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# Age-related Changes in Antioxidant Enzyme Activities and Lipid Peroxidation in Lungs of Control and Sulfur Dioxide Exposed Rats

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Antioxidant defenses within the lung are pivotal in preventing damage from oxidative toxicants. There have also been several reports with conflicting results on the antioxidant system during aging. In this study, we attempted to investigate age-related alterations in both antioxidant enzyme activities and thiobarbituric acid-reactive substances (TBARS), a product of lipid peroxidation, in the whole lung of control and sulfur dioxide ($SO_2$) exposed rats of different age groups (3-, 12-, and 24-months-old). Swiss-Albino Male rats were exposed to 10 ppm $SO_2$ 1 hr/day, 7 days/week for 6 weeks. The antioxidant enzymes examined include Cu,Zn-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST). A mixed pattern of age-associated alterations in antioxidant activities was observed. SOD, GSH-Px and GST activities were increased with age, but CAT activity was decreased. Lung SOD, GSH-Px and GST activities were also increased in response to $SO_2$. The level of TBARS was increased with age. $SO_2$ exposure stimulated lipid peroxide formation in the lung as indicated by an increase in the level of TBARS. These findings suggest that both aging and $SO_2$ exposure may impose an oxidative stress to the body. We conclude that the increase in the activities of the antioxidant enzymes of the lung during aging, could be interpreted as a positive feedback mechanism in response to rising lipid peroxidation.

*Keywords*: Aging, sulfur dioxide, antioxidant enzymes, lipid peroxidation, rat

## INTRODUCTION

A wide variety of compounds are being increasingly spread in the environment as a consequence of technological advances.[1] Industrial

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emissions and other urban air pollutants pose a major threat to human health. Sulfur dioxide ($SO_2$) is a ubiquitous air pollutant, present in low concentrations in the urban air and in higher concentrations in the working environment.[1] When inhaled it produces adverse effects on health and has been associated with an increase in respiratory morbidity and mortality.[2] $SO_2$ has all been implicated in causing damage to pulmonary tissues and in initiation of asthmatic symptoms.[3,4] A consistent decrease in lung function after $SO_2$ exposure has been described elsewhere.[2]

$SO_2$ is a highly toxic injurious agent which forms sulfuric acid in contact with moist alveolar membranes.[3,5] Within the lung $SO_2$ is detoxified through the sulfitolysis of oxidized glutathione (GSSG). GSSG is generated from reduced glutathione (GSH) through the action of glutathione peroxidase (GSH-Px) detoxifying free radical species.[3] On the other hand, $SO_2$ forms free radicals in aqueous environments. This free radical is formed by the transfer of one electron to a molecule of $SO_2$, producing the negative ion radical $SO_2^{-\bullet}$, which is produced in aqueous $SO_2$ solutions by the action of light and chemical or biochemical-reducing agents.[6,7] Free radicals cause oxidative damage to various tissues, including the lung tissue.

Many physical and biochemical changes take place during the process of aging. Several theories have been proposed to explain aging processes. Among them the free radical theory of aging is considered as a most reliable basis to ascertain the process of aging because free radicals are involved in decreasing the defense system and in causing damage to the cell structure and function.[8] There are, however, defensive enzymes like superoxide dismutase (SOD), catalase (CAT), GSH-Px and glutathione S-transferase (GST) which detoxify these oxidants to protect cells.[8]

We attempted to investigate age-related changes in both antioxidant enzymes and lipid peroxidation in the lungs of control and $SO_2$-exposed rats. The antioxidant enzymes examined includes SOD, CAT, GSH-Px and GST.

## MATERIALS AND METHODS

### Apparatus

Optical densities in the ultraviolet and visible region were measured with a Beckman 26 spectrophotometer. Tissues were sonicated in a thermally regulated sonicator (Branson Sonifier, Model 250/450). Model MRU 95/3-CD apparatus (MRU, MeBgerate für Rauchgase und Unweltschutz GmbH, Heilbronn, Germany) was used to monitor the concentration of $SO_2$ within the chamber. Spectrofluorophotometer (Shimadzu RF-5000, Kyoto, Japan) was used to measure thiobarbituric acid-reactive substances (TBARS) levels.

### Chemicals

Reduced glutathione (GSH), glutathione reductase, reduced nicotinamideadenine dinucleotide tetrasodium salt (NADPH), 1-chloro-2,4-dinitro benzene, *t*-butyl hydroperoxide, 1,1,3,3-tetramethoxypropane, 2-thiobarbituric acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). Hydrogen peroxide ($H_2O_2$) was obtained from Merck (Darmstadt, Germany). Other reagents were of the highest analytical grades available commercially.

### Animals and Preparations of Lung Homogenates

Sixty healthy Swiss-Albino Male rats (210–450 g) were used in this study. Animals were housed in a group of 4–5 rats in stainless steel cages at standard conditions ($24 \pm 2\,^{\circ}C$ and $50 \pm 5\%$ humidity) with 12 h light-dark cycle. They were separated into three equal groups according to age; young group (Y: 3 months), middle-aged group (M: 12 months) and old-aged group (O: 24 months). These groups were subdivided into two subgroups of 12 animals each: controls and those exposed to $SO_2$. So our study consisted of six groups: control (YC, MC, OC) and $SO_2$ groups ($YSO_2$, $MSO_2$, $OSO_2$).

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Ten ppm $SO_2$ was administered to the animals of $SO_2$ groups in an exposure chamber for 1 h (8.00–9.00 a.m)/day × 7 days/wk × 6 weeks. Control groups were exposed to filtered air in the same chamber for the same period of time. Animals were placed in a 1 $m^3$ exposure chamber. The gas was delivered to animals via a tube positioned at the upper level of the chamber and distributed homogeneously via a propeller in the chamber. The $SO_2$ was diluted with fresh air at the intake port of the chamber to yield the desired $SO_2$ concentration (10 ppm).

At the end of the experimental period, rats were deprived of food for 24 h and then prepared for experimental procedure under ether anasthesia.

The whole lung was immediately removed and washed with cold saline. The whole lung was sonicated in phosphate buffer pH 7.4 for 30 seconds. Each lung homogenate was divided into two portions and treated differently. The first portion of homogenate was centrifuged at 700 × g for 10 min in order to determine CAT activity.[9] In the supernatant, CAT and TBARS were measured. The second portion of the homogenate was centrifuged at 1000 × g for 10 min and the supernatant fraction was centrifuged at 10 000 × g for 20 min.[10] The resulting supernatant was used for determination of the activities of SOD, GSH-Px and GST. SOD, CAT, GSH-Px and GST activities were determined on the day of the sacrifice of animals. All centrifugations were performed at 0 to 4 °C. All assays were performed in duplicate.

## Assay of Enzymes

### *Superoxide Dismutase (Cu,Zn-SOD)*

The SOD (EC 1.15.1.1) activity was determined according to the method of Liotti *et al.*[9] The assay is based on the inhibition of the conversion of nitro-blue tetrazolium (NBT) by SOD to a blue formazan, mediated by superoxide radicals which have been generated by the xanthine oxidase system. The reduction of NBT was followed at 560 nm with a spectrophotometer. The assays were performed at 30 °C. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. All data are expressed as units of SOD per mg protein.

### *Catalase (CAT)*

CAT (EC 1.11.1.6) activity was measured at 30 °C by the method of Aebi[11] using $H_2O_2$ as a substrate. The decomposition of the substrate $H_2O_2$ was monitored spectrophotometrically at 240 nm for 30 seconds. Activity was expressed as k/mg protein (k: rate constant of the first order reaction as defined by Aebi).[11]

### *Glutathione Peroxidase (GSH-Px)*

The GSH-Px (EC 1.11.1.9) activity was measured by the method of Fecondo and Augusteyn[12] which involves coupling of glutathione peroxidase activity to glutathione reductase. Each assay consisted of 2.5 mM GSH, 0.5 mM $NaN_3$, 0.3 mM EDTA, 0.1 mM NADPH, 0.5 unit of glutathione reductase, 0.4 mM *t*-butyl hydroperoxide in 50 mM phosphate buffer (pH 7.2) and an appropriate amount of supernatant of lung homogenate in a final volume of 1.0 ml. The assays were performed at 37 °C. Reactions were initiated by the addition of *t*-butyl hydroperoxide and the change in absorbance at 340 nm was monitored. Enzymatic activity was expressed as U/mg of protein. One unit of glutathione peroxidase activity was defined as the amount of enzyme that catalyzed the transformation of 1 nmol of NADPH per minute under the defined assay conditions.

### *Glutathione S-transferase (GST)*

Activity of GST (EC 2.5.1.18) was determined by the method of Habig *et al.*[13] at 30 °C, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. This substrate may react with GST forms $\alpha$-$\varepsilon$, $\pi$ and $\mu$. The UV absorbance of the conjugated

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product was followed at 340 nm. The specific activity of GST was calculated as 1 μmol S-2,4-dinitrophenyl glutathione (DNPG) formed per mg protein in 1 min. All enzyme activities are expressed per mg of tissue protein. Protein concentration was determined by the Lowry method, using bovine serum albumin as a standard.[14]

### *Measurement of Lipid Peroxidation*

In the supernatant, lipid peroxide was ascertained by measuring TBARS content. TBARS levels were measured by a fluorometric method described by Wasowicz *et al.*[15] using 1,1,3,3-tetramethoxyropane as a standard and the results are given as nmole/g protein. Fluorescence of the butanol extract was measured in a spectrofluorometer using wavelengths of 525 nm for excitation and 547 nm for emission.

### *Statistical Analysis*

All values were expressed as mean ± SEM. The differences between values obtained from different groups were analysed by Student's *t*-test. *p*-values lower than 0.05 were judged to be significant.

## RESULTS

The data obtained from control and $SO_2$-exposed rats on SOD, CAT, GSH-Px, GST and lipid peroxidation have been summarized in Table I. In middle-aged and old groups, SOD activities were significantly higher than those in young group. The CAT activity in old rat lungs were significantly lower than those in young rat lungs. GSH-Px activity was significantly higher in MC and OC groups than in YC group. GSH-Px

TABLE I Age-related changes in the activities of SOD, CAT, GSH-Px and GST, and the level of TBARS in the lung of control and $SO_2$-exposed Swiss-Albino Male rats

| Groups | Cu,Zn-SOD (U/mg protein) $\times 10^{-3}$ | CAT (k/mg protein) $\times 10^{-3}$ | GSH-Px (U/mg protein) $\times 10^{-3}$ | GST (μmol/dk/mg protein) $\times 10^{-2}$ | TBARS (nmol/g protein) $\times 10^{-3}$ |
|---|---|---|---|---|---|
| Young control (YC) ($n=10$) | 28.00 ± 1.42 | 49.00 ± 4.43 | 1.10 ± 0.03 | 32.90 ± 2.24 | 2.90 ± 0.14 |
| Middle-aged control (MC) ($n=10$) | 110.00 ± 3.16<br>a: $p<.001$ | 41.00 ± 2.18<br>a: n.s | 6.60 ± 0.32<br>a: $p<.001$ | 45.90 ± 2.97<br>a: $p<.005$ | 6.20 ± 0.28<br>a: $p<.001$ |
| Old control (OC) ($n=10$) | 172.00 ± 5.11<br>b: $p<.001$<br>c: $p<.001$ | 37.30 ± 0.89<br>b: $p<.05$<br>c: n.s | 5.10 ± 0.14<br>b: $p<.001$<br>c: $p<.001$ | 32.90 ± 2.03<br>b: n.s<br>c: $p<.001$ | 8.30 ± 0.47<br>b: $p<.001$<br>c: $p<.001$ |
| Young + $SO_2$ ($YSO_2$) ($n=9$) | 89.00 ± 3.67<br>d: $p<.001$ | 38.00 ± 2.97<br>d: $p<.05$ | 1.70 ± 0.09<br>d: $p<.001$ | 49.00 ± 2.40<br>d: $p<.001$ | 4.20 ± 0.17<br>d: $p<.001$ |
| Middle-aged + $SO_2$ ($MSO_2$) ($n=10$) | 490.00 ± 8.09<br>d: $p<.001$<br>e: $p<.005$ | 31.00 ± 2.95<br>d: $p<.001$<br>e: $p<.001$ | 8.50 ± 0.57<br>d: $p<.05$<br>e: $p<.001$ | 50.40 ± 0.31<br>d: $p<.001$<br>e: n.s | 8.60 ± 0.32<br>d: $p<.001$<br>e: $p<.001$ |
| Old + $SO_2$ ($OSO_2$) ($n=11$) | 317.70 ± 6.71<br>d: $p<.001$<br>f: $p<.001$<br>g: $p<.001$ | 38.50 ± 1.22<br>d: n.s<br>f: n.s<br>g: $p<.001$ | 7.40 ± 0.06<br>d: $p<.001$<br>f: $p<.005$<br>g: $p<.001$ | 52.20 ± 1.73<br>d: $p<.001$<br>f: n.s<br>g: n.s | 9.40 ± 0.29<br>d: $p<.05$<br>f: $p<.001$<br>g: $<0.05$ |

a: MC vs YC, b: OC vs YC, c: OC vs MC, d: $SO_2$ groups vs control groups, e: $MSO_2$ vs $YSO_2$, f: $OSO_2$ vs $YSO_2$, g: $OSO_2$ vs $MSO_2$, n.s: non-significant, *n* represents the number of rats. Experimental procedures are as described in the text. Each value represents the mean ± SEM.

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activity was significantly lower in OC group than in MC group. Lung GST activity was increased in MC group compared with YC and OC group.

$SO_2$ exposure caused a significant increment of lung SOD, GSH-Px and GST activities in all experimental groups compared with their respective control groups. Lung CAT activity was significantly decreased in all experimental groups after $SO_2$ exposure (Table I).

TBARS levels were observed to be elevated in MC and OC groups in comparison with YC group while to be higher in OC than in MC. They were significantly increased in all $SO_2$ exposed groups compared with their respective control groups (Table I).

## DISCUSSION

It has been suggested that oxidative stress is a possible aging-accelerating factor, and, that changes in antioxidant defense are also involved in the aging phenomenon.[16,17] The rate of oxygen free radical generation could increase with age.[17] Age-related changes, so far, have been investigated in lipid peroxidation, a result of oxidative stress, and in the capacity of some antioxidant enzymes in tissues of rats.[16]

Our data clearly indicated that SOD activity was increased with age. There have been many conflicting reports on age-related changes in SOD activity of rat tissues. One earlier study that reported a significant increase in SOD in the lung of rats[18] agrees with the result of the present study. SOD activity in the lung remained unchanged in rats,[16] but one study reported a significant decrease with age.[19] Thus, changes with age in SOD activities in the lung reported in the past are extremely variable (increase, decrease and no change) depending on the animal models used.[16,18,19] GSH-Px activity of the lung was increased in the middle-age and old groups with respect to the young group. GSH-Px activity has been reported to be unchanged[16] or decreased with aging.[19,20] These discrepancies may be due to differences in methodologies for tissue preparation, enzyme determination, experimental conditions used, differences in the strains, maintenance conditions of rats or differences in sex, species and age of animals studied.

Catalase activity was observed to be decreased in old rats whereas no difference was observed in middle-aged rats compared with the young ones. Several reports on age-related changes in CAT activity showed that the lung CAT activity remained unchanged during aging.[16,19]

Several papers have been published on age-related changes in the TBARS values of tissue homogenates or organelles in rats. However, these results are quite variable. The lung TBARS level was found to be increased with age. The results obtained for TBARS levels are in agreement with the report of Matsuo *et al*.[16]

Lung SOD, GSH-Px and GST activities and TBARS levels showed a marked increase in all experimental groups following $SO_2$ exposure. CAT activity was decreased in middle-aged rats whereas there was no difference in old rats compared with the control group. The observed increase in SOD activity in response to $SO_2$, may constitute protection against superoxide anion elevation. SOD catalyzes the dismutation of superoxide anions to hydrogen peroxide. This enzyme prevents the further generation of free radicals.[21] Recently, an increased production of superoxide anions and hydrogen peroxide have been recorded in human neutrophils following $SO_2$ exposure.[22] The free radical form of $SO_2$ is produced in aqueous environments by the action of light and chemical or biochemical-reducing agents.[6] The $SO_2$ radical metabolite may be produced under aerobic conditions, but subsequently reacts with oxygen. The air oxidation of this free radical forms superoxide and regenerates bisulfite.[23] These radicals may damage all major classes of chemicals including nucleic acids, proteins, lipids and carbohydrates.[24]

It seems reasonable to ascribe the relatively high lung levels of GSH-Px to a protective

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response against increasing concentration of hydrogen peroxide. GSH-Px can utilize a variety of organic hydroperoxides as well as hydrogen peroxide as its second substrate.[25] Hydrogen peroxide production may be increased, because SOD levels showed significant increases in lungs of $SO_2$ exposured rats.

GST enzyme may protect cells against toxic agents by lowering the concentration of active compound both by direct binding and by catalyzing inactivation reactions.[26] In our study, GST activity was increased following $SO_2$ exposure. However, no comparable report is available in the literature on the activity of GST effected by $SO_2$ in the rat lung. The increased activity of GST may be due to the inactivation of $SO_2$ by GST or formation of organic hydroperoxides by sulfur dioxide radicals. In general, a variety of products of oxidative metabolism such as alkanes, epoxides, organic hydroperoxides, quinones etc. appear to be natural substrates for GST.[26]

Lipid peroxidation *in vivo* has been identified as a basic deteriorative reaction in cellular mechanisms of aging processes; in air pollution oxidant damage to cells and to the lungs.[27] It is also suggested that various environmental pollutants cause lipid peroxidation.[28] Lung TBARS levels showed marked increases in all experimental groups following $SO_2$ exposure. This finding is in agreement with the report of Haider *et al.*[28] and other studies[29,30] in which lipid peroxidative effect of $SO_2$ has been demonstrated. Although SOD and GSH-Px activities were increased following $SO_2$ exposure, the antioxidant defence system could not afford significant protection against lipid peroxidation. Because $SO_2$ exposure results in an exaggerated release of free radicals in the lung containing very low sulfite oxidase activity which is known as a detoxifying enzyme for sulfite.[31] Since free radicals have been suggested to be an important cause of aging, the results of our present study implies that $SO_2$ inhalation might potentiate the acceleration of the aging process due to its lipid peroxidative effect.

In conclusion, our results have shown that antioxidant enzyme activities do not universally decline with aging. Accordingly, the significance of changes in antioxidant enzyme activities during aging needs to be interpreted more carefully than before, bearing in mind that changes are markedly dependent on the sex of animals as well as their organs. Considering these results, the altered balance between partially reduced forms of oxygen and antioxidant enzymatic systems found in rat lung with aging could make older lungs more susceptible to free radical injury. Available data do not permit, as of now, transposition of data from experiments carried out in animals to man, since people living in urban areas are exposed to different air pollutants, the effect of which on health may be combined or superimposed.

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