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Change of Cu,Zn-superoxide Dismutase Activity of Guinea Pig Lung in Experimental Asthma

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Correlation between the level of reactive oxygen species (ROS) generated by airway inflammatory cells and superoxide dismutase (SOD) activity of pulmonary tissue during an asthma attach was investigated in a guinea pig model of allergic asthma. In addition, the influence of SOD inhibition by diethyldithiocarbamate (DDC, Cu-chelating agent) on the airway was investigated in terms of pulmonary function during an asthma attach. Relative to controls, the capacity of bronchoalveolar lavage fluid (BAL) cells to release ROS was significantly increased in guinea pigs sensitized with ovalbumin (OA) as the antigen, and significantly increased in guinea pigs with an asthma attack provoked by the inhalation of OA. SOD activity was increased significantly in the antigen-sensitized group. The asthma provocation group showed a tendency for increase in total SOD activity, compared with the sensitization group, whose increase was dependent on the increase in copper, zinc-SOD (Cu, Zn-SOD) activity. Pretreatment with DDC increased the severity and duration of the asthma attack. These results were indicated that Cu, Zn-SOD was closely involved in the asthma process, particularly in the scavenging of oxygen radicals secreted from BAL cells.

Keywords: Superoxide dismutase; Asthma; Oxygen radical; Diethyldithiocarbamate; Guinea pigs

INTRODUCTION

In recent years, asthma has been regarded generally as an inflammatory disease of the respiratory tract in which the inflammation adversely influences airway hypersensitivity.[1,2] Involvement of macrophages, neutrophils, and eosinophils in this event have been noted,[3–8] and relative oxygen species (ROS) produced by these inflammatory cells are thought to act as underlying factors in the etiology of impaired bronchial mucosa and in the increase of hypersensitivity of the airway and refractory asthma. The cells, however, produce enzymes that eliminate these ROS as part of the body's defense mechanism. One of these enzymes is superoxide dismutase (SOD).[9]

So far, little is known about the correlation between the level of ROS generated by airway inflammatory cells during an asthma attack and SOD activity of the pulmonary tissue in response to this event. Furthermore, it has not yet been elucidated whether Cu, Zn-SOD or manganese-SOD (Mn-SOD) is the more effective in eliminating superoxide during an asthma attack.

In the present study, we have investigated the correlation between the capacity of airway cells to release ROS during an asthma attack and SOD activity in response to this event. In addition, we have investigated the influence of inhibition of SOD by the Cu-chelating agent, DDC,[10] on the pulmonary function during an asthma attack.

MATERIALS AND METHODS

Experimental Animals

Four-week-old male Hartley guinea pigs weighing 250–350 g (Hokusetsu Co., Japan) were fed with standard chow and tap water *ad libitum*.

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Preparation of Experimental Asthma Model

The method of sensitizing of the experimental asthma model was essentially the same as that described by Anderson *et al.*[11] Guinea pigs were injected intraperitoneally with cyclophosphamide (30 mg/kg; Shionogi, Japan). After 2 days, sensitization was conducted by intraperitoneally injecting 1000 μg ovalbumin (OA) with 100 mg alumina gel ($Al(OH)_3$, Wako, Japan) suspended in physiologic saline. Injection (10 μg OA with 100 mg alumina gel) was repeated again after 3 weeks. Three weeks after this booster injection, these sensitized model animals were used for the studies described below.

Procedure for OA Inhalation

The sensitized model animal was placed in a cylindrical plastic chamber and the lid was closed. Airflow in the chamber was converted to an electric signal by pneumotachometer (MP45, ±2 cm H_2O; Validyne Corp., USA). The tidal volume (VT) was determined by electrical integration of the airflow signal.

Allergic bronchoconstriction (asthma attack) was provoked in sensitized guinea pigs by the 1 min. inhalation of an aerosol of OA solution (2 mg/ml) generated by an ultrasonic nebulizer (Devilibis 646, USA). VT changes were measured before OA inhalation and 0, 1, 3, 5, 10, 15, 20, 25 and 30 min. after OA inhalation.[12]

Bronchoalveolar Lavage Fluid

After the experiments, the guinea pigs were anesthetized intraperitoneally with 30 mg/kg pentobarbital sodium (Abbott Laboratories, USA), then the trachea was cannulated. Bronchoalveolar lavage fluid (BAL)[13] was performed by instillation of 5 ml/kg of physiologic saline at room temperature into the bronchus, followed by gentle aspiration. This procedure was repeated four times.

Tissue Preparation

The tissues were frozen immediately in liquid N_2 and stored at −80°C. For enzyme assays, the frozen samples were homogenized with 0.01 M phosphate buffer saline (pH 7.4) in a Polytron homogenizer, then sonicated on ice for 2 min. (30 s, four times, Bioruptor; Cosmo Bio, Japan). The sonicated samples were centrifuged at 900 g for 10 min. The supernatants were used in the NBT method to measure SOD activity. Protein concentrations of the supernatants were determined by BCA protein assay kit (Pierce, USA).

Measurement of luminol-dependent chemiluminescence of BAL cells

In the dark, 100 μl aliquots of 10^6 BAL cells suspended in Hanks balanced salt solution without phenol red (Whittaler Bioproducts, USA) were placed in luminometer vials, and the first measurement was performed before any stimulation. Next, 50 μl of luminol (Labo-Science, USA) and 50 μl or Ca ionophore A23187 (Sigma, USA) were added, and the suspension was kept at 37°C by continuous stirring during the whole experiment. LDCL was measure by a luminometer, TD-4000 (Labo-Science, USA). Measurements were taken during a period of up to 600 s. Controls were performed with cells incubated in the presence or absence of luminol or Ca ionophore.

Results were expressed as the peak value of luminol-dependent chemiluminescence (LDCL) (maximal LDCL) and integral value during the 600 s.

Measurement of Mn- and Cu, Zn-SOD Activities

In order to measure SOD activity, we sacrificed guinea pigs after conducting BAL at 3 weeks following administration of the booster in the OA-sensitized group and after conducting BAL at 3 days following OA inhalation in the OA-Challenge group, and then sampled lung tissue for use as the test sample.

The assay of SOD activity was basically the same as that described by Beauchamp and Fridovich.[14] In brief, the assay was based on the inhibition of nitro blue tetrazolium conversion by SOD into a blue tetrazolium salt, mediated by superoxide radicals which had been generated by xanthine oxidase. The assay was performed in sodium carbonate buffer (5×10^{-2} M; pH 10.2) containing EDTA (1×10^{-4} M), xanthine (1×10^{-4} M), nitro blue tetrazolium (2.5×10^{-5} M, Sigma, USA) and xanthine oxidase (2.2×10^{-9} M, Boehringer, Germany). The amount required to inhibit the rate of reduction of nitro blue tetrazolium by 50% was defined as one unit of activity. The rate of reduction was followed at 560 nm with a spectrophotometer (M-330, Hitachi, Japan). For inhibition of the Cu, Zn-SOD activity, the assay was conducted in the presence of 2 mM NaCN after preincubation for 30 min. The specific activity was expressed as units per milligram protein.

Administration of DDC

DDC[10] (1500 mg/kg, Sigma, USA) was administered intraperitoneally to guinea pigs. At specific time intervals (0, 1, 3, 6, 12, and 24 h) after administration, the guinea pigs were sacrificed, then the SOD levels in the lungs were measured using the method described above.

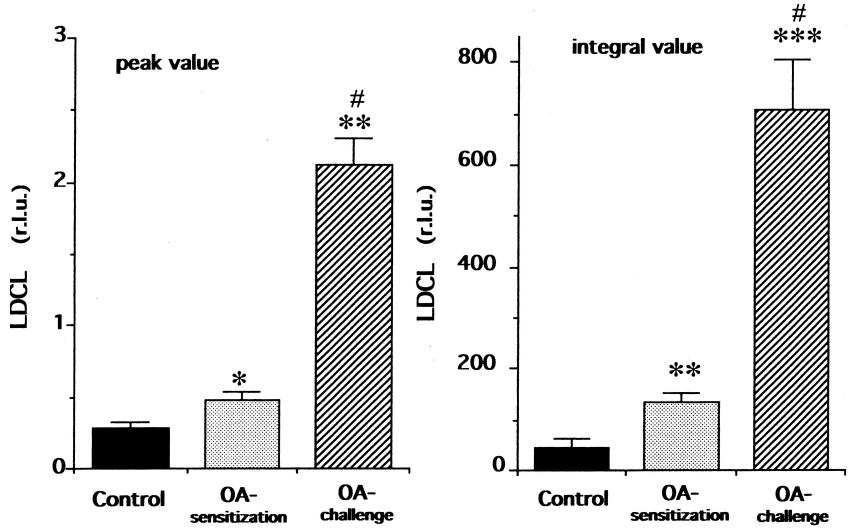

FIGURE 1 Luminol-dependent chemiluminescence (LDCL) of BAL cells. LDCL of 10^6 BAL cells suspended in Hank's balanced salt solution was measured by a luminometer (TD-4000) in the addition of luminol and Ca ionophore A23187, as described in Materials and Methods. The peak and integral values of LDCL during 600 s are expressed as a relative light unit (r.l.u.). In both peak and integral values, LDCL increased in OA-sensitization and OA-challenge. Each column represents a mean value ± SD. Control; a group of normal guinea pigs ($n = 8$). OA-sensitization; a group of guinea pigs were sensitized with OA as the antigen ($n = 14$). OA-challenge; a group of OA sensitized guinea pigs in which an asthma attack was provoked by the inhalation of OA ($n = 6$). *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$, significantly different from control, #; $P < 0.01$, significantly different from OA sensitization.

Statistical Analysis

Statistical analysis was done using the Mann-Whitney test.

RESULTS

Change of Airway Pressure After OA Inhalation

In all OA sensitized guinea pigs, VT after OA inhalation decreased more than 35% of VT before OA inhalation. These changes of VT recovered to base line within about 30 min after OA inhalation.

BAL Cell-mediated LDCL Stimulated by Ca Ionophore A23187

BAL-mediated LDCL value was expressed by relative light unit (r.l.u.). In both peak and integral values, there were differences between the control guinea pigs (control) and the sensitized guinea pigs (OA-sensitization), and between OA-sensitization

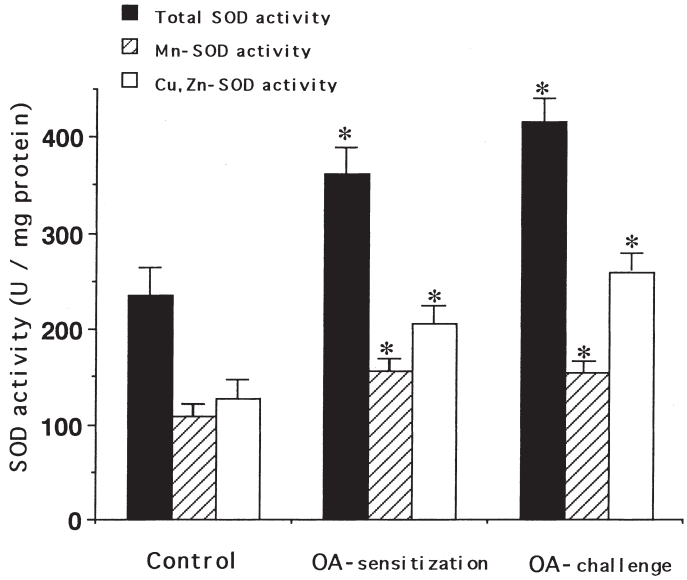

FIGURE 2 Change of SOD activities in OA sensitized or challenged guinea pigs. SOD activity of lung was measured by the NBT method as described in Materials and Methods. SODs activities increased in OA-sensitization and OA-challenge groups. Each column represents a mean value ± SD. Control; a group of normal guinea pigs ($n = 5$). OA-sensitization; a group of guinea pigs were sensitized with OA as the antigen ($n = 5$). OA-challenge; a group of OA sensitized guinea pigs in which an asthma attack was provoked by the inhalation of OA ($n = 5$). *; $P < 0.05$, significantly different from control.

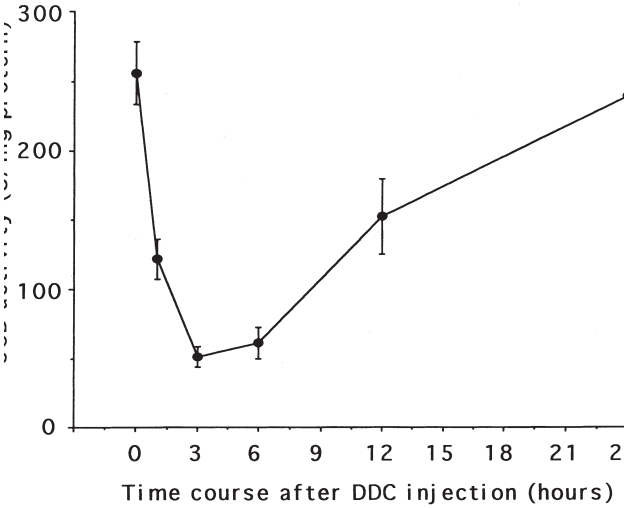

FIGURE 3 Time course for the inhibition of lung SOD by DDC in guinea pigs. DDC (1500 mg/kg) was administered intraperitoneally to guinea pigs. SOD activity of lung measured by the NBT method as described in Materials and Methods was lowest 3 h after DDC administration, but had almost recovered after 24 h. Data is expressed as a mean value ± SD for $n = 3$ in each group.

and sensitized guinea pigs after OA inhalation (OA-challenge). See Fig. 1.

SOD Activity

Total SOD activities significantly increased in OA-sensitization and OA-challenge. Compared to the control, these increased SOD activities depended on increases in Mn- and Cu Zn-SOD. However, the asthma provocation group showed a tendency for an increase in Cu, Zn-SOD activity, compared with the sensitization group. See Fig. 2.

Change of SOD Activity After DDC Injection

SOD activities were assayed at 0, 1, 3, 6, 12, and 24 h after DDC administration. Lowest SOD activity was observed at 3 h after DDC injection, therefore, measurement of airway pressure was performed 3 h after DDC administration. See Fig. 3.

Effect of DDC on VT After OA Inhalation

In sensitized guinea pigs with treatment of saline as a control, VT recovered to the value expressed before OA inhalation within 25 min. after OA inhalation. But VT of sensitized guinea pigs with pretreatment of DDC could not recover after 2 h from the onset of an asthma attack. Pretreatment of DDC prolonged the duration of the asthma attack. See Fig. 4.

DISCUSSION

The major factor underlying the disease state of bronchial asthma is hypersensitivity of the airway.[1,2] ROS produced by inflammatory cells, such as macrophages, neutrophils and eosinophils, have been considered a critical factor in inducing injury of the bronchial mucosa, increasing hypersensitivity of the airway, and inducing chronic inflammation, thereby rendering bronchial asthma refractory to therapy.[3–8]

Our present investigation demonstrated that compared with the healthy controls, the capacity of

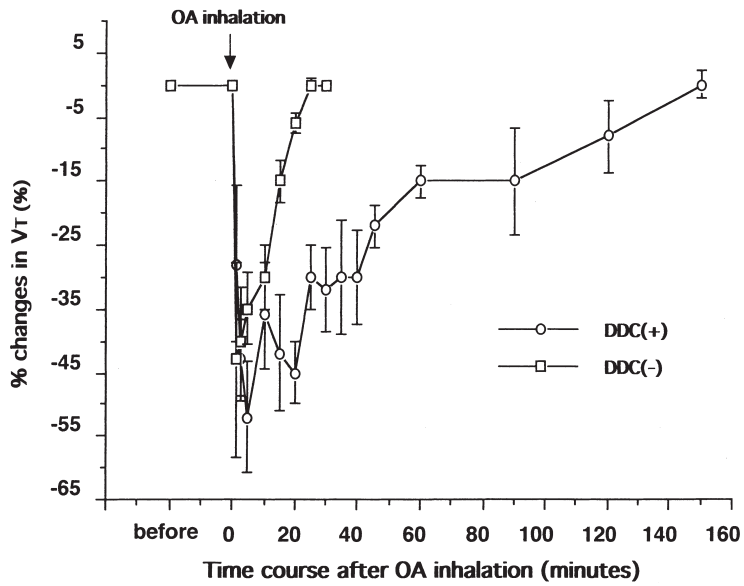

FIGURE 4 Time course for the change of tidal volume (VT) after OA inhalation in OA sensitized guinea pigs with or without pretreatment of DDC. Pretreatment of DDC (1500 mg/kg, i.p.) prolonged the duration of the asthma attack. Data is expressed as a mean value ± SD for $n = 5$ in each group.

BAL cells to release ROS was significantly increased 5.3-fold in a group of guinea pigs sensitized with OA as the antigen, and significantly increased 14.8-fold in a group of guinea pigs in which an asthma attack was provoked by the inhalation of albumin. Clunzel *et al.*[4] showed an increase in the chemilumi value of alveolar macrophages in asthmatic patients in response to stimulation with opsonin, compared with healthy individuals, and demonstrated a correlation between this increase and the degree of asthma. It has been reported that the excessive oxygen metabolites associated with leukotrienes induced the hyperreactivity of bronchial smooth muscle to histamine,[15] and that airway resistance increased by a factor of 2.5 after intrapulmonary perfusion of eosinophils stimulated with PMA.[16] On the direct influence of ROS, Katsumata *et al.*[17] reported that the inhalation of xanthine and xanthine oxidase by nonsensitized cats to artificially induce the formation of ROS resulted in a 10-fold increase in airway resistance; in combination with our results, this finding suggests the possibility that ROS are involved in airway hypersensitivity during an asthma attack.

It has been reported that relative to healthy controls, eosinophils and monocytes in patients with asthma showed an increased capacity to release ROS even in an asthma-free state,[4–6,8] suggesting that cytokines may activate eosinophils, thereby acting as a trigger for increase in oxygen metabolism. Similarly, our finding that sensitization with intraperitoneal injection of OA caused a 5.3-fold higher capacity to release ROS compared with the healthy controls suggests close involvement of cytokines that accelerate the proliferation of neutrophils and eosinophils at antigen sensitization in the development of a state prior to the release of ROS. Blocking these routes of oxygen activation may serve as an effective therapeutic strategy in treating asthmatic patients, pointing to the need for more detailed study in the future.

In contrast, pulmonary cells retain enzymes that eliminate ROS as part of the body's defense mechanism. In particular, SOD is important because superoxide radicals generate other ROS (H_2O_2, OH^-, etc.). However, it has not been clarified how SOD in the pulmonary cells changes in asthma, and which form, Mn-SOD or Cu, Zn-SOD, plays the major role in these changes. Measurement of SOD activity during the present experiment revealed that the antigen-sensitized group showed a significant increase in SOD activity, compared with the healthy control group. Since Mn-SOD is induced by TNF-alpha and IL-1,[18–20] an increase in SOD in response to such antigen sensitization may have been a consequence of the influence of cytokines in association with antigen sensitization. Moreover, OA contains lipopolysaccharide. It is well known that lipopolysaccharide induces Mn-SOD, but not Cu, Zn-SOD.[20] However, Owen *et al.*[8] reported the occurrence of increased peroxidation of serum lipids in patients with asthma, and the present experimental results showed an increase of not only Mn-SOD, but also Cu, Zn-SOD by antigen sensitization and an actual increase in the chemiluminescence level in BAL cells by a factor of 5.3. These findings suggest that an increase in SOD in pulmonary cells may have corresponded to increased generation of oxygen radicals by the BAL cells, rather than cytokines. However, there was a tendency for an increased total SOD activity in the group of subjects whose asthma attack was provoked by OA inhalation and an apparent attack was induced at the time of provocation by OA inhalation, suggesting that markedly increased generation of oxygen radicals by the airway cells clearly exceeded the scavenging capacity of SOD. Although it has not yet been elucidated whether it is Mn-SOD or Cu, Zn-SOD that is the major scavenger of oxygen radicals in pulmonary cells, we noted in the present asthma model that the inhalation provocation group showed a tendency for an increase in total SOD activity, compared with the sensitization group, whose increase was dependent on an increase in Cu, Zn-SOD activity. It is well known that an increase in cytokines or radicals can induce Mn-SOD. However, in the current investigation, an increase in Mn-SOD did not appear to result from an asthma attack. The interaction of NO with the superoxide anion produces the peroxynitrite anion. The nitration of tyrosine residues of SOD by peroxynitrite has been reported, and Mn-SOD is inactivated on the nitration.[21] Actually, asthmatic patients have higher amounts of NO in the expired air, due to the inflammation. In the present study, we measured the activity values and believe that this topic, including a comparison with protein levels, is a useful area for future investigation.[22] In addition, as the present study was designed to measure the activity values of lung tissue, the localization remained unknown.

Accordingly, we have administrated DDC to investigate its effect on the pulmonary function at the time of OA inhalation provocation. In the group pretreated with DDC, VT changed from −42% to −52%, showing enhanced severity of their attacks. Moreover, the time course required for the increased airway resistance after antigen inhalation to return to the normal level was prolonged from 25 min to two and a half hours by administration of DDC, showing a marked prolongation of the duration of an asthma attack. Since DDC is a Cu-chelating agent but not a specific inhibitor of Cu, Zn-SOD, other Cu-associated enzymes must be taken into account. However, these results suggested that Cu, Zn-SOD was closely involved in the asthmatic process, particularly in the scavenging of oxygen radicals secreted from BAL

cells, thereby playing an important role in reducing airway spasm and hypersensitivity.

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