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Inhibition of Cellular Antioxidants: A Possible Mechanism of Toxic Cell Injury

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Cells that utilize molecular oxygen generate highly reactive oxygen-derived free radicals. Endogenous cellular oxidants inactivate oxidant free radicals and protect aerobic cells from oxidant injury. Glutathione, glutathione reductase, and superoxide dismutase are key components of this antioxidant defense. Inhibition of antioxidant components would be expected to result in cell injury. Using exposure to oxygen at high pressure to increase the level of oxidant free radicals, evidence is presented to support the hypothesis that inhibition of cellular antioxidants renders organisms more susceptible to oxygen toxicity. Diethyldithiocarbamate at doses of 250, 500 and 1000 mg/kg inhibited rat brain superoxide dismutase activity and shortened onset time to seizures in a dose-related manner in 4 ATA oxygen. Carmustine at doses of 12.5, 25 and 50 mg/kg inhibits glutathione reductase activity in rat brain in proportion to the dose. Time to onset of seizures of rats pretreated with carmustine prior to exposure to 4 ATA oxygen was shortened, and oxidized glutathione levels were increased in the cortex and subcortex. These data suggest that inhibition of antioxidant components results in organisms becoming more sensitive to oxygen toxicity. Compounds that inhibit cellular antioxidants may produce toxic cell injury by permitting intracellular oxidant free radicals to attack essential cell constituents.

Antioxidant Defense Mechanisms

All cells that utilize molecular oxygen (O_2) for metabolic or respiratory purposes are at risk of being damaged by activated oxygen and other O_2 derived free radicals. The most prevalent form of this species is thought to be superoxide anion ($O_2^{\cdot-}$), which is formed when elemental O_2 is utilized as an electron acceptor during cellular metabolism or respiration. Superoxide anion represents the product of a one-electron reduction of elemental O_2 (*1*). The aerobic cell possesses many potential sources of $O_2^{\cdot-}$. Enzymes that catalyze the incorporation of molecular O_2 to organic molecules, such as xanthine oxidase, aldehyde oxidase, monoamine oxidase, NADPH-cytochrome c reductase, NADPH-cytochrome P_{450} reductase, certain hydroxylases, and several flavoproteins generate $O_2^{\cdot-}$. Certain auto-oxidation reactions, such as those of ubisemiquinone, catechols, ferrodoxins, and hemoproteins, as well as autooxidation of sulfhydryl groups may result in production of $O_2^{\cdot-}$ (*2*). Certain metallic ions (e.g., ferrous ions) catalyze the reduction of elemental O_2 to $O_2^{\cdot-}$ (*3*).

$O_2^{\cdot-}$ will react with electron acceptors in the immediate vicinity. Reactants for $O_2^{\cdot-}$ are sulfhydryl groups such as those located at enzyme-active sites, and unsaturated fatty acids such as those located in membrane lipids. Indiscriminate and unfocused reactions of this type could result in inactivation of enzymes and damage to membranes. Maintenance of normal cell structure and function in the presence of the constant production of highly reactive oxidant species depends upon the presence of an effective system for inactivating them. Most O_2-utilizing cells contain a multicomponent antioxidant defense mechanism for this purpose.

The endogenous cellular antioxidant mechanism, as currently recognized in aerobic cells, consists of a number of components. Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2. In mammalian tissues this enzyme is known to exist in two forms: a cytosolic, Cu–Zn enzyme, and a mitochondrial Mn enzyme (*4*).

In mammalian systems, SOD activity tends to be highest in tissues that have high O_2 utilization (*5*). In addition, tissue SOD content has been shown to vary with tissue p_{O_2} in normal air-breathing mice (*6*). This suggests that the function of SOD is to prevent toxic cell damage from a normal metabolic by-product of O_2.

Glutathione (GSH), a sulfhydryl containing tripeptide (γ-glutamyl-cysteinylglycine), appears to play a multifaceted role as an antioxidant in this system. Glutathione maintains the reduced state of the sulfhydryl groups of a number of sulfhydryl-dependent enzymes (*7*). Endogenously generated hydroperoxides and other O_2 intermediates (*8*) are inactivated via the enzyme GSH peroxidase, which utilizes GSH as a proton donor. Because GSH has a strong reducing potential, it can also act as a nonspecific, nonenzymatic scavenger of free radicals produced by O_2 (*9*). Whether GSH interacts with

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oxidants enzymatically or nonenzymatically, the by-product is oxidized glutathione (GSSG).

The reduction of GSSG is catalyzed by the enzyme GSH reductase utilizing NADPH as an electron donor. NADPH is supplied by the enzyme glucose-6-phosphate dehydrogenase (G-6-PDH). Like SOD, GSH levels tend to be highest in those tissues with the highest utilization of O_2 (*7*).

Adequate levels of GSH reductase and NADPH are critical to the maintenance of GSH in the reduced state which is necessary for the defense of the cell against attack by oxidant free radicals (*10*). Increased G-6-PDH activity has been demonstrated in the lungs of rats exposed to hyperoxic gaseous environments (*11*).

Other components of the cellular antioxidant defense system are tocopherol (vitamin E) and ascorbate (vitamin C). Tocopherol can act as a lipid antioxidant (*2*). Ascorbate appears to be able to reduce the tocopherol free radical that is formed when tocopherol reduces an oxidant substance. This interaction may explain, in part, the synergistic antioxidant effect of tocopherol and ascorbate in suppressing lipid peroxidation in an *in vitro* system (*12*). The intracellular antioxidant defense system whose components have been described above, has evolved to prevent toxic cell injury from oxidant free radicals produced during normal metabolic and respiratory processes.

Table 1. Effect of carmustine (25 mg/kg, IV) and exposure to 4 ATA 100% oxygen for 1 hr on oxidized glutathione (GSSG) content of different regions of the rat brain.

Brain region	Oxidized glutathione content, nmole/g protein[a]		Change, %
	Vehicle control	Carmustine treated	
Cerebral cortex	103 ± 12	143 ± 8*	+ 39.3
Brainstem	111 ± 18	114 ± 17	+ 1.1
Cerebellum	105 ± 10	99 ± 11	− 5.6
Subcortical	89 ± 11	128 ± 11†	+ 44.8

[a]Values are means ± SE of six determinations.
*$p < 0.02$ when compared with vehicle controls.
†$p < 0.05$ when compared with vehicle controls.

Table 2. Effect of carmustine (25 mg/kg, IV) and exposure to 4 ATA 100% oxygen for 1 hr on the GSH/GSSG ratio in different regions of the rat brain.

Brain region	Ratio of oxidized to reduced glutathione[a]		Change, %
	Vehicle control	Carmustine treated	
Cerebral cortex	151 ± 13	95 ± 7*	− 36.9
Brainstem	114 ± 19	107 ± 3	− 6.0
Cerebellum	132 ± 13	146 ± 12	+ 10.0
Subcortical	161 ± 25	101 ± 9†	− 37.1

[a]Values are means ± SE of six determinations.
*$p < 0.01$ when compared with vehicle controls.
†$p < 0.05$ when compared with vehicle controls.

Cellular Oxidant Damage

Much of the evidence indicating that oxidant species can adversely affect cellular constituents has been obtained by exposure of animals to environments containing elevated levels of oxidant substances. Inspiration of O_2 at increased partial pressures has been demonstrated to produce toxicity in both man and experimental animals. This toxicity occurs as a result of the increased production of oxidant free radicals (e.g., $O_2^{\cdot}$, hydroxyl radicals and peroxides) secondary to the increased intracellular O_2 tension. For certain enzymatic reactions, the production of $O_2^{\cdot}$ increases as the partial pressure of O_2 increases (*13*). Oxygen toxicity in intact animals is demonstrated most frequently as effects on the lung and central nervous system.

According to the theory of intracellular antioxidant defenses, the intracellular concentration of oxidant free radicals occurring as a result of exposure to hyperoxia exceeds the capacity of the antioxidant defense mechanisms to inactivate and detoxify these reactive species. Evidence in support of this proposed sequence of events is provided by the characteristic lag or onset time observed between the initiation of the exposure of the animal to the hyperoxic environment and the appearance of the manifestations of toxicity (*14*). The time to onset of gross toxicity is inversely related to the pressure of O_2 to which the animal is exposed (i.e., the higher the O_2 dose, the shorter the time to onset). This relationship has been observed in the manifestations of O_2 toxicity in whole animals (*14*), as well as in the toxic effects of O_2 on isolated tissues (*15*) and cells in culture (*16*). The time required for the toxic effects of O_2 to occur as a function of oxygen pressure can be interpreted as an indication of the antioxidant defense capacity of a particular tissue or cell. From this view, the time to onset of toxic effect would represent the time required for that quantity of O_2 to overcome the antioxidant capacity of the cell and begin to attack cellular constituents which then give rise to the toxic effect.

Auto-oxidation of both nonprotein (*17*) and protein sulfhydryls (*18*) by reactive O_2 intermediates with subsequent loss of enzyme activity has been postulated as a key mechanism of O_2 toxicity. Exposure of animals to high pressure O_2 inhibits sulfhydryl dependent enzymes, *in vivo*, e.g., glutamate decarboxylase (*19,20*) and lactate dehydrogenase (*21*). Exposure of tissue preparations to high O_2 tensions results in inhibition of many enzymes thought to be sulfhydryl-dependent, e.g., succinic dehydrogenase (*22*), Na^+,K^+-ATPase (*15,23*). An important aspect of the interaction of sulfhydryls with toxic O_2 intermediates is that GSH cannot only prevent O_2 inactivation of certain enzymes by being preferentially oxidized but can also reactivate O_2 inactivated enzymes (*22*). Both of these protective and/or restorative functions of GSH depend upon the maintenance of GSH in the reduced form by GSH reductase and the NADPH provided by G-6-PDH.

Interaction of the $O_2^{\cdot}$ or other reduction products of O_2 with unsaturated tissue fatty acids may result in the

formation of lipid free radicals (*24*). In the presence of O_2, the lipid free radical can undergo auto-oxidation to a lipid peroxide. The lipid peroxide thus formed can react with other unsaturated lipids to generate a lipid hydroperoxide and the regeneration of another oxidant lipid radical. This results in a cyclical reaction and the continuous reformation of lipid peroxides (*11,25*). The significance of lipid peroxidation is twofold; first, progressive lipid peroxidation may result in loss of cellular membrane integrity; second—at least *in vitro*—inactivation of enzymes by lipid peroxides has been demonstrated, and thus loss of enzyme function may occur (*26*).

The rate of oxidation of these cellular constituents is dependent upon the level of O_2 available to provide oxidant free radicals and the quantity and level of activity of the antioxidant defense components which must first be exhausted.

Impairment of Endogenous Antioxidants

Evidence that impaired antioxidant defense mechanisms could result in adverse effects on cell function is exemplified by the clinical syndrome associated with G-6-PDH deficiency. Patients having erythocytes deficient in this enzyme exhibit decreased red blood cell survival time and increased sensitivity to drugs which present the red cell with an increased level of oxidants (*27*). The nature of the cell damage indicates a primary target to be the lipids of the red cell membrane (*27*). Experimentally, impairment of the endogenous antioxidants has been attempted by using two methods: (1) directly by pharmacologic manipulation of one or more of the endogenous antioxidants or (2) indirectly by affecting the nutritional status of the experimental animal resulting in a deficiency of an antioxidant constituent. Experimental nutritional deficiency in rats has been shown to affect susceptibility to O_2 toxicity. Rats maintained on a cysteine-deficient diet develop pathologic changes indicative of pulmonary O_2 toxicity earlier and have a decreased survival time when exposed to 98% O_2 at 1 ATA (atmospheres absolute). Addition of 3% cysteine to their diet reversed both effects. Presumably, this effect was due to a deficiency of lung GSH. Exposure of cysteine-supplemented rats to 97% O_2 for 60 hr resulted in a significant increase in lung GSH content when compared to the cysteine-deprived rats similarly exposed (*28*).

Dietary deprivation of selenium renders rats more susceptible to O_2 pulmonary damage. This effect was associated with a decrease in lung GSH-peroxidase activity (*29*).

Exposure of tocopherol-deficient mice to 45 psia (pounds per square inch absolute) O_2 for 1 hr results in a higher incidence of seizures. In these studies, tocopherol deficiency is associated with increases in brain content of peroxide and lipid peroxides following O_2 exposure. As with the cysteine-deprived rats, these effects were reversible upon addition of tocopherol to the diet (*30,31*).

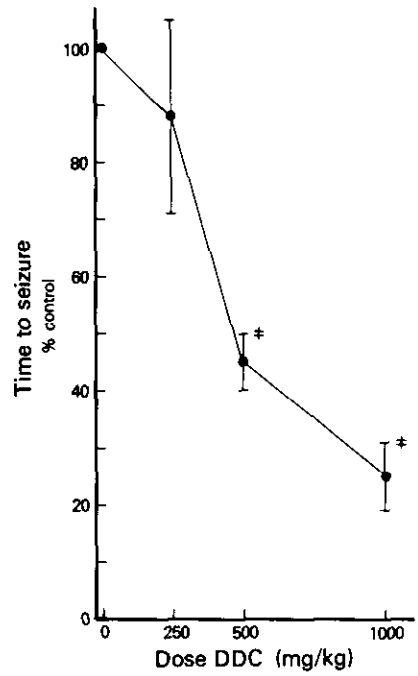

FIGURE 1. Effect of diethyldithiocarbamic acid (DDC) on time to seizure of rats exposed to 4 ATA 100% oxygen. Two hours after treatment with DDC or vehicle (saline) the rats were exposed to 4 ATA 100% oxygen until seizure. Values are expressed as the mean ± SE percent of control. The control mean ± SE time to seizures were 138 ± 15, 156 ± 8 and 136 ± 15 min at the 250, 500 and 1000 mg/kg DDC points, respectively. The number of rats in each group was six to eight. $^*p < 0.01$ as determined by one-way ANOVA.

Pharmacological manipulation of the endogenous antioxidants has been attempted using several agents. Diethyldithiocarbamic acid (DDC) is an inhibitor of the Cu–Zn SOD (*32*). Treatment of rats with this compound has been demonstrated to potentiate pulmonary damage in rats exposed to 95% O_2 at 1 ATA (*33*) and to decrease survival time of rats exposed to 95% O_2 at 1 ATA (*33*) or 2 ATA 100% O_2 (*34*).

In our laboratory, experiments with DDC have been conducted to ascertain the relationship of central nervous system SOD inhibition in brain on the rate of development of CNS O_2 toxicity. Treatment of rats with DDC at doses of 250, 500 and 1000 mg/kg, IP, results in a dose-dependent decrease in time to seizure in rats exposed to 4 ATA 100% O_2. The effect on time to seizure was in the presence of a dose-dependent decrease in brain Cu–Zn SOD activity (*35*).

This laboratory has also been examining the effect of inhibition of GSH reductase on rate of development of CNS O_2 toxicity. 1,3-Bis(2-chloroethyl)-1-nitrosourea or carmustine was first demonstrated to produce a generalized inhibition of GSH reductase by Frischer and Ahmad (*36*). Treatment of rats with carmustine intravenously, at doses of 12.5, 25 and 50 mg/kg, produces a

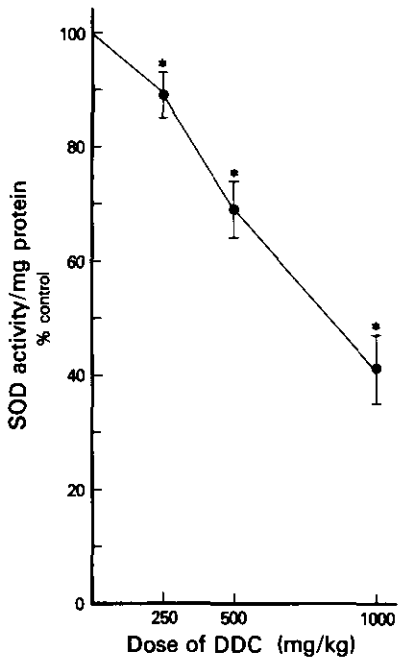

FIGURE 2. Effect of diethyldithiocarbamic acid (DDC) on rat brain superoxide dismutase (SOD) activity. Two hours after treatment with DDC or vehicle (saline) whole brain SOD activity was determined. Values are expressed as mean ± SE percent of control. Mean ± SE control SOD activities were 11.2 ± 0.5, 12.5 ± 0.7 and 8.1 ± 0.4 units/mg protein at the 250, 500 and 1000 mg/kg DDC points, respectively. The number of rats in each group was seven to eight. $^{*}p < 0.05$ as determined by one-way ANOVA.

dose-dependent decrease in time to seizure at 4 ATA 100% O_2. These doses of carmustine also resulted in a dose-dependent inhibition of brain GSH reductase activity. Furthermore, the decreased time of onset of seizures was associated with increased GSSG content of the cortex and subcortex. This suggests that inhibition of brain GSH reductase in the presence of an oxidizing environment alters the GSH status of neuronal tissue and thus increases the sensitivity of this tissue to the toxic effects of O_2 (*37*).

In all the experiments cited above, evidence of tissue damage was demonstrated in an environment which exposed cells to significantly elevated levels of O_2. The cellular antioxidant defenses normally function to protect cellular constituents from endogenously derived oxidant free radicals generated in cells exposed to 0.2 ATA O_2 in sea level air. Inhibition of antioxidant components would be expected to result in O_2-mediated cell injury even in normoxic environments. According to the O_2 dose-toxic response relationship the rate of development of oxidant toxic cell injury would be dependent upon the extent as well as the duration of antioxidant inhibition, since O_2 dose would remain constant. Based on the existing onset times for the various manifestations of O_2 toxicity, measurable normoxic O_2 toxicity may be expected to require days or perhaps weeks to occur. The ability of exogenous substances to impair cellular antioxidants may represent a significant mechanism of toxic cell injury by endogenously generated oxidant species.

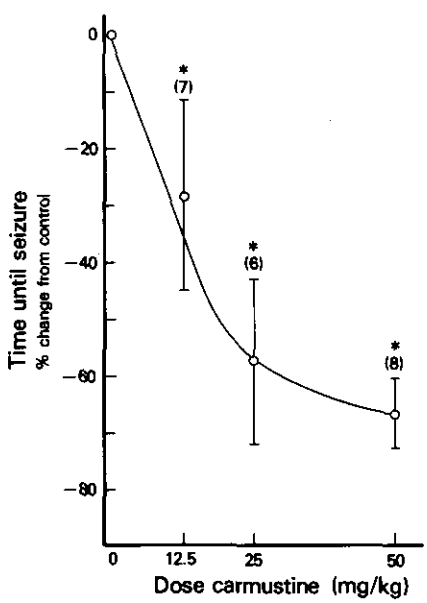

FIGURE 3. Effect of carmustine on time to seizure of rats exposed to 4 ATA 100% oxygen. One hour after treatment with carmustine or vehicle (50% ethanol/saline) the rats were exposed to 4 ATA 100% oxygen until seizure. Values are expressed as the mean ± SE percent change from control. The pooled control mean ± SE time to seizure was 137 ± 8 min. The number of rats in each group was six to eight. $^{*}p < 0.001$ as determined by one-way ANOVA.

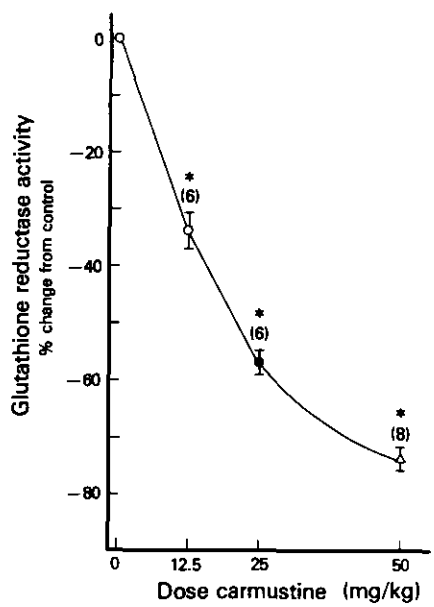

FIGURE 4. Effect of carmustine on rat brain glutathione reductase activity. One hour after treatment with carmustine or vehicle (50% ethanol/saline) whole rat brain glutathione reductase activity was determined. Values are expressed as mean ± SE percent change from control. The control mean ± SE values of glutathione reductase were (○) 8.2± 0.3, (●) 5.0 ± 0.1 and (Δ) 4.8 ± 0.3 units. The number of rats in each group was six to eight. $^{*}p < 0.001$ as determined by one-way ANOVA.

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