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Characterization of Antioxidant Enzymes in the Lung of Rat Exposed to Cigarette Smoke

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흡연한 흰쥐 폐조직 항산화효소들의 특성

이영구, 손형옥, 임흥빈, 이동욱, 박준영

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초 록

공기중에 존재하는 여러 산화성 물질들은 호흡기의 손상과 관련이 있는 것으로 알려지고 있으며 이와같은 산화성 물질에 의한 손상은 폐에 존재하는 항산화 물질이나 항산화 효소들에 의해 감소 또는 예방될 수 있다. 저자들은 폐의 항산화방어 기전에 대한 흡연의 영향을 흰쥐에서 관찰하였다.

흰쥐를 6개피의 담배연기에 일일 20분씩 90일간 전신 폭로했을때 조직내의 catalase와 superoxide dismutase(SOD)의 활성이 유의하게 증가되었다($p<0.05$). 그러나 glutathione peroxidase의 활성도는 변화되지 않았고 thiol 화합물의 함량은 흡연 시작후 15일에 44%까지 감소되었으나 그후 정상으로 회복되었다. 한편, 흰쥐를 1, 3, 5, 10 및 20개피의 담배연기에 같은 방법으로 15일간 노출시켰을때, catalase는 개피수에 따라서 증가되었고 총 SOD의 활성도는 5개피 이하에서만 특이하게 증가되었으며 대부분 Mn-SOD이었다. 폐에는 한 종의 Cu, Zn-SOD (pI 4.9)와 CN에 내성이 있는 두종의 Mn-SOD(pI 4.7, pI 7.9)가 존재하였고, 등전점이 4.7인 Mn-SOD가 주된 동위효소로써 흡연에 의해 유도되는 형태였다.

이 결과들은 흡연으로부터 폐의 보호는 초기에는 항산화 물질들의 소모로, 그리고 만성 흡연의 경우는 항산화 효소들의 유도로 이루어지며, 특히 Mn-SOD (pI 4.7)와 catalase가 중요한 역할을 하는 것으로 사료된다.

ABSTRACT

Oxidants in environment or cigarette smoke are known to be implicated in the oxidative damages of pulmonary system. Such cellular damages are prevented by the presence of adequate levels of antioxidants in the tissue. In the present study, we investigated the influences of smoking duration and concentration of smoke on lung antioxidant defense in rats.

Subchronic exposure of rats to smoke generated from 6 cigarettes per day for 90 days caused the activities of catalase and superoxide dismutase (SOD) to increase. However, glutathione peroxidase (GPXase) was not significantly changed. Total sulfhydryl compounds (Total-SH) in the lung homogenates from the rats inhaled with cigarette smoke for 15 days was decreased by 44%, thereafter it was returned to the level of normal rats. On the contrary, when rats were daily exposed to a different concentration of smoke generated from 1 to 20 cigarettes per day for 15 days, the activity of catalase was increased gradually with dose, but total SOD activity was increased only in the rats of low dose groups less than 5 cigarettes. Three types of SOD (one Cu, Zn-SOD with pI 4.9, and two Mn-SOD with pI 4.7 and 7.9)were detected in the lung homogenates and Mn-SOD with pI 4.7 was the major and cigarette-smoke inducible form.

These results indicate that the protection of lung against oxidants from cigarette smoke seems to be accomplished by the induction of catalase and SOD, especially a cyanide resistant Mn-SOD with pI 4.7, following the consumption of antioxidants such as GSH in the beginning of inhalation period.

INTRODUCTION

Reactive oxygen species (ROS) are considered to be involved in the development of many degenerative diseases such as emphysema (1), cancer (2) and atherosclerosis (3). Emphysema, a predominant disease in smokers, is known to be caused by an imbalance between protease and antiprotease in low respiratory tract. This biochemical events are closely associated with *in vivo* antioxidant capacity. Oxidatve inactivation of α_1-protease inhibitor plays a major role in the protease-antiprotease imbalance (4−6). Cigarette smoke contains a remarkable amount of free radicals including ROS, and induces ROS generation from polymorphoneuclear leukocytes or macrophages in the lungs (7−9).

Pulmonary system is a target organ and primary defense mechanism against environmental toxins. Enzymatic and nonenzymatic antioxidant defense mechanisms undoubtedly protect the lung from oxidants even endogenous oxidative stress. Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPXase) are the major enzymes responsible for scavenging ROS. Mn-SOD is induced sensitively under hyperoxic conditions, and plays a pivotal role in the protection of organism from ROS-inducible tissues damage (10). Reduced glutathione (GSH) is also an important cellular thiol

reducing the electrophilic molecular species or free radicals and its intracellular concentration is very high (11). The levels of these major antioxidants in RBC from smokers are generally higher than those from nonsmokers (12, 13). Such increased levels of antioxidants might be indicative of an adaptive response to oxidative stress imposed by smoking.

It is, however, not reasonable to predicate that cigarette smoking has solely influence upon all the free radical associated pathogenesis, because hosts have various antioxidative defense mechanisms as well as their capacities are not the same one another. Therefore, a balance between smoke-mediated oxidants and antioxidant activity of the host may be a major factor determining *in vivo* oxidative stress (14), and the induction and replenishment of antioxidants are equally important to restrict the oxidative tissue damage. However, little is known on the systematic effects of various concentration of cigarette smoke and duration of smoking in human or animals.

In the present study, we investigated the effects of smoking duration and different concentration of cigarette smoke on the major antioxidants in rat lungs. We also determined the effects of acute inhalation of smoke with its different concentration on SOD isozymes in the lungs.

MATERIALS AND METHODS

Chemicals

Riboflavine, 2-thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), cumene hydroperoxide, brilliant cresyl blue, cytochrome c, and NADPH were obtained from Sigma Chemical Co. All other chemicals used were of analytical grade purity.

Cigarette Smoke Exposures

Two inhalation experiments, one for duration effect and the other for dose effect, were conducted. To observe the duration effect of cigarette smoking, male Sprague-Dawley rats (150−160g) exposed whole-body to the diluted mainstream smoke (1 : 5) were subjected to a continuous flow of smoke generated from 6 commercial filter cigarettes for 20 min per day. A round cylinderical polycarbonate chamber (D 46 cm×H 33cm) contained 12 separate compartments, 10 cm wide and each housing one rat was used for the study (Fig. 1). The experimental animals were exposed every other day for 15, 30 or 90 days and seven rats were used for an experimental group. For all exposures, cigarettes were smoked using CORESTA (Cooperation Center for Scientific Research Relative to Tobacco) standard condition (35 ml puff of 2 sec duration taken once per minute from each cigarette) by automatic smoking machine (Heiner Borgbaldt). Cigarettes used for the study delivered 17 mg of tar under this condition. During a 20 min experiment 12 cigarettes were burned using 6 cigarettes at a time, and each cigarette gave 10 puffs. In order to evaluate the dose effect of cigarette smoking, 6 experimental groups of 7 rats each were exposed to the

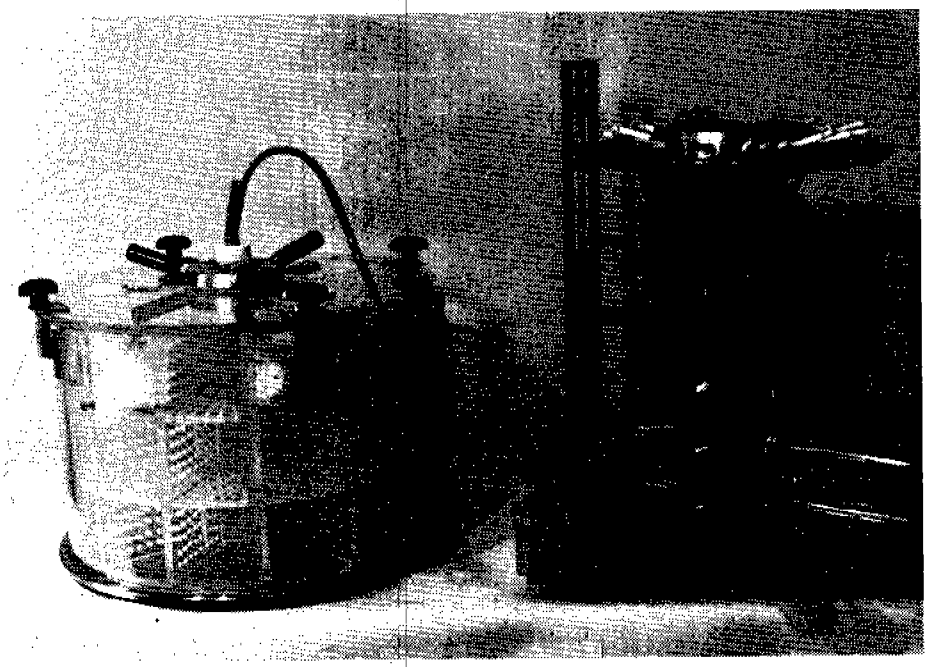

Fig. 1. Smoke exposure system

smoke generated from 0, 1, 3, 5, 10 or 20 cigarettes per day for 15 days as above.

Preparations of Lung Homogenates

Blood was removed by cardiac puncture to reduce its contamination into lung tissues. The rinsed lungs were torn by a cell disrupter (Polytron, Blinkman) and homogenized in a teflon pestle homogenizer at 4℃. Homogenates and cytosolic fractions were prepared by differential centrifugation and stored it in -70℃(15). Thereafter then the relative levels of biochemical markers were determined.

Determination of Antioxidant Activity

Total antioxidant activity (AOA) in lung homogenates was assayed by measuring its inhibitory capacity on autooxidation of ox-brain homogenates (16). The contents of TBA reactive substances was determined by the method of Yu *et al.* (17).

Assays of Enzyme Acitivities

SOD activity was determined by its ability to inhibit the reduction of cytochrome c by superoxide (18). Catalase was assayed by measuring hydrogen peroxide decomposition at 240 nm (19). The activity of GPXase was determined by measuring the oxidation of NADPH at 340 nm using cumene hydroperoxide as substrate (20). The contents of total-SH was determined by the methods of Sedlak and Lindsey (21).

Isoelectric Focusing Electrophoresis

Isoelectric focusing electrophoresis for SOD isozymes was carried out by the method of Winter *et al.* (22) using an Ampholine polyacrylamide gel plates (LKB 80−1124−80, pH 3.5−9.5) under the condition of 2000 V and 50 W for 90 min. SOD isozymes were visualized with a riboflavine and NBT solution, and for the detection of Mn-SOD, the gels were pretreated with 3 mM KCN to inactivate Cu, Zn-SOD (23).

RESULTS

To evaluate the change of antioxidant capacity in the lung of rat inhaled with cigarette smoke time and dose dependently, the major antioxidant enzymes and the possible markers for oxidative stress were assayed. The rats exhibited no discernible clinical signs or symptoms within the experimental period, and food intake and body weight gain of the rats were also not changed by cigarette smoking.

Effect of Smoking Duration on Lung Antioxidant Activity

Fig 2. shows the changes in activities of SOD, catalase and GPXase. The exposure of rats to cigarette smoke resulted the significant induction of lung SOD with duration dependently. SOD was remarkably increased from the 30th day after beginning of inhalation and it in the 90 days inhalation group was reached to 1.7 fold of normal rat and 70% of the activity was cyanide resistant. Catalase activity in the lungs of both of normal and smoke inhaled rats were gradually increased with age, but it was constantly higher in the smoke inhaled rats

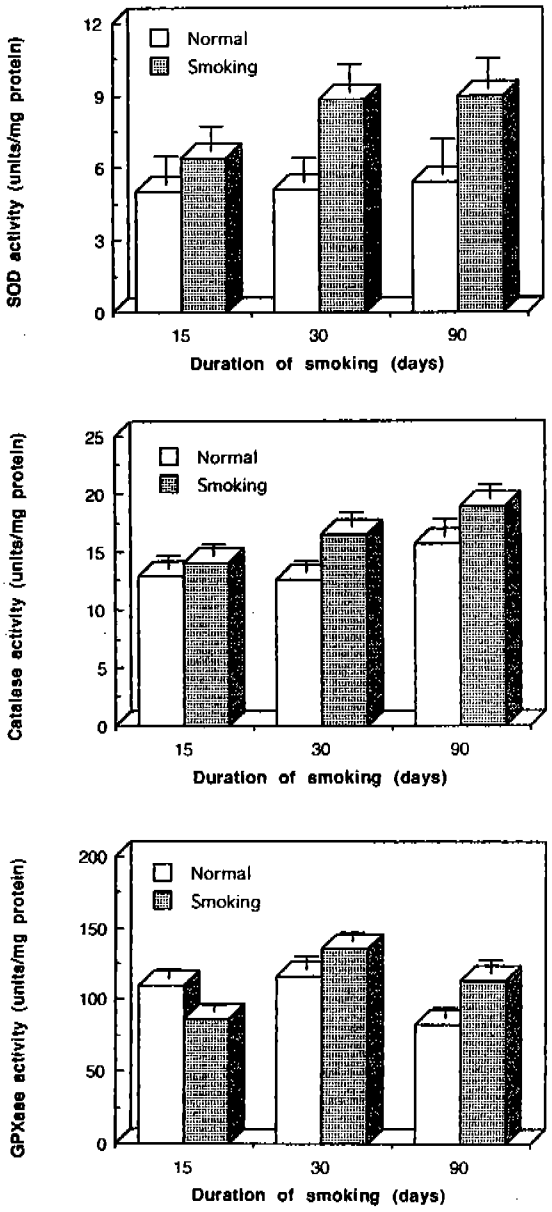

Fig. 2. Effect of duration of cigarette smoking on the activities of SOD, catalase and GPXase in rat lungs

Table 1. Effect of smoking duration on the levels of total sulfhydryl compounds and TBA reactive substances in lung homogenates

Periods	Total-SH (μmoles/g of tissue)		TBARS[a] (A_{532})	
(day)	N	SM	N	SM
15	4.8± 0.5	2.7± 0.7*	0.11± 0.01	0.12± 0.01
30	5.1± 0.4	5.4± 0.2	0.11± 0.03	0.14± 0.02
90	4.9± 0.5	4.5± 0.7	0.21± 0.07	0.32± 0.10

N : Normal, SM : Smoking

a : TBA reactive substances

* Significantly different from normal rats($P<0.05$)

than normal one. However, GPXase was decreased in the 15 days inhalation group and then it was gradually increased until the 30th day, thereafter it was maintained constantly. But there was no significant differences. The contents of total-SH in the lung homogenates was significantly decreased at the 15th day after inhalation ($P<0.05$) and it was returned to the level of normal rats in the 30 days inhalation group as shown in Table 1. On the other hand, the contents of TBA reactive substances in lung homogenates was gradually increased with the smoking duration.

Effect of Smoke Concentration on Lung Antioxidant Activity

Fig 3. shows the dose dependent response of catalase and GPXase activities in the lung. The activity of catalase was not changed with dose until 5 cigarettes a day, but it was increased by 25% of normal rats in the rats inhaled with smoke from

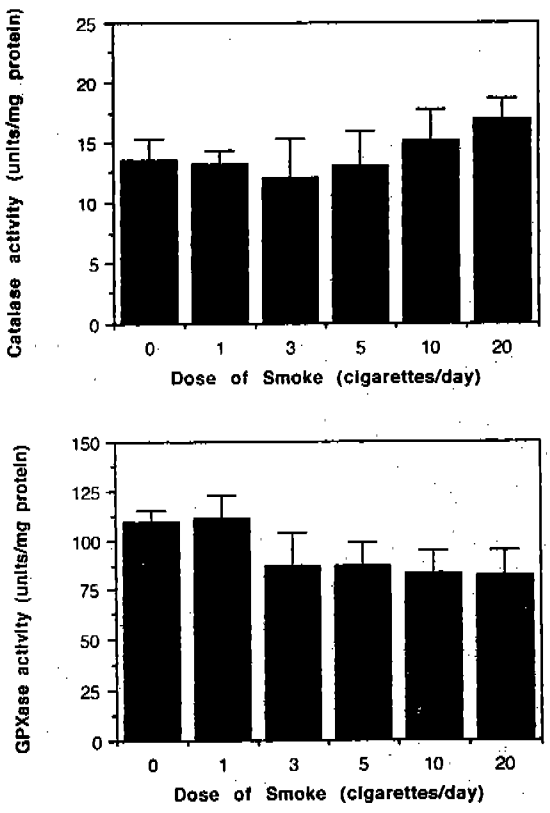

Fig. 3. Effect of smoke concentration on the activities of catalase and GPXase in the lungs

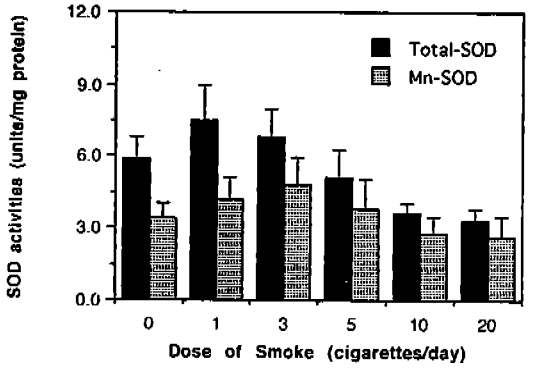

Fig. 4. Effect of inhalation amount of cigarette smoke on the activity of SOD in the lungs

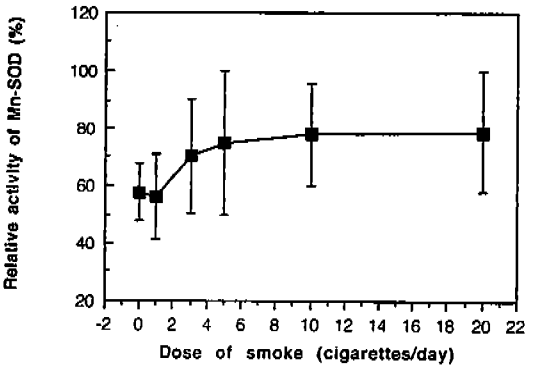

Fig. 5. The change in the relative activity of Mn-SOD in the lung with dose of cigarette smoke

20 cigarettes. GPXase was, however, decreased by 30% by higher dosage than 3 cigarettes and it showed an interesting alteration as shown in Fig 4. Namely, the activities of both total and Mn-SOD were increased by only low dose less than 5 cigarettes. The enhancement of the enzyme activity was slightly decreased in the high dose groups more than 5 cigarette. The relative activity of Mn-SOD was, however, slightly increased (Fig 5). The conteuts of total-SH was decreased by the increase of smoke concentration but TBA reactive substances in the lung homogenates was not significantly changed as shown in Table 2.

Fig 6. shows AOA of lung homogenates. The inhibitory capacity of lung homogenates of the rats on autooxidation of ox-brain homogenates was decreased with amount of smoke inhalation, but it was slightly attenuated in 10 and 20 cigarettes inhalation groups.

Table 2. Effect of inhalation of cigarette smoke on the levels of total sulfhydryl compounds and TBA reactive substances in lung homogenates

Dose (cigarettes/day)	Total−SH (μ moles/g of tissue)	TBARS (A_{532})
0	4.8± 0.4	0.08± 0.01
1	4.5± 0.3	0.11± 0.01
3	4.2± 0.4	0.12± 0.03
5	4.2± 0.2	0.09± 0.01
10	4.1± 0.1	0.12± 0.02
20	4.6± 0.4	0.11± 0.03

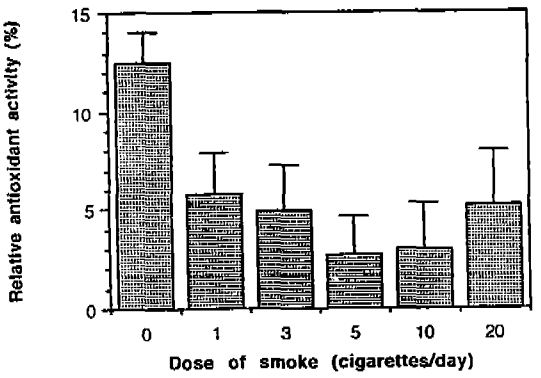

Fig. 6. Effect of inhalation amount of cigarette smoke on the inhibitory capacity of lung homogenates on oxidation of ox brain homogenates

Characteristics of Lung SOD

Since cigarette smoking with different doses resulted an interesting change in lung SOD activity as shown in Fig 4, we attempted further investigations on the characteristics of this enzyme. SOD isozymes in the lung homogenates were separated by isoelectric focusing electrophoresis and determined their isoelectric points (pI). Three active protein bands with different pIs of 4.7, 4.9 and 7.9 were observed(Fig 7.). SOD isozyme with pI 7.9 showed very weak activity, while two other isozymes with pI 4.7 and 4.9 appeared as more intense bands. Most activity of SOD isozyme with pI 4.9

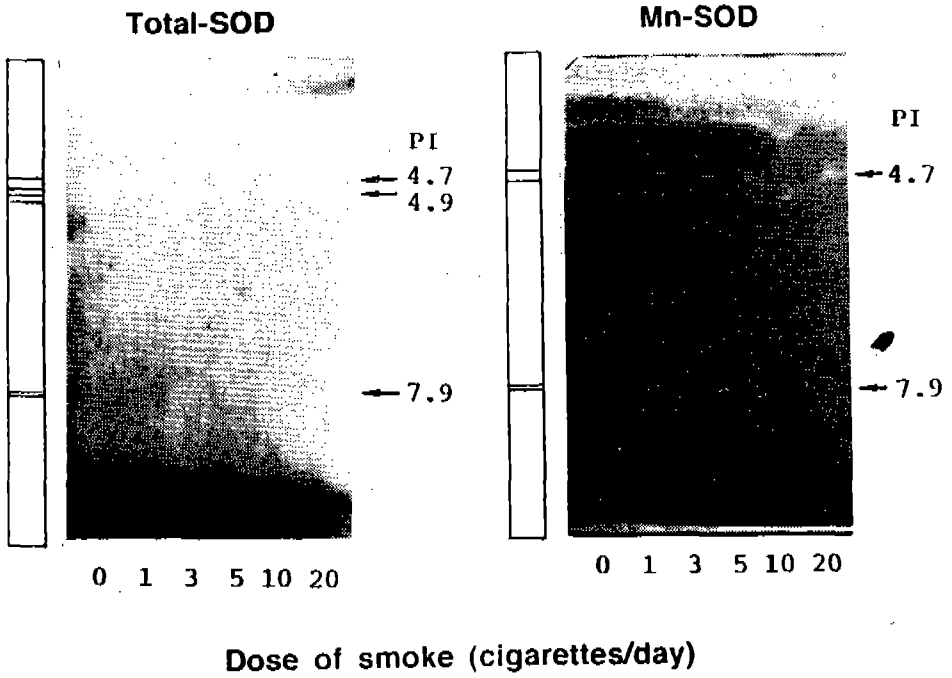

FIg. 7. Polyacrylamide gel isoelectric focusing electrophoresis of lung homogenates. SOD activity was visualized by NBT

was disappeared by pretreatment with 3 mM KCN. An isozyme with pI 4.7 exhibited higher activity band in cigarette smoke inhalation groups than that in normal animals.

DISCUSSION

Depletion of antioxidants and the induction of various antioxidant enzymes are normal responses of self-protection against oxidants. However, if cellular antioxidants are remarkably depleted or *in vivo* oxidants level is ultimately enhanced, cellular toxicity should be occur.

The main implication of this work is to furnish a possible explanation how or which of antioxidant defenses are changed by cigarette smoke with the duration and dose of smoking and what is a primary antioxidant(s) responsible for the restriction of smoke-mediated oxidative damage. The data in the present study can be considered as two aspects, one of which is the induction of antioxidant enzymes and the other is the depletion of sulfhydryl compounds. However, the changes of such biochemical markers exhibited somewhat complicate aspect dependent on exposing period and dose of smoking.

Our first finding is that the major antioxidant enzymes are induced in the lungs with different manner dependent on the dose or time exposed. Previous studies have reported contradictional results that cigarette smoke induces SOD and catalase in rat lung (24), or it resulted no effect on these enzymes activity (25). Such biphasic response of the enzymes activities against cigarette smoking seem to be due to the differences in the duration or dose of smoking. As an evidence, acute inhalation of rats with the moderate amount of cigarette smoke did not induce SOD and catalase, but low dose of it was caused significant induction of SOD in the lung (Fig 3 and 4) and induction patterns of these enzymes by smoke were quite differed each other. However, the depletion of sulfhydryl compounds was observed in the early period of smoke inhalation in the both case (Table 1 and 2). These suggest that the consumption of antioxidants such as sulfhydryl compounds during the smoking by lung cells might be due to a direct reactions of them with oxidants in smoke or with those formed by smoke-mediated reactions and indicate also that those are rapidly replenished. Thiols seem to act as an important role for the prevention of oxidative tissue damage by smoking. However, since pulmonary system has a relatively low rate of GSH synthesis, replacement of them might be attained by diffusion via plasma from liver (26).

SOD and catalase activities in the lung of rats inhaled smoke for 90 days were elevated by about 70% and 25% of normal, respectively, but GPXase was not (Fig 2). Catalase and GPXase appear to be of very consequence in scavenging hydroperoxides, however, catalase has appreciable reductive activity only for small molecules such as hydrogen peroxide, and GPXase complements catalase and

catalyzes actively hydrogen peroxide in low concentration (27−29). On the basis of this, the apparent differences in the activity of the enzymes may reflect that hydrogen peroxide concentration in the lungs was increased by smoking, Generally, the change of catalase activity is closely related to SOD levels because the rate of the reaction of superoxide dismutation catalyzed by SOD to hydrogen peroxide is much faster than that of spontanseous dismutation reaction in physiological condition (30). Therefore, the results indicate that cigarette smoking caused the increase of ROS formation in lung and predominant ROS might be hydrogen peroxide which possesses a relatively long life-time and can be readily penetrated to the cell membrane (31).

In the case of acute inhalation with different concentration of smoke, these enzymes showed different response as shown in Fig 2, 3 and 4. Such selective induction of SOD and catalase by cigarette smoking may reflect the differences in their role or in concentration of their substrates *in vivo*. Especially, as the decreased SOD activity in the high dose groups is a peculiar phenomenon, it might be due to the direct suppression of SOD by some components in cigarette smoke because it contains many other oxidants besides ROS (7).

Our second finding is the dose and time dependent changes of AOA and TBA reactive substances levels in lung homogenates. Both AOA and TBA reactive substances can be utilized as a convenient marker for *in vivo* oxidative stress (32). Although lung Mn-SOD and catalase activities were slightly increased, AOA of lung homogenates was rather decreased. AOA exhibited a very similar pattern to that of thiol contents (Fig 6 and Table 2). This indicates that thiols play the major role as antioxidant defense especially in early period of smoke inhalation. In spite of the depletion of thiols, there was no significant differences in TBA reactive substances contents in lung with the exception of the 90 days inhalation group. These results suggest that rats can be protected from cigarette smoke-mediated oxidants by consumption of cellular antioxidants without more induction of antioxidant enzymes in the case of acute inhalation under this condition.

The other consequence of our findings is that cigarette smoke induces cyanide resistant SOD in the lung. Mn-SOD exists usually in inner membrane space of mitochondria and it is induced easily under oxygen rich conditions (16). In this study, the relative activity of Mn-SOD was gradually increased by cigarette smoking with dose although there was no significant differences. This indicates that Mn-SOD plays a more pivotal role than Cu, Zn-SOD for the scavenging smoke-inducible ROS or that Cu, Zu-SOD is more susceptible to oxidants mediated by smoke than Mn-SOD. We have further investigated the characteristics of its isozymes to manifest which is the cigarette smoke inducible form. Three SOD isozymes were found in the lung homogenates and two of them were cyanide resistant form and the other was cyanide sensitive one.

Especially, Mn-SOD with pI 4.7 was the major form induced by cigarette smoking. Lung SOD was different in the number of its isozymes from brain or liver (33).

Putting together all of the results it is concluded that antioxidant enzymes are induced selectively in the lungs dependent on the concentration of smoke and duration of cigarette smoking and that protection of lungs against oxidants from cigarette smoke seems to be accomplished by the induction of catalase and cyanide resistant SOD following the consumption of antioxidants such as GSH in the early period.

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