 faster than with α_1 -AP. The rate of the reaction of AChE with HOCl is very low. Thus, the relative reaction rates of the functional groups in the proteins, respectively SH, SMe and S-S, with HOCl are the same for the enzymes and the small organic molecules. As shown by the effects of GSSG, which does not protect GST P1-1 and α_1 -AP but is effective for protecting AChE, the difference in reaction rate of the target has consequences for the efficacy of scavengers.

Rapid and convenient test tube assays have been developed to screen the HOCl scavenging activity of a wide variety of compounds. These assays are based on the competition between detector molecules and scavengers for HOCl. The detectors that have been employed include thionitrobenzoic acid (TNB, reduced Ellman's reagent) (16) and bovine serum albumin (BSA) (17). In TNB the aromatic sulphhydryl group is oxidized by HOCl which leads to a reduced absorption at 412 nm. Inhibition of this decolorization by a compound gives its HOCl scavenging ability. The major drawback of this assay is the fact that GSH and other thiols cannot be evaluated in this assay since thiols are capable of regenerating TNB from oxidized TNB. In the albumin assay, electrophoresis is used to quantify HOCl induced oxidation of BSA. Scavenging potency is reflected by the concentration of compound reducing 50% of BSA oxidation. Results obtained by Ching *et al.* (16) using the TNB assay and Yan *et al.* (17) using the albumin assay are displayed in Table 2. The results have been displayed in a similar fashion as used in Table 1 to facilitate comparison. Scavenging potencies differ greatly between the TNB and albumin assay. GSSG has no antioxidant activity in the TNB assay, while it has an activity compa-

table to that of LA and GSMe in the albumin assay. The explanation is similar to that provided for the different antioxidant activity of GSSG for the three enzymes tested. The reaction rate of the aromatic sulphhydryl group is relatively high, this could lead to an underestimation of antioxidant activity. GSMe and LA, which are effective protectors of the three tested enzymes, are qualified as relatively poor HOCl scavengers in the TNB assay. The functional group in BSA that reacts with HOCl has not been identified (17). Based on the protective effect of GSSG in this assay, it can be predicted that the reactivity of this group is relatively low and comparable to an unstrained S-S moiety. Due to this low reactivity the HOCl scavenging potency of compounds can easily be overestimated. Both in the TNB assay and in the albumin assay, artefacts easily arise: False negative results in the TNB assay and false positive in the albumin assay.

Table 2. Protection of TNB and albumin against oxidation by HOCl.

Compound	Detector Molecule	
	TNB	Albumine
GSH	n.d.	0.2
GSSG	>200	0.6
GSMe	14	0.5
Lipoic acid	2.8	0.5

The protective efficacy of the sulfur containing compounds in the TNB assay (16) and the albumine assay (17). The ratio EC_{50} / HOCl concentration is given.

The present study has demonstrated that the *in vitro* efficacy of an antioxidant depends on the choice of detector molecule. An antioxidant is defined as a compound which, in a relatively low concentration, protects other compounds (biological compounds as a rule) against oxidation (by oxygen free radicals, reactive oxygen species or oxidants in general). Scavengers of reactive oxygen species protect by competing with the biological compound for the reactive oxygen species. Of great importance is the rate of reaction of the protecting compound with HOCl relative to the rate of reaction of the biological target to be protected with HOCl. The present study has demonstrated that GSSG is capable of competing with AChE for HOCl, offering antioxidant activity to a compound thought to be devoid of antioxidant activity until now.

The consequences of this finding for the *in vivo* situation is that the efficacy of an antioxidant depends on the biological target which the antioxidant is meant to protect. The biological target in turn is determined by the location where the reactive species is formed. In the upper airways (trachea), it has been shown that the biological target for HOCl is AChE resulting in an increased basal smooth muscle tension (5). In the lower airways, the biological target for HOCl is α_1 -AP, resulting in unbridled elastase activity. The HOCl concentrations employed in the *in vitro* studies can be reached *in vivo*, albeit very locally (2). Formation of HOCl could be responsible for damage in the upper airways resulting in altered airway function. The present results demonstrate that GSSG might be beneficial in reducing damage in the upper airways, however, the low endogenous concentrations of GSSG render its participation in the antioxidant scale of these tissues probably a minor one. Because GSSG cannot protect α_1 -AP, GSSG is not effective further down along the airway tree. In general, the question whether a compound is a good antioxidant should be replied as: 'That depends, on the location where you want it to act and what target needs to be protected.'

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CHAPTER VIII

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Summary

In **Chapter 1**, the general introduction to the thesis, an outline concerning $O_2^{\cdot-}$ and the family of SOD enzymes is given. Superoxide ($O_2^{\cdot-}$) and superoxide dismutase (SOD) are Janus-faced molecules. $O_2^{\cdot-}$ is formed in a wide variety of (patho)physiological processes, including ischemia-reperfusion, inflammation and biotransformation. $O_2^{\cdot-}$ has many toxic actions of its own, induction of membrane lipid peroxidation, oxidation of catecholamines, reaction with, and thus inactivation of, nitric oxide, and influencing cellular processes by activation of protein kinases and transcription factors. Furthermore, $O_2^{\cdot-}$ is the precursor of a number of reactive oxygen species that can cause further damage to the cell. The toxic effects of $O_2^{\cdot-}$ are beneficial when the body has to be defended against invading microorganisms. Consequently, the inability to generate $O_2^{\cdot-}$ is a life-threatening condition. $O_2^{\cdot-}$ production is therefore necessary at times, but has to take place under tight control. This important task resides on the shoulders of the SODs, which are found in the cytosol (SOD1), mitochondria (SOD2) and in the extracellular space (SOD3). However, in many circumstances SOD appears to be overwhelmed by the amount of $O_2^{\cdot-}$ which it encounters. In these circumstances, it appears rational to raise SOD levels, either by administration of (chemically modified) SOD or SOD mimetics or by increasing SOD expression. This has been tested in a wide variety of diseases and proved helpful. There are however several problems associated with SOD. The most striking of these problems is the additional peroxidase activity that SOD1 possesses. The combination of hydrogen peroxide, one of the products of SOD, with SOD1 results in peroxidation of biological compounds, e.g. lipid peroxidation. The neurodegeneration observed in patients with Down's syndrome has been ascribed to overexpression of SOD1. The gene encoding for SOD1 is located on chromosome 21. Just like the production of $O_2^{\cdot-}$ must be tightly controlled, the use of SOD1, though rational and effective, ought to be surrounded with great care. In **Chapter 2**, some background information is given on two classes of drugs that have toxic effects on the heart, the anthracyclines and the tricyclic antidepressants. Both classes of drugs have effects on the ECG, most notably a lengthening of the ST interval, indicating that the repolarization of the ventricles is compromised. Especially in people who have been treated with anthracycline antitumor drugs this is a possible life-threatening situation. These ECG effects can be used to study the effectiveness of compounds that protect the heart against the toxic ef-

fects of these drugs in laboratory animals. The absence of cardiotoxic effects in analogs of the cardiotoxic anthracyclines or tricyclic antidepressants can also be assessed using ECG measurements in animals. The telemetry method has been used in the past to determine the efficacy of flavonoids to protect against doxorubicin-induced cardiotoxicity. We have improved the telemetry method in two ways. The transmitter has been implanted subcutaneously, an operation that does less harm to the animals. Additionally, the data evaluation has been simplified. Signal averaging and automatic detection of the intervals in the ECG make the experiment more accurate and less time-consuming and less prone to inter-experimentator variation. One of the cardiotoxic anthracyclines is doxorubicin and the cardiotoxicity is the main factor that limits the therapeutic application of this otherwise very effective drug. Doxorubicin generates $O_2^{\bullet -}$ by redox-cycling and is generally accepted that $O_2^{\bullet -}$ generated by doxorubicin is responsible for the cardiotoxic effects. Reducing the $O_2^{\bullet -}$ concentration might therefore prevent the occurrence of this severe side-effect. In **Chapter 3**, the efficacy of PC-SOD, lecithinized SOD1, to protect against doxorubicin-induced cardiotoxicity is described. It was found that PC-SOD inhibited the doxorubicin-induced lengthening of the ST interval in the mouse. Thus, the compromised ventricular repolarization normally observed after treatment with doxorubicin, is not detected when this drug is given together with PC-SOD. Furthermore, the combination of doxorubicin with PC-SOD did not influence the antitumor effect of doxorubicin. We can therefore conclude that PC-SOD protects the heart against the noxious effects of doxorubicin, but does not reduce the effects of doxorubicin on the tumor. The use of PC-SOD seems safe and solid in this pathophysiological process. How about that negative side of SOD? In **Chapter 4**, the effects of SOD1 and PC-SOD were investigated in a superoxide producing environment in a test tube. *In vitro*, it was demonstrated that a coumaric acid derivative is hydroxylated when the concentration of SOD1 or PC-SOD becomes high during $O_2^{\bullet -}$ generation by xanthine and xanthine oxidase. At low concentrations SOD1 and PC-SOD dose-dependently protects nitroblue tetrazolium which is reduced by $O_2^{\bullet -}$. Summarizing, it can be said that *in vitro* SOD1 and PC-SOD give a U-shaped concentration-damage curve. *In vivo*, the redox cycling compound doxorubicin was used to generate $O_2^{\bullet -}$. PC-SOD was administered in three doses, 5,000, 20,000 and 80,000 U.kg⁻¹. The damage, caused by $O_2^{\bullet -}$, was monitored by ECG measurements. *In vivo*, no toxicity was observed at the highest dose. Thus, the U-shaped curve which was observed *in vitro* was not found *in vivo*. The

explanation for this discrepancy came from the pharmacokinetics of PC-SOD. These results show that the concentration of PC-SOD found in plasma after administration of the highest dose of PC-SOD is not as high as the concentration that caused the toxic effects *in vitro*. Doxorubicin not only affects the heart, it also causes damage to the blood vessels. In **Chapter 5**, another enzyme which might be capable of one-electron reduction of doxorubicin is more closely investigated. Endothelial nitric oxide synthase, normally responsible for the production of nitric oxide in the vasculature was reported to be switched to the production of superoxide radicals in the presence of doxorubicin in cultured endothelial cells. This switch to superoxide production could have fargoing consequences *in vivo*. The remaining nitric oxide is prone to inactivation by $O_2^{\cdot -}$, resulting in the cytotoxic peroxynitrite. Thus, vasodilation becomes impaired due to decreased NO production, while peroxynitrite damages vascular tissue. We investigated the effects of doxorubicin on the isolated aorta in an organ bath study. In contrast to the known eNOS inhibitor L-NAME, doxorubicin did not attenuate the carbachol-mediated relaxation of phenylephrine precontracted aortic rings. The dismutation of $O_2^{\cdot -}$ yields H_2O_2 and O_2 . However, in the presence of activated neutrophils, H_2O_2 is not a final product. Myeloperoxidase turns H_2O_2 into HOCl, which is much more efficient in killing bacteria than H_2O_2 is. In **Chapter 6**, we show that HOCl can inactivate SOD1. A consequence of the inactivation of SOD1 is that the H_2O_2 concentration decreases. Since H_2O_2 drives the oxidation of Cl⁻ by MPO, this means that HOCl production is decreased. Therefore, the inactivation of SOD1 by HOCl leads to a shift in toxicity from the HOCl mediated to $O_2^{\cdot -}$ mediated damage. Furthermore, inactivation of SOD1 probably results in the release of Cu^{2+} from the active site of the enzyme. The free Cu^{2+} can catalyze the formation of $HO^{\cdot}$ radical, thus causing further oxidative damage. **Chapter 7** explores inactivation of acetylcholinesterase (AChE) by HOCl. It was found that HOCl inactivates AChE *in vitro*. Furthermore, when HOCl was added to an electrically stimulated tracheal preparation in an organ bath, the amplitude of the contractions was increased, an effect very similar to that of physostigmine, a known inhibitor of AChE. The effect of HOCl on AChE might explain at least part of the hyperreactivity of airway smooth muscle, observed in inflammation of the higher airways. Finally, **Chapter 8** is concerned with the question how antioxidants, and especially the sulfur containing ones, can protect against the HOCl that is formed. Using our newly discovered target for HOCl, AChE, we tested the efficacy of HOCl scavenging by sulfur containing com-

pounds. An astonishing discovery was made. Glutathione disulfide (GSSG), a compound previously thought to be devoid of any antioxidant activity was found to be able to protect AChE against HOCl. Other well-investigated targets (e.g. α_1 antiprotease) for HOCl were not protected by GSSG. This led us to answer the question 'what makes an antioxidant a good antioxidant?' as 'That depends on where you want the antioxidant to act or what the antioxidant has to protect'. In the lower airways, where HOCl is likely to play a role during the development of emphysema, GSSG will not provide protection. However, if the inflammatory process takes place in the higher airways, GSSG might have a beneficial effect.

We can conclude by stating that SOD indeed acts as a toxicity modulator. *In vivo*, we were able to demonstrate that a lecithinized form of SOD completely inhibits the cardiotoxicity caused by doxorubicin, thus modulating the extent and severity of toxicity. *In vitro*, specific detectors for $O_2^{\cdot -}$ radicals could be protected by SOD. However, raising the concentration of SOD beyond that needed for protection created another form of toxicity mediated by $HO^{\cdot}$ radicals, here both the location and mechanism of superoxide toxicity are modulated. In general, when considering to use an antioxidant as a therapeutic agent, one should consider the question where the antioxidant should act and what biological target it should protect and which oxidant is involved. Therefore, the site of oxidative damage should be precisely determined before optimal antioxidant therapy is feasible.

Samenvatting

In **Hoofdstuk 1**, de algemene inleiding van dit proefschrift, wordt de rol van superoxide anionen ($O_2^{\cdot -}$) en de familie van superoxide dismutase enzymen (SODs) geschetst. Zowel $O_2^{\cdot -}$ als de SODs zijn moleculen met een Janus gezicht. $O_2^{\cdot -}$ wordt gevormd in verschillende (patho)fysiologische processen, waaronder ischemie-reperfusie, ontsteking en biotransformatie. $O_2^{\cdot -}$ bezit een aantal toxische eigenschappen. $O_2^{\cdot -}$ induceert peroxidatie van membraanlipiden, oxideert catecholamines, reageert en inactieveert $NO^{\cdot}$ waarbij tegelijkertijd het sterk oxiderende peroxynitriet gevormd wordt. Verder beïnvloedt $O_2^{\cdot -}$ cellulaire processen door activatie van proteïne kinases en transcriptie factoren. Daarnaast is $O_2^{\cdot -}$ de voorlopervan een aantal reactieve zuurstof deeltjes die de cel verder kunnen beschadigen. De toxische eigenschappen kunnen voor een goed doel aangewend worden. Met name als het lichaam beschermd dient te worden tegen microorganismen die het lichaam binnendringen speelt $O_2^{\cdot -}$ een cruciale rol. Het niet kunnen produceren van $O_2^{\cdot -}$ is derhalve een levensbedreigende aandoening. Het moge duidelijk zijn dat $O_2^{\cdot -}$ productie soms noodzakelijk is, maar dat het in ieder geval gecontroleerd dient te gebeuren. De controlerende taak rust op de schouders van een familie van enzymen, de superoxide dismutases (SODs), die gevonden worden in het cytosol (SOD1), de mitochondriële matrix (SOD2) en in de extracellulaire ruimte (SOD3). Er zijn echter omstandigheden waarin de SODs overweldigd raken door de hoeveel $O_2^{\cdot -}$ waarmee ze geconfronteerd worden. In deze omstandigheden lijkt het rationeel om de hoeveelheid SOD te verhogen, hetzij door toediening van (gemodificeerd) SOD of SOD mimetica, hetzij door het verhogen van de expressie van de SODs. Deze methoden zijn uitgebreid getest in een veelheid van ziekten en gebleken is dat het werkt. Echter, er zijn verschillende problemen met SOD in verband gebracht. Het meest opvallende van deze problemen is de additionele peroxidase activiteit die SOD1 bezit. De combinatie van waterstofperoxide (H_2O_2), een van de producten van SOD1, en SOD1 resulteert in de peroxidatie van biomoleculen, met name membraan lipiden. De neurodegeneratie, die vaak gezien wordt bij patiënten met het syndroom van Down, is in verband gebracht met overexpressie van SOD1. Het gen dat codeert voor SOD1 bevindt zich namelijk op chromosoom 21. Dus, net zoals de productie van $O_2^{\cdot -}$ binnen de perken gehouden dient te worden moet het verhogen van SOD niveaus met de grootste zorg omringd worden. In **Hoofdstuk 2**, wordt enige achtergrondinformatie over twee klassen van geneesmiddelen, de

anthracyclines en de tricyclische antidepressiva, welke cardiotoxische bijwerkingen hebben, gegeven. Beide klassen veroorzaken veranderingen in het electrocardiogram waarbij de verlenging van het ST interval het meest in het oog springt. Deze verandering in het ECG duidt op een verstoring van de repolarisatie van de kamers. Voor patiënten die met anthracycline antitumor geneesmiddelen behandeld zijn, kan deze beschadiging van het hart levensbedreigend zijn. De ECG veranderingen kunnen tevens als een maat voor cardiotoxiciteit worden aangewend. Met behulp van ECG metingen kan dus de effectiviteit van stoffen die het hart zouden kunnen beschermen getest worden. Verder zou van analoga van de genoemde geneesmiddelen de mate van cardiotoxiciteit bepaald kunnen worden. Door middel van telemetrie kan in een wakker en vrij bewegend proefdier het ECG gemeten worden. In het verleden is met het telemetrie model de cardioprotectieve eigenschappen van een aantal flavonoïden tijdens doxorubicine behandeling in de muis onderzocht. Wij hebben het telemetrie model op twee punten verbeterd. In de nieuwe methode wordt de zender onder de huid geïmplanterd in plaats van in de buikholte, een procedure die minder belastend voor het proefdier is. Naast de implantatie van de zender is ook de gegevensverwerking verbeterd. Het middelen van de ECG's en het automatisch detecteren van de verschillende intervallen in het ECG maken het experiment betrouwbaarder, minder tijdrovend en minder onderhevig aan interexperimentator variatie. Een van de cardiotoxische anthracyclines is doxorubicine. Het zijn de cardiotoxische eigenschappen van dit zeer effectieve antitumor geneesmiddel die de therapeutische toepassing ervan ernstig hinderen. Via redox-cycling produceert doxorubicine $O_2^{\cdot -}$ radicalen en het is algemeen aanvaard dat het deze $O_2^{\cdot -}$ radicalen zijn die het hart beschadigen. Vermindering van de hoeveelheid $O_2^{\cdot -}$ radicalen zou derhalve de beschadiging van het hart moeten voorkomen. In **Hoofdstuk 3** wordt het onderzoek naar de effectiviteit van PC-SOD, gelecithineerd SOD1, om te beschermen tegen de door doxorubicine veroorzaakte cardiotoxiciteit beschreven. Reeds de laagste dosis van PC-SOD die onderzocht werd, 5000 units per kilogram lichaamsgewicht, bleek in staat de door doxorubicine veroorzaakte verlenging van het ST interval te voorkomen. Dus als doxorubicine vlak na PC-SOD wordt toegediend, wordt de verstoorde kamer repolarisatie die normaliter na een behandeling met doxorubicine wordt gezien niet meer waargenomen. Verder beïnvloedt het combineren van doxorubicine met PC-SOD niet het antitumor effect van doxorubicine. Geconcludeerd kan dus worden dat PC-SOD het hart beschermt tegen de schadelijke effecten van doxorubicine maar het effect

van doxorubicine op de tumor ongemoeid laat. Het gebruik van PC-SOD lijkt dus veilig en solide als adjuvant bij doxorubicine. De vraag komt nu op hoe het met het negatieve aspect van SOD zit. In **Hoofdstuk 4** worden de effecten van SOD1 en PC-SOD in een $O_2^{\cdot-}$ producerende omgeving in een reageerbuis beschreven. *In vitro* konden we laten zien dat een coumarine zuur derivaat gehydroxyleerd werd in de aanwezigheid van hoge concentraties SOD1 of PC-SOD in een systeem waar $O_2^{\cdot-}$ geproduceerd werd door het koppel xanthine en xanthine oxidase. Bij lagere concentraties geven SOD1 en PC-SOD een concentratie-afhankelijke bescherming van nitroblauw tetrazolium, een stof die gereduceerd wordt door $O_2^{\cdot-}$. Samenvattend kan men zeggen dat *in vitro* SOD1 en PC-SOD een U-vormige concentratie-schade curve geven. Bij lage concentraties voorkomen SOD1 en PC-SOD schade terwijl deze schade weer opkomt, zij het in een andere vorm, op het moment dat de concentratie boven een bepaalde waarde komt. *In vivo* werd doxorubicine gebruikt om $O_2^{\cdot-}$ te produceren. PC-SOD werd toegediend in drie doseringen: 5.000, 20.000 en 80.000 U.kg⁻¹. De schade die door $O_2^{\cdot-}$ wordt veroorzaakt werd bepaald aan de hand van ECG metingen. Net als in het *in vitro* assay werd bescherming door PC-SOD waargenomen. Bij de hoogste dosering werd echter geen toxiciteit waargenomen. De U-vormige concentratie-schade curve die *in vitro* werd gevonden werd *in vivo* niet waargenomen. Een verklaring voor dit verschil kwam uit experimenten waarin de farmacokinetiek van PC-SOD werd bepaald. Deze resultaten toonden aan dat de maximale PC-SOD concentratie *in vivo* lang niet zo hoog was als de PC-SOD concentratie die *in vitro* voor de toxische effecten (hydroxylering) verantwoordelijk was. Doxorubicine is niet alleen schadelijk voor het hart, het beschadigt ook de bloedvaten. In **Hoofdstuk 5** wordt meer aandacht besteed aan een ander enzym dat een 1-electron reductie van doxorubicine kan katalyseren. Volgens verschillende groepen zou het endotheliale stikstofmonoxide (NO[•]) synthase (eNOS) in gekweekte endotheel cellen onder invloed van doxorubicine kunnen overgaan op $O_2^{\cdot-}$ productie. Dit zou ten koste gaan van de NO[•] productie die het enzym normaliter katalyseert. NO[•] speelt een cruciale rol bij de relaxatie van glad spierweefsel in de bloedvaten. Deze omschakeling van NO[•] naar $O_2^{\cdot-}$ productie kan vergaande consequenties hebben *in vivo*. Het resterende NO[•] kan door $O_2^{\cdot-}$ worden omgezet in het cytotoxische peroxynitriet. De verminderde productie van NO[•] verstoort dus de vasorelaxatie, terwijl peroxynitriet het vaatweefsel beschadigt. In een orgaanbadstudie werd het effect van doxorubicine op de relaxatie van geïsoleerde aortaringetjes bepaald. Er bleek echter dat doxorubicine, in tegenstelling

tot de bekende eNOS remmer L-NAME, de carbachol geïnduceerde relaxatie van aorta ringetjes, welke met phenylephrine gecontraheerd waren, niet beïnvloedt. Een effect van doxorubicine dat dus duidelijk aantoonbaar was in een reageerbuis of in gekweekte aorta cellen bleek op het meer fysiologische niveau van de geïsoleerde aorta weinig of niet relevant te zijn. De dismutatie van $O_2^{\cdot -}$ levert H_2O_2 en O_2 op. In de aanwezigheid van geactiveerde neutrofielen is H_2O_2 niet het eindproduct. Het enzym myeloperoxidase (MPO) zet H_2O_2 om in hypochloorzuur (HOCl), wat een veel efficiëntere bacterie-dodende stof is dan H_2O_2 . In **Hoofdstuk 6** laten we zien dat HOCl SOD1 inactieveert. Een consequentie hiervan is dat de H_2O_2 concentratie afneemt. Omdat H_2O_2 de oxidatie van Cl^- door MPO aandrijft betekent dit dat ook de HOCl productie afneemt. De inactivatie van SOD1 door HOCl leidt dus tot een verschuiving in toxiciteit van de HOCl zijde naar de $O_2^{\cdot -}$ zijde. Tevens zal de oxidatieve inactivatie van SOD1 waarschijnlijk resulteren in de vrijzetting van Cu^{2+} ionen uit de actieve plaats van het enzym. Het vrije Cu^{2+} kan vervolgens de vorming van $HO^{\cdot}$ radicalen katalyseren en op die manier de oxidatieve schade verergeren. **Hoofdstuk 7** behandelt de inactivatie van acetylcholinesterase (AChE) door HOCl. *In vitro* werd gevonden dat HOCl AChE inactieveert. Vervolgens kwam aan het licht dat als HOCl wordt toegevoegd aan een electrisch gestimuleerd trachea preparaat in een orgaanbad de amplitudes van de contracties toeneemt. Dit effect lijkt sterk op dat van fysostigmine, een bekende remmer van AChE. Het effect van HOCl op AChE kan wellicht ten dele de waargenomen hyperreactiviteit van het gladde spierweefsel in de luchtwegen, die wordt waargenomen tijdens ontstekingen van de luchtwegen, verklaren. Tot slot wordt in **Hoofdstuk 8** ingegaan hoe antioxidanten, en in het bijzonder antioxidanten die zwavelatomen bezitten, kunnen beschermen tegen het gevormde HOCl. Gebruik makend van ons recentelijk ontdekte doelwit van HOCl, AChE, werd de beschermende activiteit van een aantal zwavel bevattende antioxidanten bepaald. Een verrassende ontdekking werd gedaan. Glutathiondisulfide (GSSG), een stof waaraan tot nu toe geen antioxidant activiteit werd toegeschreven, bleek in staat AChE te beschermen tegen inactivatie door HOCl. Andere, reeds goed onderzochte, doelwitten van HOCl (bijv. α_1 -antiprotease) werden niet door GSSG beschermd. Deze vinding bracht ons er toe de vraag 'wat een antioxidant een goede antioxidant maakt' te beantwoorden met 'dat ligt eraan waar de antioxidant zijn beschermende werking moet uitvoeren of wat hij moet beschermen'. In de lagere luchtwegen, waar HOCl een rol lijkt te spelen bij het ontstaan van longemfyseem door het beschadigen van

het α_1 -antiprotease, zal GSSG geen bescherming bieden. Als het inflammatoire proces zich hoger in de luchtwegen afspeelt kan GSSG wel degelijk een gunstige rol spelen. Samenvattend kan geconcludeerd worden dat SOD inderdaad kan optreden als een toxiciteitsmodulator. *In vivo* kon gedemonstreerd worden dat een gelecithineerde vorm van SOD1 de cardiotoxiciteit van doxorubicine compleet kon voorkomen. Dus SOD1 moduleert zowel de ernst als de locatie van toxiciteit. *In vitro* kon gedemonstreerd worden dat SOD1 specifieke detectoren voor $O_2^{\cdot -}$ kon beschermen. Bij het verhogen van de SOD1 concentratie boven de concentratie die voor bescherming benodigd was kwam een andere vorm van toxiciteit, gemedieerd door $HO^{\cdot}$ radicalen, aan het licht. Wederom wordt dus de mate van toxiciteit gemoduleerd door SOD, maar nu ook de locatie en het mechanisme. In het algemeen kan gesteld worden dat als overwogen wordt een antioxidant als therapeutikum toe te passen, men zich moet afvragen welk doel in welk gedeelte van het lichaam tegen welke oxidant beschermd moet worden. Deze vragen moeten zeer precies beantwoord worden alvorens men tot antioxidant toediening overgaat.

Nawoord

Zonder de hulp van velen zou dit proefschrift niet tot stand zijn gekomen. Allereerst wil ik mijn beide promotoren, prof. dr. A. Bast en prof. dr. W.J.F. van der Vijgh bedanken voor de buitengewoon deskundige en, bovenal, geduldige begeleiding die ik van hun mocht ontvangen. Uiteraard ben ik jullie ook dankbaar voor het feit dat jullie mij hebben overgehaald om naar Maastricht te gaan en mij de mogelijkheid hebben gegeven om het onderzoek met PC-SOD uit te voeren. Mijns inziens is het niet overdreven te stellen dat de verhuizing naar Maastricht mijn leven een andere wending heeft gegeven. Mijn co-promotor, dr. G.R.M.M. Haenen, ben ik dank verschuldigd voor de enorme lading ideeën die hij produceert dan wel induceert. Veel van de gedachten over antioxidanten zoals geventileerd in de laatste 2 hoofdstukken van dit proefschrift zijn, zoals de kenners van dit werk direct herkennen, van hem afkomstig. De leden van de commissie onder leiding van prof. dr. Ph. Lambin die mijn proefschrift beoordeeld heeft wil ik hartelijk bedanken voor de tijd die zij geïnvesteerd hebben in het lezen van het manuscript. Zonder de vaardigheden van Nicole Bitsch, Leo Baars, Gregorio Fazzi in Maastricht en die van het team van dr. E. Boven in Amsterdam: Caroline Erkelens, Hennie Schlüper en Saskia Hulscher waren de experimenten zoals beschreven in de hoofdstukken 3 en 4 min of meer onuitvoerbaar geweest; ik wil ze dan ook nadrukkelijk bedanken voor hun assistentie. De studenten, Erik Vegt (Nijmegen), David Jaspers (Eindhoven), Karim Benzian (Heerlen) en Agnes Boots (Maastricht), ben ik dank verschuldigd voor hun waardevolle bijdragen aan respectievelijk hoofdstuk 4, 2, 6 en 5. Els Geurts en Mia Hogenboom voor alle onmisbare assistentie bij administratieve activiteiten. Mijn paranimfen, Barbara van Veen en Ilonka van Hoof, ben ik minstens een aantal diners schuldig nu ze in mijn 'uur van de waarheid' naast me willen staan. Mijn collega's bij Humane Toxicologie en alle andere leden van de capaciteitsgroep Farmacologie en Toxicologie bedank ik voor de prettige contacten en de vele hulp en goede tips die ik mocht ontvangen. Natuurlijk mogen de collega's van de capaciteitsgroep Gezondheidsrisico Analyse en Toxicologie en de medewerkers van de Centrale Proefdier Voorzieningen ook niet vergeten worden bij de dankbetuigingen.

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Curriculum Vitae

Gerardus Johannes Martinus (Gertjan) den Hartog was born on Sunday February 11th 1973 in Harderwijk, the Netherlands. In the same town, from 1985 to 1991, he attended highschool (VWO) at the Christelijk College Nassau-Veluwe. In 1991, he moved to Amsterdam to study Pharmacochemistry at the Vrije Universiteit. The principle subject was Molecular Pharmacology, 'The role of tachykinins in ozone-induced airway hyperreactivity' under the supervision of prof. dr. A. Bast, dr. H.-P. Voss and dr. H.J.M. van Hoof. The subsidiary subject was Organic Synthesis, 'Synthesis of leukotriene D₄ antagonists' under the supervision of dr. W.P.M.B. Menge, dr. M.Q. Zhang and dr. M. Zwaagstra. The M. Sc thesis had the diazepam receptor as subject, was written under the supervision of prof. dr. H. Timmerman and was awarded with the Taeke Bultsma Award for best pharmacochemical paper. During his study he worked for 1½ years as a researcher at the department of Head and Neck Surgery at the VU Medical Center in Amsterdam, where he continued to do research on the use of antioxidants in upper airway infections after obtaining his M. Sc. ('met veel genoegen') in 1998. After a short interlude as an assistant teacher at the Department of Pharmacochemistry, Faculty of Chemistry, he moved to Maastricht where the investigations described in this thesis were carried out.