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European Journal of Pharmacology 409 (2000) 179–183

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Effects of lecithinized superoxide dismutase and a neutrophil elastase inhibitor (ONO-5046) on hyperoxic lung injury in rat

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Received 30 August 2000; accepted 14 September 2000

Abstract

Reactive oxygen and neutrophil metabolites have been implicated in the development of hyperoxic lung injury. We determined the protective effects of either a superoxide dismutase or neutrophil elastase inhibitor and the combination of both agents on the development of hyperoxic lung injury in rats. Two drugs (lecithinized superoxide dismutase and ONO-5046) were used in the present study. Lecithinized superoxide dismutase, a lecithin derivative bound to recombinant CuZn superoxide dismutase, has a higher affinity for cells such as polymorphonuclear leukocytes and endothelial cells than recombinant human superoxide dismutase. *N*-[2-[4-2,2-dimethylpropionyloxy) phenylsulfonylamino] benzoyl]} aminoacetic acid (ONO-5046), a specific neutrophil elastase inhibitor, which was developed as a low-molecular weight inhibitor, showed protective effects against various lung injuries. Rats were exposed to over 90% oxygen for 72 h, and bronchoalveolar lavage was performed to evaluate the permeability and neutrophil accumulation in the lungs. Rats were treated with lecithinized superoxide dismutase (30,000 U/day, intravenously $n = 7$) or ONO-5046 (10 mg/kg, intramuscularly twice a day, $n = 7$) or a combination of both drugs ($n = 7$). Albumin concentration and neutrophil counts in bronchoalveolar lavage fluid were compared between animals with and without drug treatment. Either lecithinized superoxide dismutase or ONO-5046 treatment significantly decreased albumin concentration and neutrophil counts in bronchoalveolar lavage fluid compared to those in the animals of the hyperoxia-alone group ($n = 9$). However, albumin leakage and neutrophil accumulation in the rat lung treated with combined agents were identical to that of either the lecithinized superoxide dismutase or ONO-5046 treatment. These findings suggest that lecithinized superoxide dismutase and ONO-5046 are useful drugs to protect against hyperoxic lung injury in rats. However, there were no additive effects by the combination in preventing hyperoxic lung injury.

Keywords: Hyperoxia; Lecithinized superoxide dismutase; Neutrophil elastase; ONO-5046; Lung injury

1. Introduction

Superoxide anion acts as an exacerbating factor in developing lung injury and producing substantial amount of oxygen metabolites such as hydrogen peroxide and hydroxyl radicals (Turrens et al., 1982; Barnes, 1990). Superoxide dismutase, an enzyme that scavenges superoxide anions, has been suggested as a possible therapeutic tool for preventing several types of lung injury (Freeman et al., 1983; Turrens et al., 1984; White et al., 1989). However, the effects of unmodified superoxide dismutase have been limited by its low cell membrane or tissue affinity and its rapid metabolism (Crapo et al., 1977; Turrens et al., 1984; Igarashi et al., 1992). One way to overcome this disadvantage is to incorporate superoxide dismutase into a drug delivery system. Lecithinized superoxide dismutase, a lecithin derivative bound to recombinant CuZn superoxide dismutase, has a higher affinity for cells such as polymorphonuclear leukocytes and endothelial cells than recombinant human superoxide dismutase (Igarashi et al., 1992, 1994). Synthesized lecithinized superoxide dismutase resulted in increased radical scavenging ability, higher cell affinity and more prolonged half-life in plasma than unmodified superoxide dismutase (Igarashi et al., 1992, 1994). It has been demonstrated that lecithinized superoxide dismutase can attenuate several injuries, including colitis (Hori et al., 1997), bleomycin-(Yamazaki et al., 1997) and phorbol myristate acetate-induced (Miyahara et al., 1998) lung injury, and traumatic

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00725-1

brain injury (Yunoki et al., 1997). Thus, lecithinized superoxide dismutase has been examined as a potential therapeutic drug for various diseases.

On the other hand, it is well known that oxygen radicals inactivate antiproteases and enhance the toxicity of neutrophil elastase (Carp and Janoff, 1979; Weiss and Regiani, 1984; Weiss et al., 1986; Iwamura et al., 1993). Indeed, hyperoxic exposure of lung epithelial and pulmonary artery endothelial cells renders them more susceptible to injury by phorbol myristate acetate-stimulated neutrophils in vitro (Suttorp and Simon, 1982). In addition, in vivo experimental models, reactive oxygen metabolites and neutrophils and/or the derived elastase synergistically cause acute edematous lung injury (Krieger et al., 1985; Baird et al., 1986). Therefore, we suggested that neutrophil elastase and superoxide anion have synergistical interaction in the development of lung injury. In the present study, we focused on hyperoxic lung injury, where reactive oxygen metabolites and neutrophils were thought to be implicated (Barry and Crapo, 1985; Jackson, 1985). We examined the effects of lecithinized superoxide dismutase or neutrophil elastase inhibitor or the combination of both drugs on the permeability and neutrophil accumulation in the rat lungs exposed to hyperoxia. We used *N*-[2-[4-(2,2-dimethylpropionyloxy) phenylsulfonylamino] benzoyl] aminoacetic acid (ONO-5046), a specific neutrophil elastase inhibitor (Kawabata et al., 1991), which was developed as a low-molecular weight inhibitor. ONO-5046 has been shown to attenuate several types of acute lung injury, including that induced by endotoxin (Kubo et al., 1994; Sakamaki et al., 1996), phorbol myristate acetate- (Wang et al., 1999), ischemic-reperfusion (Ohwada et al., 1996), and leukotriene B_4-induced (Yoshimura et al., 1994) lung injury.

2. Methods

2.1. Animal and drugs

Six-week-old Sprague–Dawley rats weighting 250–300 g were purchased from Japan SRC Ins. (Hamamatsu). Lecithinized superoxide dismutase and ONO-5046 were provided by Seikagaku (Tokyo, Japan) and Ono Yakuhinn (Tokyo, Japan), respectively.

2.2. Experimental protocol

A total of 36 rats underwent one of the following treatments: Experiment 1: exposed to over 90% oxygen with intravenous administration with vehicle or lecithinized superoxide dismutase (30,000 U/day, intravenously, $n = 7$). Experiment 2: exposed to over 90% oxygen with intramuscularly administration with vehicle or ONO-5046 (10 mg/kg, intramuscularly twice a day, $n = 7$). Experiment 3: exposed to over 90% oxygen with vehicle or given combination of lecithinized superoxide dismutase and ONO-5046 ($n = 7$). Administration dose and schedule of lecithinized superoxide dismutase and ONO-5046 were the same as in experiments 1 and 2, respectively. Experiment 4: exposed to room air with vehicle ($n = 6$). Lecithinized superoxide dismutase and ONO-5046 were dissolved in 5% mannitol and normal saline, respectively. An equal volume of 5% mannitol or normal saline was used as the control vehicle. The data in rats exposed to hyperoxia with vehicle ($n = 9$) were collected from the results in experiments 1–3. The hyperoxygen exposure was performed in a specific (gas-tight) chamber with double-locked subchamber and soda-sorb carbon dioxide. A 90–95% oxygen mixture was continuously blown into the chamber at a flow rate of 5 l/min. The gas outflow from the chamber passed through a long narrow tube so as to keep the chamber inflated. The partial oxygen and carbon dioxide tension in the chamber were monitored by oxygen and carbon dioxide analyzer (Respina IH26, San-ei, Tokyo) to exceed to 90% oxygen and to decrease partial carbon dioxide tension below 2 Torr. Every 12 h during oxygen exposure, the chamber was opened for 5–10 min to allow administration of multiple drug treatments. Rats were given standard food and water ad libitum.

2.3. Measurements

Bronchoalveolar lavage was performed 72 h after the start of oxygen breathing. Total cell, neutrophil counts and albumin concentrations in bronchoalveolar lavage fluid were measured. Lungs in each animal were lavaged with 20 ml of normal saline. Fluid recovery was always above 90% and there were no significant differences in the fluid recovery rate among the groups. Total cell counts were measured by a hemocytometer. All counts were in quadruplicate. For differential counting of cells in bronchoalveolar lavage fluid, cell monolayers were prepared from bronchoalveolar lavage fluid by cytocentrifugation. Differential counts were performed on 200 cells from smears stained with Wright's stain. To assess capillary permeability, albumin concentration was determined by nephelometric immunoassay.

2.4. Statistical analysis

All values are expressed as means ± S.E.M. One-way analysis of variance and Fisher's multiple range test was used to determine statistical significance. A P value < 0.05 was considered as an indication of significant difference between the means.

3. Results

The total cell and neutrophil counts in bronchoalveolar lavage fluid are shown in Figs. 1 and 2. The total cell

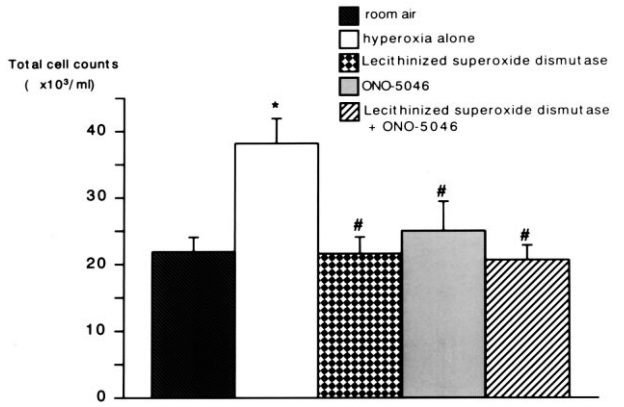

Fig. 1. Total cell counts in bronchoalveolar lavage fluid of rats exposed to room air ($n = 6$), hyperoxia ($n = 9$), hyperoxia + lecithinized superoxide dismutase ($n = 7$), hyperoxia + neutrophil elastase inhibitor (ONO-5046) ($n = 7$), and hyperoxia + combined treatment with lecithinized superoxide dismutase and neutrophil elastase inhibitor (ONO-5046) ($n = 7$). $^{*}P <$ 0.05 vs. room air, $\#P < 0.05$ vs. hyperoxia group.

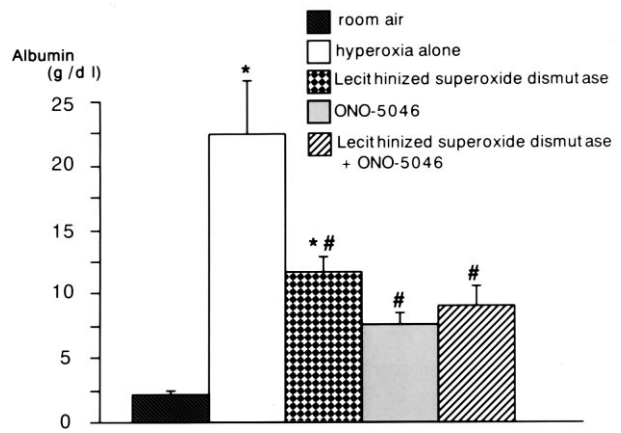

Fig. 3. Albumin concentrations in bronchoalveolar lavage fluid of rats exposed to room air ($n = 6$), hyperoxia ($n = 9$), hyperoxia + lecithinized superoxide dismutase ($n = 7$), hyperoxia + neutrophil elastase inhibitor (ONO-5046) ($n = 7$), and hyperoxia + combined treatment with lecithinized superoxide dismutase and neutrophil elastase inhibitor (ONO-5046) ($n = 7$). $^{*}P < 0.05$ vs. room air, $\#P < 0.05$ vs. hyperoxia group.

counts in the hyperoxia group were significantly increased compared to that of the room air control group. The increased cells in the hyperoxia group consisted mainly of neutrophils (Fig. 2). Either lecithinized superoxide dismutase or ONO-5046 treatment significantly decreased total and neutrophil cell counts in bronchoalveolar lavage fluid compared with the hyperoxia-alone group. However, inflammatory cells in the lungs of the animals of the combined treatment group were almost identical to those of either the lecithinized superoxide dismutase or ONO-5046 group. Likewise, albumin concentration in the bronchoalveolar lavage fluid receiving hyperoxia-alone group was significantly higher than that in room air rats, suggesting an increased lung permeability (Fig. 3). Although both lecithinized superoxide dismutase and ONO-5046 significantly attenuated albumin concentration in bronchoalveolar lavage fluid induced by hyperoxia, the combined effect on albumin leakage was similar to that of either lecithinized superoxide dismutase or ONO-5046 treatment.

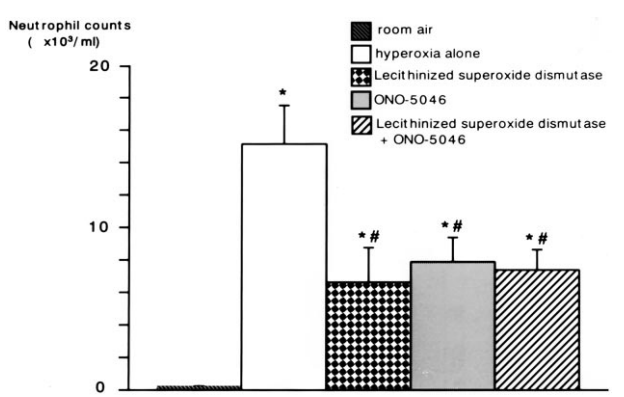

Fig. 2. Neutrophil counts in bronchoalveolar lavage fluid of rats exposed to room air ($n = 6$), hyperoxia ($n = 9$), hyperoxia + lecithinized superoxide dismutase ($n = 7$), hyperoxia + neutrophil elastase inhibitor (ONO-5046) ($n = 7$), and hyperoxia + combined treatment with lecithinized superoxide dismutase and neutrophil elastase inhibitor (ONO-5046) ($n = 7$). $^{*}P < 0.05$ vs. room air, $\#P < 0.05$ vs. hyperoxia group.

4. Discussion

In the present study, we found that either lecithinized superoxide dismutase or ONO-5046 attenuated neutrophil infiltration and protein leakage in rat lungs exposed to hyperoxia. However, there were no additive effects in the combined lecithinized superoxide dismutase and ONO-5046 on neutrophil infiltration and protein leakage in rat lung exposed to hyperoxia.

Lecithinized superoxide dismutase used in the present study has 4 to 20 times greater affinity in vitro for neutrophils and endothelial cells than unmodified superoxide dismutase, and also has a high cellular uptake by these cells. Igarashi et al., 1994 showed that lecithinized superoxide dismutase is 100 times more potent than unmodified superoxide dismutase in human vascular endothelial cell damage caused by superoxide anions generated by phorbol myristate acetate-stimulated polynuclear cells in vitro. Miyahara et al. (1998) found that lecithinized superoxide dismutase attenuated phorbol myristate acetate-induced lung injury in dog more strongly than unmodified superoxide dismutase. In addition, lecithinized superoxide dismutase has a more prolonged half life than that of unmodified recombinant human superoxide dismutase, because of the increase in molecular weight due to lecithinization (Igarashi et al., 1992, 1994). We administered the drug every 24 h in the present study, although unmodified recombinant human superoxide dismutase was usually infused continuously in a previous study (Koyama et al., 1992). Thus, the pharmacological activities of lecithinized superoxide dismutase seem to be over 200 times greater than those of

unmodified recombinant human superoxide dismutase (Igarashi et al., 1992, 1994). As it has been shown that lecithinized superoxide dismutase has protective effects in various experimental models (Hori et al., 1997; Yamazaki et al., 1997; Yunoki et al., 1997), the agent is promising for clinical application.

Lecithinized superoxide dismutase consists of lipid microspheres made of soybean oil and lecithin. The lipid microspheres have the same pharmacokinetic properties as liposomes (Mizushima and Igarashi, 1991). Lipid supplement has been reported to influence hyperoxic toxicity. Liposomes made from saturated fatty acid-containing phospholipids have a small but significant protective effect on oxygen-induced cell damage (Freeman et al., 1983). On the contrary, enrichment of polyunsaturated fatty acids improved survival in hyperoxia exposure in premature infants (Sosenko et al., 1991). Thus, attachment of lipids may have some beneficial and protective outcomes in hyperoxic situations. As the interactions between lecithization and the basement membrane of cells were unknown, further pharmacokinetic study was needed to clarify the role of enrichment by lecithinization to the attenuation in hyperoxic lung injury.

In the present study, oxygen toxicity including neutrophil infiltration and protein leakage was not completely blocked by lecithinized superoxide dismutase. As a reason, we have to consider the possibility of the roles of other reactive oxygen species, such as hydrogen peroxide, hypochlorous acid, and hydroxyl radicals. These have been reported to be generated and to participate in the development of hyperoxic lung injury. A 10-fold increase of hydrogen peroxide in lung mitochondria and microsomes was observed after 100% oxygen exposure (Turrens et al., 1982). Indeed, several studies demonstrated that a combination of superoxide dismutase and catalase synergestically attenuated the hyperoxic lung injury (Freeman et al., 1983; Turrens et al., 1984).

The influx of neutrophils into the lung is a histological feature of hyperoxic lung injury. Shasby et al. (1982) showed a significant correlation between the number of neutrophils accumulation and severity of histological injury after exposure to hyperoxia. Thus, neutrophils have been shown to contribute to lung injury in hyperoxia. Treatment with ONO-5046 attenuated hyperoxic lung injury in the present study, suggesting that neutrophil-derived elastase contributed to the development of hyperoxic lung injury.

It is well known that oxygen radicals inactivate antiproteases and enhance susceptibility to neutrophil elastase (Carp and Janoff, 1980; Rodell et al., 1987). Weiss et al. (1986) showed that reactive oxygen metabolites enhanced the susceptibility of the microvasculature to neutrophil elastase. Rodell et al. showed in an isolated lung that lungs with previous exposure to hyperoxia were more susceptible to elastase-induced microvascular injury, and that inactivation of xanthine oxidase reversed the hyperoxia-induced sensitivity to neutrophil elastase. These findings supported the presence of a synergic interaction between reactive oxygen metabolites and neutrophil elastase. However, we found in the present study that inactivations of both superoxide and neutrophil elastase productions resulted in little additive protection against hyperoxic lung injury in rats.

The reason was unknown in the present study. Matrix degradation by phorbol myristate acetate-stimulated neutrophils was more strongly inhibited by catalase (a scavenger of hydrogen peroxide) than superoxide dismutase (Weiss and Regiani, 1984). Suttorp and Simon (1982) also found that although superoxide dismutase at high concentrations had a weak protective effect, catalase fully reversed the neutrophil-mediated cytotoxicity in endothelial cells. These studies suggested that the main oxygen radical species by neutrophil-mediated cytotoxicity was hydrogen peroxide. In addition, radical-scavenging agents themselves may influence the neutrophil elastase activity. In in vitro experiment of stimulated neutrophils induced by phorbol myristate acetate, superoxide dismutase and catalase significantly reduced elastase activity. However, the effect of catalase was stronger than that of superoxide dismutase (Iwamura et al., 1993). As it has not been reported that either neutrophil elastase or ONO-5046 has a direct effect on reactive oxygen metabolite production, different interactions with neutrophil elastase may exist among reactive oxygen species. Taken together, our present results suggested that injurious interplay between superoxide anions and neutrophil products seemed to be little in the development of hyperoxic lung injury in rats.

In summary, lecithinized superoxide dismutase and ONO-5046 are useful drugs to protect against hyperoxic lung injury in rats. However, there were no additive effects in the combination of those for the prevention of hyperoxic lung injury in rat.

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